Review Article

Association between eNOS 4b/a Polymorphism and the Risk of Diabetic Retinopathy in Type 2 Diabetes Mellitus: A Meta-Analysis

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Many studies have assessed the association between eNOS-4b/a polymorphism and the risk of diabetic retinopathy (DR) among type 2 diabetic subjects. However, the results are inconsistent. In order to derive a more precise estimation of the association, a meta-analysis was conducted. Fifteen studies with 3, 183 cases and 3, 410 controls were enrolled by searching the databases of Pubmed, Embase, China National Knowledge Infrastructure (CNKI), and Chinese Wanfang Database. Summary odds ratios (ORs) with 95% confidence intervals (CIs) were calculated. The main analysis indicated no significant association between eNOS-4b/a polymorphism and the risk of DR in overall population [allelic model: OR = 0.94 (0.79–1.11); additive model: OR = 0.91 (0.73–1.14); recessive model: OR = 1.01 (0.81–1.25); dominant model: OR = 0.91 (0.75–1.09)]. Subgroup analysis by ethnicity also indicated no significant association. In conclusion, the current meta-analysis did not observe any association between the polymorphism of eNOS 4b/a and the risk of DR among type 2 diabetic subjects. However, larger well-designed studies are required to confirm this finding.

1. Introduction

Diabetic retinopathy (DR) is one of the most common microvascular complications of diabetes mellitus (DM) and a leading cause of adult blindness worldwide [1, 2]. Although long duration of diabetes and poor control of glycemia have been considered as the major risk factors for the development of DR, accumulated evidences suggest a genetic influence on susceptibility to this complication [3]. A number of genes have been suggested as candidate genes of diabetic retinopathy, for example, methylenetetrahydrofolate reductase gene, endothelial nitric oxide synthase gene (eNOS), vascular endothelial growth factor gene, and so on [4–6].

The eNOS gene is located on chromosome 7q35-36 and includes 26 exons, spanning 21 kb. The polymorphism of eNOS 4b/a gene consists of the two alleles of eNOS 4a with 4 tandem 27-repeats and eNOS 4b with 5 repeats [7]. NO is produced through the oxidation of L-arginine by eNOS [8]. NO can regulate endothelial function and is an important factor in the maintenance of homeostasis. NO can contribute to vasodilatation, increase local blood flow, and decrease vascular resistance in ocular circulation. Studies on humans and animal models have suggested that eNOS plays an essential role in retinal vascular function and disequilibrium in its production can lead to the development of DR [9–11]. 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 DR.

To date, many case-control studies have been carried out to investigate the relationship between eNOS-4b/a polymorphism and the risk of DR 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 DR [4, 12], while some others suggested there was no significant association [13, 14]. 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 DR among type 2 diabetic subjects, including subgroup analyses based on different ethnicities.

2. Materials and Methods

2.1. Literature Search Strategy. We searched the literature databases including Pubmed, Embase, China National Knowledge Infrastructure (CNKI), and Chinese Wanfang Database. The last updated search was performed on November 20, 2013. The search used the following terms: "endothelial nitric oxide synthase or eNOS or 4b/a" in combination with "mutation or polymorphism or variant" and in combination with "diabetic retinopathy or DR." We also manually searched all the references of included studies to further identify additional relevant studies. Unpublished studies were not sought. For overlapping or republished studies, only the larger sample size or the most recent published papers were included in this meta-analysis. Publication language and publication date were not restricted in our search.

2.2. Inclusion and Exclusion Criteria. Studies included in this meta-analysis must meet the following criteria: (1) case-control or cohort studies; (2) studies evaluating the association between eNOS-4b/a polymorphism and DR risk; (3) subjects in the control group having type 2 diabetes but being free of DR; (4) human studies; and (5) having detailed data to calculate the odds ratio (OR) and 95% confidence interval (CI).

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

2.3. Data Extraction. The following data were collected from each study: first author, publication date, region, ethnicity, sample size of cases and controls, genotype and allele frequencies of cases and controls, and genotyping methods. All the data were extracted independently by two investigators (Zejun Ma and Hui-Zhu Ren), according to the inclusion criteria above. Disagreements about eligibility were resolved through a discussion between the two investigators.

2.4. Statistical Analysis. In this meta-analysis, we evaluated the relationship between eNOS-4b/a polymorphism and DR risk using the allelic model (a versus b), the additive model (aa versus bb), the dominant model (aa + ab versus bb), and the recessive model (aa versus ab + bb) (see Figure 5). The alleles and genotypes between patients and control subjects were compared with OR and corresponding 95% CIs. A chi-square based Q statistics test and I^2 test were used to evaluate the heterogeneity between the studies ($P < 0.10$ and $I^2 > 50\%$ indicated the evidence of heterogeneity) [15]. The fixed effects model (FEM) was used when there was no statistical heterogeneity among the included studies; otherwise, the random effects model (REM) was used. Subgroup analysis was conducted according to different ethnicity. Sensitivity analysis was performed for estimating the stability of the

meta-analysis. First, sensitivity analysis was carried out by exclusion of studies which failed the HWE test. Another analysis was done by omitting one study at a time to examine influence of one study on the overall summary estimate. Begg's funnel plot and Egger's test were carried out to assess possible publication bias [16, 17]. Asymmetric plot or the P value of Egger's test less than 0.05 suggested possible publication bias. Meta-analyses were performed using the statistical software Review Manager (version 5.2 for Windows, Cochrane Collaboration) and STATA software (version 12.0; Stata, College Station, TX), using two-sided P values.

3. Results

3.1. Characteristics of the Studies. Based on our search strategy, 61 potentially relevant articles were identified in Pubmed, Embase, CNKI, and Chinese Wanfang Database. A flow chart of study selection was shown in Figure 1. Of these, 46 were excluded because they did not meet the criteria or were overlapping publications. Finally, a total of 15 studies published between 2004 and 2012 met our inclusion criteria, involving 3,183 cases and 3,410 controls. The main characteristics of these studies were listed in Table 1. Of the ethnicity among all studies, four studies were performed on Caucasians [4, 13, 14, 18], ten were performed on Asian [12, 19–27], and one study was performed on West African [28]. The sample size in these studies varied considerably (ranging from 166 to 1446 individuals). The genotype and allele distributions for each study and HWE in controls were summarized in Table 2. Two genotyping methods were used to check genotypes in the studies including polymerase chain reaction (PCR) or PCR-restriction fragment length polymorphism (PCR-RFLP).

The distribution of the eNOS 4b/a genotype in control group was consistent with the HWE, except for two studies [23, 26]. Because excluding these two studies did not materially affect the results, they were still included in this analysis.

3.2. Meta-Analysis Results. The main results of meta-analysis for eNOS 4b/a polymorphism with the risk of DR were shown in Table 3. No significant association between eNOS 4b/a polymorphism and susceptibility to DR was identified in any of the genetic models (allelic model: OR = 0.94, 95% CI: 0.79–1.11; additive model: OR = 0.91, 95% CI: 0.73–1.14; the recessive model: OR = 1.01, 95% CI: 0.81–1.25; and the dominant model: OR = 0.91, 95% CI: 0.75–1.09) (Figures 2, 3, and 4).

In the subgroup analysis by ethnicity, similarly, there was still no significant association detected in all genetic models among Asians and Caucasians. The results of the subgroup analyses were shown in Table 3.

3.3. Heterogeneity and Publication Bias. Some heterogeneity was found during the course of the study. Hence, the random effects model was used. Begg's funnel plot and Egger's test were performed to assess the publication bias of the eligible literatures in this meta-analysis. The shapes of the funnel plots in all genetic models did not reveal any evidence of obvious asymmetry. The Egger's test further confirmed the absence
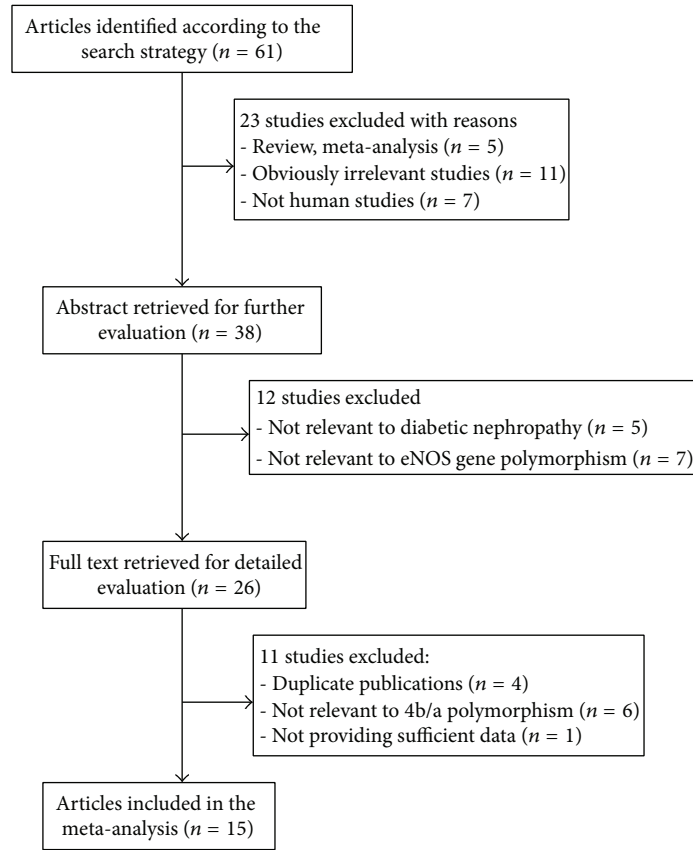
FIGURE 1: Flow chart of included studies.

Study or subgroup	Experimental		Control		Weight	Odds ratio M-H, random, 95% CI	Odds ratio M-H, random, 95% CI
	Events	Total	Events	Total			
Awata et al. [12]	27	234	30	218	5.2%	0.82 [0.47, 1.43]	
Cheema et al. [20]	518	1684	402	1208	11.0%	0.89 [0.76, 1.04]	
Chen et al. [28]	37	136	232	602	7.1%	0.60 [0.39, 0.90]	
Cilenšek et al. [4]	81	344	140	810	8.7%	1.47 [1.08, 2.01]	
de Syllos et al. [14]	18	112	33	228	4.6%	1.13 [0.61, 2.11]	
Ezzidi et al. [19]	149	766	246	978	9.9%	0.72 [0.57, 0.90]	
Li et al. [26]	23	174	25	158	4.7%	0.81 [0.44, 1.49]	
Mehrab-Mohseni et al. [21]	11	82	61	358	4.0%	0.75 [0.38, 1.51]	
Petrovič et al. [18]	123	566	58	286	8.0%	1.09 [0.77, 1.55]	
Santos et al. [13]	135	814	63	368	8.3%	0.96 [0.69, 1.34]	
Suganthalakshmi et al. [22]	41	240	24	180	5.4%	1.34 [0.78, 2.31]	
Sun et al. [24]	42	294	23	310	5.5%	2.08 [1.22, 3.55]	
Uthra et al. [23]	68	374	59	376	7.5%	1.19 [0.81, 1.75]	
Yue et al. [27]	31	308	53	334	6.2%	0.59 [0.37, 0.95]	
Zhang et al. [25]	11	238	35	406	4.0%	0.51 [0.26, 1.03]	
Total (95% CI)		6366		6820	100.0%	0.94 [0.79, 1.11]	
Total events	1315		1484				

Heterogeneity: $\tau^2 = 0.06$; $\chi^2 = 38.38$, df = 14 ($P = 0.0005$); $I^2 = 64\%$
Test for overall effect: $Z = 0.70$ ($P = 0.48$)
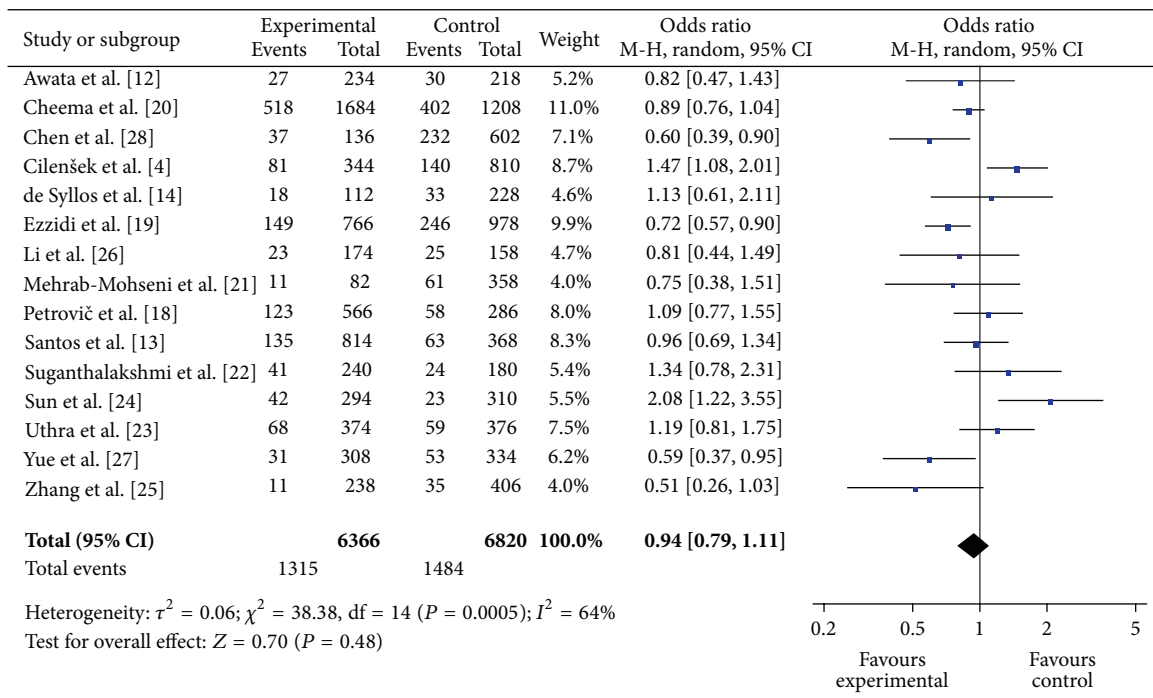
FIGURE 2: The forest plot of a versus b of eNOS polymorphism and overall DR risk.

TABLE 1: Main characteristics of included studies in the meta-analysis.

Author	Year	Country	Ethnicity	Case/control	Genotyping methods
Awata et al. [12]	2004	Japan	Asian	117/109	PCR
Cheema et al. [20]	2012	Indian	Asian	842/604	PCR-RFLP
Chen et al. [28]	2007	Ghana and Nigeria	West African	68/301	PCR
Cilenšek et al. [4]	2012	Slovenia	Caucasians	172/405	PCR
de Syllos et al. [14]	2006	Brazil	Caucasians	56/114	PCR
Ezzidi et al. [19]	2008	Bahrain	Asian	383/489	PCR-RFLP
Li et al. [26]	2010	china	Asian	87/79	PCR
Mehrab-Mohseni et al. [21]	2011	Iran	Asian	41/179	PCR
Petrovič et al. [18]	2008	Slovenia	Caucasians	283/143	PCR
Santos et al. [13]	2012	Brazil	Caucasians	407/184	PCR
Suganthalakshmi et al. [22]	2006	India	Asian	120/90	PCR-RFLP
Sun et al. [24]	2004	china	Asian	147/155	PCR-RFLP
Uthra et al. [23]	2007	India	Asian	187/188	PCR
Yue et al. [27]	1994	china	Asian	154/167	PCR
Zhang et al. [25]	2005	china	Asian	119/203	PCR

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	
Awata et al. [12]	1	25	91	3	24	82	27	207	30	188	Yes
Cheema et al. [20]	87	344	411	66	270	268	518	1166	402	806	Yes
Chen et al. [28]	10	17	41	48	136	117	37	99	232	370	Yes
Cilenšek et al. [4]	15	51	106	13	114	278	81	263	140	670	Yes
de Syllos et al. [14]	1	16	39	3	27	84	18	94	33	195	Yes
Ezzidi et al. [19]	17	115	251	33	180	276	149	617	246	732	Yes
Li et al. [26]	5	13	69	7	11	61	23	151	25	133	No
Mehrab-Mohseni et al. [21]	12	111	284	5	53	126	135	679	63	305	Yes
Petrovič et al. [18]	16	91	176	7	44	92	123	443	58	228	Yes
Santos et al. [13]	12	111	284	5	53	126	135	679	63	305	Yes
Suganthalakshmi et al. [22]	7	27	86	3	18	69	41	199	24	156	Yes
Sun et al. [24]	3	36	108	1	21	133	42	252	23	287	Yes
Uthra et al. [23]	7	54	126	1	57	130	68	306	59	317	No
Yue et al. [27]	2	27	125	4	45	118	31	277	53	281	Yes
Zhang et al. [25]	0	11	108	2	31	170	11	227	35	371	Yes

HWE: Hardy-Weinberg equilibrium, $P < 0.05$ was considered significant.

of publication bias for any of the four genetic models in this meta-analysis ($P = 0.277$ for dominant model, $P = 0.313$ for recessive model, $P = 0.287$ for additive model, and $P = 0.764$ for allelic model, resp.).

3.4. Sensitivity Analysis. Sensitivity analyses were performed to assess the stability of the results. Although two studies [23, 26] did not follow the HWE, the summary ORs were not materially altered including or excluding the studies (data shown in Table 3). Moreover, no other single study influenced the overall results (data not shown), which indicated that our results were statistically reliable and robust.

4. Discussion

The polymorphism of eNOS-4b/a gene has been associated with many vascular diseases including hypertension, diabetic retinopathy, and diabetic nephropathy in various populations [29, 30]. Variable results have been reported for the association of eNOS-4b/a polymorphism with DR [13, 20, 21]. In a systematic meta-analysis study [3], no relationship of the eNOS 4b/a polymorphism was found with DR development regardless of ethnicity. However, a recent meta-analysis study found that eNOS 4b/a has a protective effect against DR [31]. The present meta-analysis of 15 studies, including 3,183 cases and 3,410 controls, provided the most comprehensive analysis on the association of the eNOS 4b/a polymorphism

TABLE 3: Meta analysis of the association of eNOS-4b/a gene polymorphism with DR in type 2 diabetes.

Genetic model	Populations	Studies (n)	Number of cases/controls	Heterogeneity Q test P-value	I^2 (%)	OR (95% CI)	P value
a versus b	All	15	3183/3410	0.0005	64	0.94 (0.79–1.11) (REM)	0.48
	Asian	10	2265/2564	0.006	61	0.90 (0.73–1.11) (REM)	0.32
	Caucasians	4	918/846	0.30	18	1.17 (0.97–1.40) (FEM)	0.10
	HWE (yes)	13	2909/3143	0.0003	67	0.93 (0.77–1.12) (REM)	0.44
aa versus bb	All	15	3183/3410	0.04	43	0.91 (0.73–1.14) (FEM)	0.40
	Asian	10	2265/2564	0.21	24	0.78 (0.61–1.01) (FEM)	0.06
	Caucasians	4	918/846	0.26	25	1.64 (0.98–2.73) (FEM)	0.06
	HWE (yes)	13	2909/3143	0.06	41	0.88 (0.70–1.11) (FEM)	0.28
aa + ab versus bb	All	15	3183/3410	0.002	60	0.91 (0.75–1.09) (REM)	0.30
	Asian	10	2265/2564	0.0004	61	0.82 (0.65–1.04) (REM)	0.11
	Caucasians	4	918/846	0.58	0	1.13 (0.91–1.40) (FEM)	0.26
	HWE (yes)	13	2909/3143	0.0007	65	0.89 (0.72–1.10) (REM)	0.30
aa versus bb + ab	All	15	3183/3410	0.21	22	1.01 (0.81–1.25) (FEM)	0.96
	Asian	10	2265/2564	0.49	0	0.90 (0.70–1.15) (FEM)	0.39
	Caucasians	4	918/846	0.28	21	1.60 (0.97–2.65) (FEM)	0.07
	HWE (yes)	13	2909/3143	0.30	14	0.98 (0.78–1.23) (FEM)	0.88

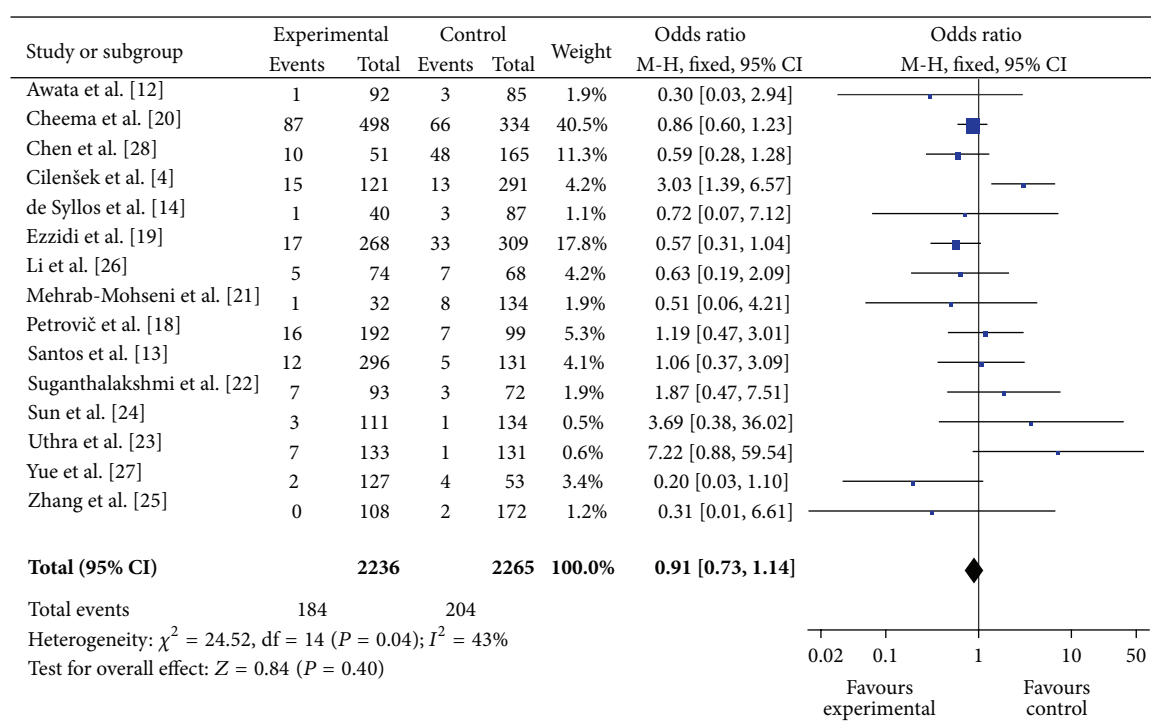OR: odds ratio; CI: confidence interval.

FIGURE 3: The forest plot of aa versus bb of eNOS polymorphism and overall DR risk.

with the risk of diabetic retinopathy. The results indicated that the eNOS 4b/a polymorphism was not associated with an increased risk of DR in the overall studied population. These findings were consistent with most of the studies that were included in our meta-analysis [13, 14, 23]. The lack of association between eNOS 4b/a polymorphism and diabetic retinopathy suggested that genetic variations in the eNOS 4b/a gene did not predict the risk of diabetic retinopathy in T2DM patients. It is possible that eNOS-derived NO plays a minor role in the development of diabetic retinopathy. In the subgroup analysis according to ethnicity, no significant association was observed in Asian and Caucasians in all genetic models. The results demonstrated that ethnic difference in genetic background and the living environment
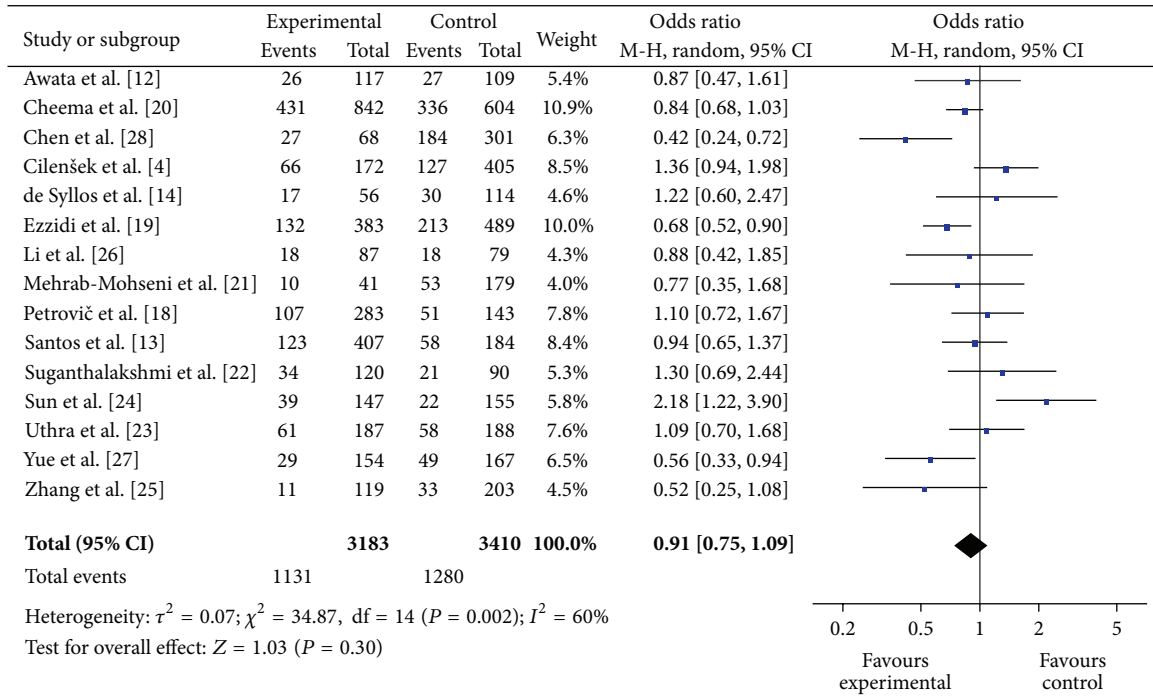
| Study or subgroup | Experimental | | Control | | Weight | Odds ratio | Odds ratio |
	Events	Total	Events	Total		M-H, random, 95% CI	M-H, random, 95% CI
Awata et al. [12]	26	117	27	109	5.4%	0.87 [0.47, 1.61]	
Cheema et al. [20]	431	842	336	604	10.9%	0.84 [0.68, 1.03]	
Chen et al. [28]	27	68	184	301	6.3%	0.42 [0.24, 0.72]	
Cilenšek et al. [4]	66	172	127	405	8.5%	1.36 [0.94, 1.98]	
de Syllos et al. [14]	17	56	30	114	4.6%	1.22 [0.60, 2.47]	
Ezzidi et al. [19]	132	383	213	489	10.0%	0.68 [0.52, 0.90]	
Li et al. [26]	18	87	18	79	4.3%	0.88 [0.42, 1.85]	
Mehrab-Mohseni et al. [21]	10	41	53	179	4.0%	0.77 [0.35, 1.68]	
Petrovič et al. [18]	107	283	51	143	7.8%	1.10 [0.72, 1.67]	
Santos et al. [13]	123	407	58	184	8.4%	0.94 [0.65, 1.37]	
Suganthalakshmi et al. [22]	34	120	21	90	5.3%	1.30 [0.69, 2.44]	
Sun et al. [24]	39	147	22	155	5.8%	2.18 [1.22, 3.90]	
Uthra et al. [23]	61	187	58	188	7.6%	1.09 [0.70, 1.68]	
Yue et al. [27]	29	154	49	167	6.5%	0.56 [0.33, 0.94]	
Zhang et al. [25]	11	119	33	203	4.5%	0.52 [0.25, 1.08]	
Total (95% CI)		3183		3410	100.0%	0.91 [0.75, 1.09]	
Total events	1131		1280				

Heterogeneity: $\tau^2 = 0.07$; $\chi^2 = 34.87$, df = 14 ($P = 0.002$); $I^2 = 60\%$
Test for overall effect: $Z = 1.03$ ($P = 0.30$)

0.2   0.5   1   2   5
Favours experimental   Favours control

FIGURE 4: The forest plot of aa + ab versus bb of eNOS polymorphism and overall DR risk.

| Study or subgroup | Experimental | | Control | | Weight | Odds ratio | Odds ratio |
	Events	Total	Events	Total		M-H, fixed, 95% CI	M-H, fixed, 95% CI
Awata et al. [12]	1	117	3	109	1.9%	0.30 [0.03, 2.97]	
Cheema et al. [20]	87	842	66	604	43.1%	0.94 [0.67, 1.32]	
Chen et al. [28]	10	68	48	301	9.4%	0.91 [0.43, 1.90]	
Cilenšek et al. [4]	15	172	13	405	4.4%	2.88 [1.34, 6.19]	
de Syllos et al. [14]	1	56	3	114	1.2%	0.67 [0.07, 6.62]	
Ezzidi et al. [19]	17	383	33	489	17.3%	0.64 [0.35, 1.17]	
Li et al. [26]	5	87	7	79	4.3%	0.63 [0.19, 2.06]	
Mehrab-Mohseni et al. [21]	1	41	8	179	1.8%	0.53 [0.06, 4.40]	
Petrovič et al. [18]	16	283	7	143	5.5%	1.16 [0.47, 2.90]	
Santos et al. [13]	12	407	5	184	4.2%	1.09 [0.38, 3.13]	
Suganthalakshmi et al. [22]	7	120	3	90	2.0%	1.80 [0.45, 7.15]	
Sun et al. [24]	3	147	1	155	0.6%	3.21 [0.33, 31.20]	
Uthra et al. [23]	7	187	1	188	0.6%	7.27 [0.89, 59.70]	
Yue et al. [27]	2	154	4	167	2.4%	0.54 [0.10, 2.97]	
Zhang et al. [25]	0	119	2	201	1.2%	0.33 [0.02, 7.01]	
Total (95% CI)		3183		3408	100.0%	1.01 [0.81, 1.25]	
Total events	184		204				

Heterogeneity: $\chi^2 = 17.97$, df = 14 ($P = 0.21$); $I^2 = 22\%$
Test for overall effect: $Z = 0.05$ ($P = 0.96$)

0.02   0.1   1   10   50
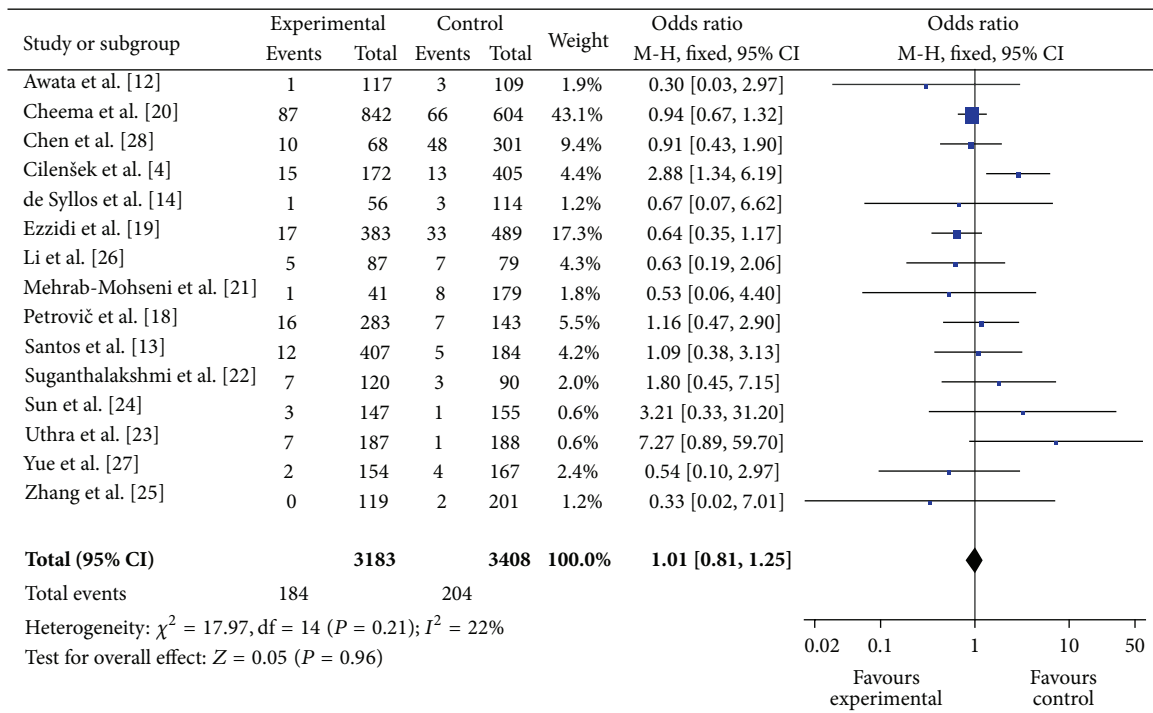Favours experimental   Favours control

FIGURE 5: The forest plot of aa versus bb + ab of eNOS polymorphism and overall DR risk.

did not play an obvious role in the association between the eNOS 4b/a polymorphism and the risk of DR. Although the available genetic data do not implicate the eNOS 4b/a polymorphism as a determinant of DR susceptibility in Asian and Caucasians, further studies are needed to see if the eNOS

4b/a polymorphism can confer a risk of DR in other ethnic populations.

Publication bias is an important factor affecting 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 that the results observed should be stable. Sensitivity analyses did not significantly alter the results, also suggesting that our results were statistically reliable and stable.

Our study has several limitations that need to be taken into consideration when interpreting the results. First, significant between-study heterogeneity was detected in some comparisons and might distort the meta-analysis. Second, only published studies in English or Chinese were included for data analysis; some potential studies with other languages or unpublished could be missed. Third, this meta-analysis was based predominantly on Asian research. Only 4 studies involving Caucasians and one study involving Africans were included. No study from other parts of the world was found. This may develop a partial result. Fourth, our meta-analysis was based on unadjusted OR estimates. Despite these limitations, our study provides a better understanding of the association between eNOS-4b/a gene polymorphisms and risk of DR in type 2 diabetes.

In summary, this meta-analysis indicates that the eNOS 4b/a polymorphism is not associated with an increased risk of DR among type 2 diabetic subjects. 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 confirm this finding.

Conflict of Interests

All authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Ze-jun Ma and Li-ming Chen conceived and designed the experiments. Ze-jun Ma, Xin Guo, and Rui Chen carried out the literature searching and data extraction. Ze-jun Ma, Hui-Zhu Ren, Jun Guo, and Li-ming Chen analyzed the data. Ze-jun Ma, Xin Guo, Rui Chen, and Jun Guo contributed reagents/materials/analysis tools. Ze-jun Ma, Rui Chen, and Li-ming Chen wrote the paper.

Acknowledgment

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