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Original Article

Association between eNOS 4b/a polymorphism and susceptibility of pulmonary hypertension: a meta-analysis of 6 studies

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Abstract: Background: Pulmonary hypertension (PH) is one of the most common types of vascular diseases. Endothelial nitric oxide synthase (NOS3) is suspected to be related to PH risk. Recently, many studies have investigated the relationship between NOS3 4b/a polymorphism and PH risk, but the results were controversial and inconclusive. Method: A meta-analysis was performed by searching electronic databases and bibliographies of related articles until October 2015. Odds ratios (OR) and 95% confidence intervals were determined by fixed or random-effects model according to the respective heterogeneity. Subgroup analysis and sensitivity analysis were employed to examine potential source of heterogeneity. Egger's and Begg's tests were done to detect publication biases. Results: Six case-control studies, containing 198 cases and 250 controls, were included in this meta-analysis. A significant difference was found in the homozygote comparison (4a/a vs 4b/b) by fixed-effects model. The pooled OR was 3.83 (95% CI=1.33-11.02, P_{OR}=0.01, $P_{heterogeneity}$=0.63, I^2=0.0%). When comparing the recessive model (4a/a vs 4b/b+4b/a), a tendency of increasing risk of PH to the individuals with the genotype of 4a/a compared to those with the genotype 4b/b or 4b/a was found. The pooled OR by fixed-effects mode was 2.55 (95% CI=0.95-6.88, P_{OR}=0.06, $P_{heterogeneity}$=0.89, I^2=0.0%). However, no statistically significant differences were observed for 4b/a vs 4b/b or (4a/a+4b/a) vs 4b/b. Conclusion: An increasing susceptibility between NOS3 4b/a polymorphism and PH risk was found in this study. Individuals with the genotype of 4a/a have an obviously higher risk of developing PH than those with the genotype of 4b/b.

Keywords: Pulmonary hypertension, NOS3, polymorphism, meta-analysis

Introduction

Pulmonary hypertension (PH) is a hemodynamic and pathophysiological condition defined as mean pulmonary arterial pressure (mPAP) more than 25 mmHg at rest assessedby right heart catheterization [1, 2]. It contains five clinical subtypes with different etiologies [3, 4]. Patients with PH often suffer from shortness of breath, fatigue, weakness, angina, syncope, even sudden death with low exercise capacity, poor quality of life and compromised right ventricular function [2]. The pathogenesis of PH remains unclear. An imbalance between vasodilators and vasoconstrictors may play a role [5]. Nitric oxide (NO), a potent vasorelaxant, synthesized in human body from L-arginine by nitric oxide synthase (NOS), exerts a vasoprotective effect during the progressing of PH [6]. Inhibition or reduction of NO, thus, may be detrimental in this process. NO production has been known to be influenced by several polymorphisms of the NOS gene [7], of which the endothelial nitric oxide synthase (eNOS or NOS3) polymorphism may be a risk factor of PH.

NOS3, encoded by the NOS3 gene, is a key regulator of vascular NO production. NOS3 gene is located on chromosome at 7q35-q36, spans 21 kb, and consists of 26 exons [8]. There are many mutations in the promoter region, introns, and exons of this gene, such as G894T (rs1799983), T786C (rs2070744) and 4b/a (Ensembl Gene IDENSG00000164867), of which the eNOS 4b/a polymorphism is one of

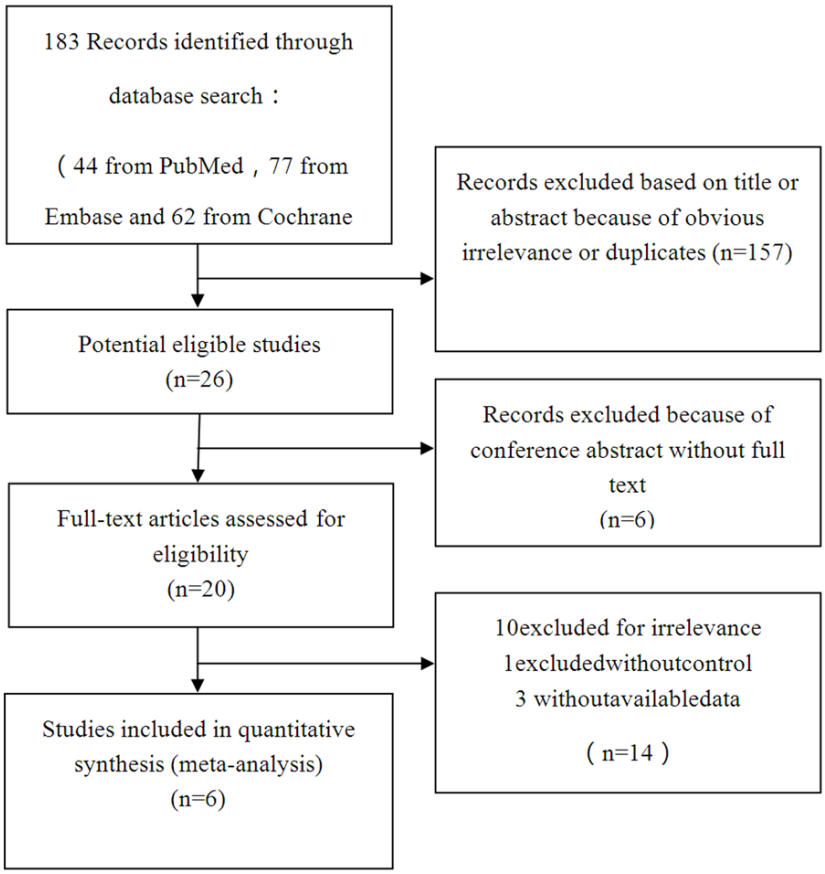

Figure 1. PRISMA flow chart. A total of 183 references were searched and 6 articles were identified for this meta-analysis.

the most common type to have functional consequence [9].

NOS3 4b/a polymorphism, characterized by the variable number of tandem repeats (VNTR) polymorphism located in intron 4 of eNOS gene, is significantly associated with plasmanitric oxide concentration [10]. There are two alleles, 4b and 4a, identified in intron 4 of the NOS3 gene. 4b is the larger one, consisting of five tandem 27-bp repeats and 4a is the smaller one, possessing four of the same repeats. Last decade, several studies have researched the relationship of NOS3 4b/a polymorphism and the risk of PH. However, the results in controversial and inconsistent. Yildiz P [11] reported that patients with NOS3 4b/a polymorphism were more susceptible to the risk of HP. His conclusion were supported by Gao Y [12] and Tantawy AA [13]. While those associations were not observed by some other studies [14-16].

To determine the association of NOS3 4b/a polymorphism and PH, we performed a meta-analysis of all related case-control studies to obtain a conclusive result.

Materials and methods

Study selection

We searched the case-control studies by electronic databases (PubMed, EMBASE, the Cochrane library and China National Knowledge Infrastructure (CNKI)) and manually searched the references of relative articles and reviews, using key words of “pulmonary hypertension, pulmonary arterial hypertension or pulmonary pressure” and “endothelial nitric oxide synthase, eNOS or NOS3” and “polymorphism*, mutation* or variant*”. The last search data was October 10th, 2015 without restriction to language. And if there were studies describing the same groups of subjects or controls, the largest available published data would be included. We finished this meta-analysis according to the PRISMA guideline [17].

Inclusion and exclusion criteria

The inclusion criteria were as follows: (1) case-control study; (2) the association between the NOS3 4b/a polymorphism and risk of HP were reported; (3) detailed genotypes data (4b/b, 4b/a and 4a/a) in cases and control groups could be gotten. Exclusion criteria were as follows: (1) duplicated studies; (2) studies topic ofother type of NOS3 polymorphisms, such as G894T or T786C polymorphism; (3) genotype frequencies not accessible.

Data extraction and quality assessment

Two reviewers extracted the main data independently from the included studies. To obtain enough information, the study authors would be contacted if necessary. If any disagree-

Table 1. Characteristics of 6 case-control studies eligible for this meta-analysis

First author/ref	Year	Country	Ethnicity	NO. of case/control	Gender distribution (M/F)		Age	
					Case	Control	Case	Control
Yildiz P	2003	Turkey	Asian	24/14	NG	NG	62	57
Gao Y	2006	China	Asian	61/104	NG	NG	59	53
Ulrich S	2010	Switzerland	Caucasian	27/22	14/13	17/5	70	66
Ulrich S	2010	Switzerland	Caucasian	32/24	13/19	15/9	65	67
Ulasli SS	2013	Turkey	Asian	30/50	36/41	60/40	67	64
Tantawy AA	2015	Egypt	African	24/36	NG	NG	12	12

Note: M, male; F, female; NG, data not given.

Table 2. Distributions of NOS3 4b/a genotype and some other related characteristics

Study/year	Case group			Control group			HWE (Y/N)	PH clinical classification
	4b/b	4b/a	4a/a	4b/b	4b/a	4a/a		
Yildiz P 2003	20	4	0	7	7	0	Y	3
Gao Y 2006	43	17	1	89	14	1	Y	3
Ulrich S 2010	17	9	1	14	8	0	Y	3
Ulrich S 2010	21	10	1	16	7	1	Y	1 or 4
Ulasli SS 2013	28	1	1	44	6	0	Y	3
Tantawy AA 2015	5	12	7	25	7	4	Y	5

Note: Y, yes; N, no; HWE: Hardy-Weinberg equilibrium; '3' indicates PH secondary to respiratory disease; '5' PH secondary to some other disease; '1 or 4' PH secondary to idiopathic etiology or pulmonary embolism.

Table 3. Pooled results of meta-analysis

NOS3	NO. of studies	Test of association			Test of heterogeneity	
		Model	OR (95% CI)	P_{OR}	P_h	I^2 (%)
aa/bb	5	F	3.83 (1.33, 11.02)*	0.01	0.63	0.0
ab/bb	6	R	1.16 (0.43, 3.15)	0.76	0.00	73.0%
aa+ba/bb	6	R	1.27 (0.49, 3.25)	0.62	0.00	73.5%
aa/ba+bb	5	F	2.55 (0.95, 6.88)	0.06	0.89	0.0

Note: NOS3: endothelial nitric oxide synthase; NO.: number of included study; P_h: *P* value for heterogeneity; OR: odds ratio; 95% CI: 95% confidence interval; P_{OR}: *P* value for OR; I^2: variation in OR attributable to heterogeneity; F: fixed-effects model; R: random-effects model; '*' indicated the difference was statistically significant.

ments, discussion would be done till consensus was achieved between two reviewers,the following information was extracted: first authors, year of publication, age, ethnicity, the number of case and control, clinical characteristics, Hardy-Weinberg equilibrium (HWE) information, genotypic distributions in case and control. We used an online program (http://ihg.gsf.de/cgi-bin/hw/hwa1.p1) [18] to test the Hardy-Weinberg equilibrium in the controls and a *p* value less than 0.05 suggested statistically significance.

Statistic methods

The strength of the association between the eNOS 4b/a and PH was measured by odds ratios (OR) and the corresponding 95% confidence intervals (CI). The overall HP risk was evaluated in four comparison models: homozygote comparison (4a/a vs. 4b/b), heterozygote comparison (4b/a vs. 4b/b), recessive model (4a/a vs. 4b/a+4b/b) and dominant model (4a/a+4b/a vs. 4b/b). Individual study effect on pooled results was tested by sensitivity analysis.

Heterogeneity across studies was tested by the-Cochran Q-test and I^2-test. $P<0.1$ or $I^2>50\%$ was considered to be heterogeneous. If those tests showed non-significant heterogeneity, a fixed-effects model (Mantel-Haenszel approach) would be used to calculated the pooled ORs; or else, a random-effects model (Der-Simonian method)would be employed [19-22]. Publication bias was investigated by funnel plot; Begg's test and Egger's test were also conducted to calculate potential publication bias, and a $P<0.05$ was considered statistically significance [23]. All statistical analyses were performed with the software STATA (Stata Corporation, College Station, TX, version 13.0).

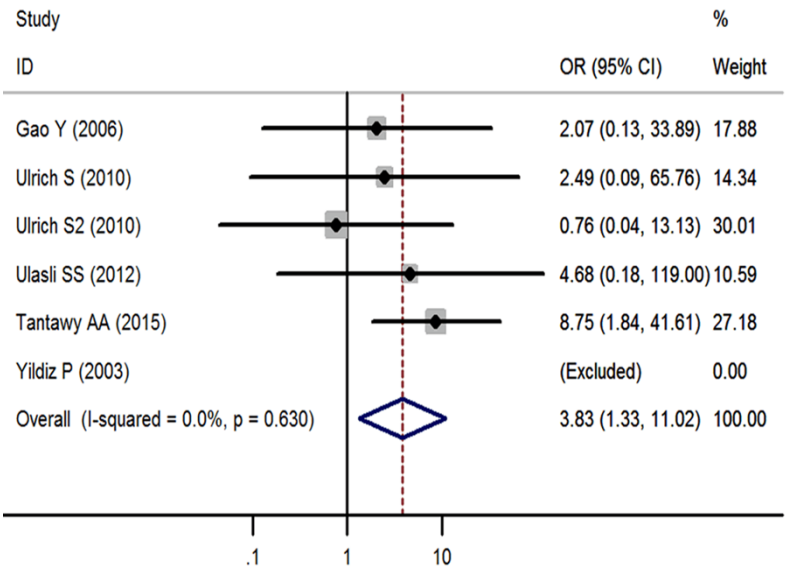

Figure 2. Forest plot of homozygote comparison (4a/a vs. 4b/b) for overall comparison by fixed-effects model (OR=3.83, 95% CI=1.33-11.02, P_{OR}=0.01, Pheterogeneity=0.63, I^2=0.0%).

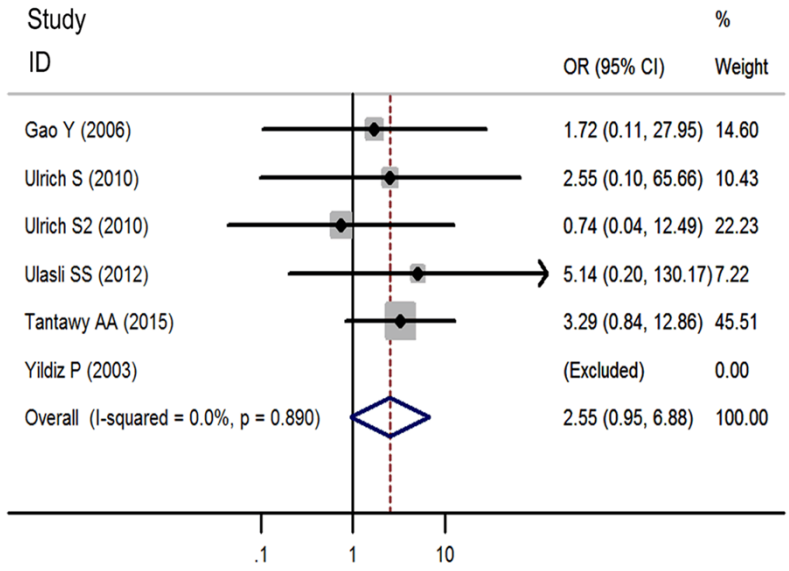

Figure 3. Forest plot of recessive model (4a/a vs. 4b/a+4b/b) for overall comparison by fixed-effects model (OR=2.55, 95% CI=0.95-6.88, P_{OR}=0.06, Pheterogeneity=0.89, I^2=0.0%).

And all *P* values were two-side with a significance level of 0.05.

Results

Characteristics of eligible studies

A total of 6 studies with 198 cases and 250 controls, who were genotyped for investigating the association between the NOS 4b/a polymorphism and PH were identified according to the inclusion and exclusion criteria. The research details and screening process was showed in **Figure 1**, the demographic characteristics of included studies were listed in **Table 1**, and a summary of the NOS 4b/a genotype distributions and some other related characteristics were shown in **Table 2**. There were three studies performed in Asian, two were performed in Caucasian and one in African. HWE in the controls was tested by the online program as previous described with all included studies [24] in accord with HWE.

Result of meta-analysis

6 studies were included in the meta-analysis with 198 PH patients and 250 control subjects. The pooled results of this meta-analysis are shown in **Table 3**. A significant difference was found in the homozygote comparison (4a/a vs 4b/b) by fixed-effects model. The pooled OR was 3.83 (95% CI=1.33-11.02, P_{OR}=0.01, $P_{heterogeneity}$=0.63, I^2=0.0%, **Figure 2**). The results abovesuggested that individuals with the 4a/a genotype had a higher risk of having PH than those with the 4b/b genotype. When comparing the recessive model (4a/a vs. 4b/b+4b/a), we found that there was a tendency of increasing risk of PH to the individuals with the genotype of 4a/a compared to those with the genotype 4b/b or 4b/a. The pooled OR by fixed-effects mode was 2.55 (95% CI=0.95-6.88, P_{OR}=0.06, $P_{heterogeneity}$=0.89, I^2=0.0%, **Figure 3**).

While when comparing PH subjects and controlswith heterozygote comparison and domi-

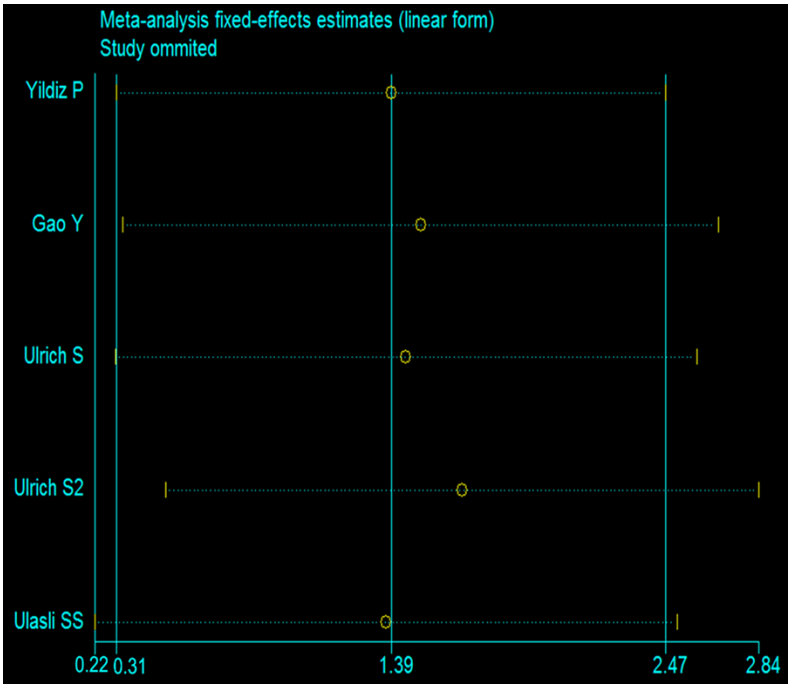

Figure 4. Sensitivity analysis for homozygote comparison (4a/a vs 4b/b) to evaluate the effect of single study to the pooled result.

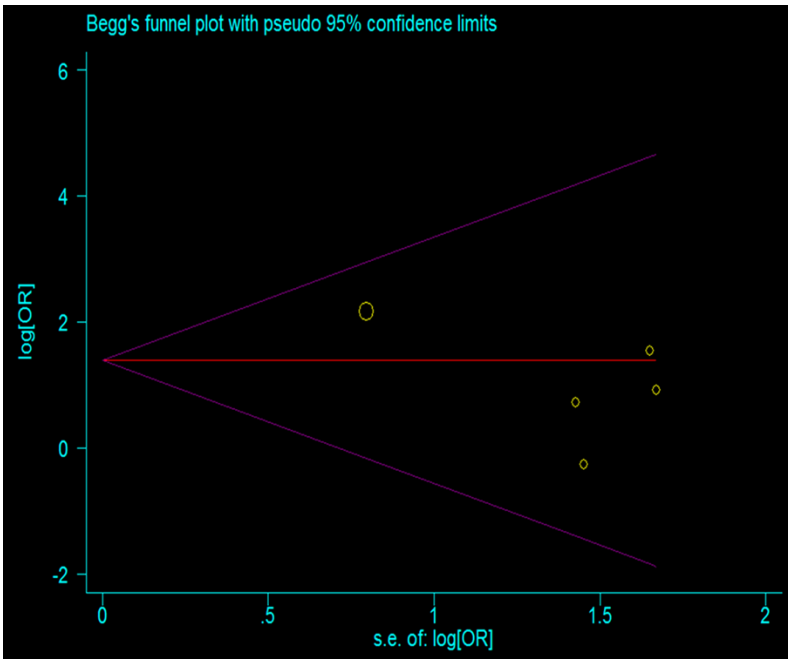

Figure 5. Begg's funnel plot for homozygote comparison (4a/a vs 4b/b). Begg's test: P_{Begg}=0.76. OR: odds ratio.

nant model, a significant heterogeneity was found (I^2=73% and I^2=73.5% respectively). So we employed the random-effects model to evaluate the pooled results. Yet no statistically significant differences were observed. The pooled OR by random-effects model were OR 4b/a vs.4b/b=1.16 (95% CI=0.43-3.15, P_{OR}=0.76) and OR 4a/a+4a/b vs. 4b/b=1.27, 95% CI=0.49-3.25, P_{OR}=0.62).

Subgroup analysis for ethnicity suggested that a NOS3 4b/a polymorphism is associated an increasing risk of PH to non-Caucasian population in the homozygote comparison and recessive model by fixed effects model. The pooled OR were 3.84 (95% CI=1.51-9.73, P_{OR}=0.005, I^2=0.0%) and 2.67 (95% CI=1.00-7.10, P_{OR}=0.049, I^2=0.0%) respectively. While in heterozygote comparison and dominant model, the difference became non-significance. The pooled OR by random-effects model were 1.17 (95% CI=0.23-5.93, P_{OR}=0.85, I^2=82.6%) and 1.34 (95% CI=0.30-5.91), P_{OR}=0.70, I^2=82.8%) respectively. In Caucasian population, no significant relationship was found between NOS3 4b/a polymorphism and the risk of PH in all comparing models (data not shown).

Heterogeneity and sensitivity analysis

In the homozygote comparison and recessive model, heterogeneity was non-significant in overall analysis (**Table 3**). We performed sensitivity analysis to evaluate single included study's influence on the total results in the above two genetic models (**Figure 4**). The sensitivity analysis showed nothing either the pooled effect or the test of heterogeneity was statistically signifi-

cant when deleting a single included study alternately.

Test for potential publication bias

Publication bias was assessed by Begg's funnel plot and Egger's test. The results showed no evidence of publication bias in all tested models (homozygote comparison: P_{Begg}=0.76; heterozygote comparison: P_{Begg}=0.55; dominant model: P_{Begg}=0.45, recessive model P_{Begg}=0.806) (**Figure 5** showed the funnel plot of homozygote comparison).

Discussion

NO, as a potent vasorelaxant substance, play an important role in the pathogenesis of PH [25]. A state of reduced NO bioavailability is confirmed by decreased eNOS expression, oxidative stress and inhibition of NO synthesis in nearly all kinds of PH [26, 27]. Whether NOS3 4b/a polymorphism will interfere the function of eNOS, thus resulting in the reduction of nitric oxide output and the increasing risk of PH, is controversial [28].

This is the first meta-analysis to explorer the association of NOS3 4b/a polymorphism to the risk of PH. A total of six studies including 198 PH subjects and 250 controls were analyzed and an increasingsusceptibility of PH risk was found in the individuals with 4a/a genotype compared to the ones carrying genotype of 4b/a or 4b/b. Our study proved that the 4a/ahomozygote carriers raised the risk of PH at least 2.5 times as against their counterparts.

Subgroup analysis by ethnicity suggested that non-Caucasian population carriers with 4a/a gene might have high risk of PH. The risks increased 3.84 times as compared to the subjects with 4b/b gene.However, in Caucasian population, no significant difference was found in any of the four genetic models. This result indicated that discrepancy of genetic susceptibility of NOS3 4b/a polymorphism to risk of PH might exist between Caucasian population and non-Caucasian counterpart. More related studies with larger participants specialized for the Caucasian should be conducted to confirm this conclusion.

For heterogeneity, in overall analysis, the results showed no significant heterogeneity both in homozygote comparison and recessive model. Begg's funnel plot and Egger' test also showed no publication bias existed. All of the above indicated that the conclusion derived from this meta-analysis was of high credibility and reliability. However, in the heterogeneity comparison and dominant mode, significant heterogeneity was found. Subgroup analysis revealed that the heterogeneity mainly derived from the non-Caucasian population. The reasons were as follows: first, the individuals in this subgroup came from the different country where difference in genetic susceptibility might exist; second, the age scope of the included subjects varied obviously; third, different clinical classification of PH were include in this subgroup which might also contributed to the heterogeneity. Once again, more studies should be conducted to further clarify this problem.

In this meta-analysis, we included all eligible studies about the association between NOS 4b/a polymorphism and risk of HP. No language restriction was given when searching, and no publication bias was found in all the testing models. However, this meta-analysis should be interpreted within the context of its limitations. First, HP is a multifactorial disease. Several interfering factors, such as familial history or comorbidities, may affect the result, so individual data should be needed to achieve a precise estimate. Second, HP contains five clinical subtypes. Meta-analysis directed at one kind of PH classification should be done. Third, only six case-control studies were included, which is limited. More studies and more subjects were needed for a greater testing power [29]. Fourth, we only evaluated the association between the eNOS 4b/a polymorphism and PH; we did not evaluate other types of polymorphisms in this gene (such as eNOS T786C, eNOS G894T) or other targeted genes (such as endothelin-1 gene polymorphism, angiotensin converting enzyme I allele etc.) that might be associated with PH. Possibility is that the potential role of the eNOS 4b/a polymorphism is somewhat diluted, concealed or interfered by other gene-gene or gene-environment interactions. More studies are needed to explore this interfering effect.

In conclusion, an increasing susceptibility between eNOS 4b/a polymorphism and PH risk was found in this study. Individuals with the genotype of 4a/a have an obviously higher risk of developing PH than those with the genotype of 4b/b.

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Disclosure of conflict of interest

None.

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