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Review Article

Plasma total homocysteine and risk of hypertension in pregnancy: a mendelian randomization study

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Abstract: Background: Several studies had observed the levels of homocysteine (Hcy) increased in the patients with hypertension in pregnancy (HIP). However, as the problems of confounding and reverse causation, the results of the observational studies had become less compelling. Therefore, we performed a Mendelian randomization (MR) study to evaluate a causal relationship between plasma total Hcy and HIP in Chinese. Methods: We used the *MTHFR* C677T polymorphism influencing Hcy levels as an instrumental variable. Estimates of this polymorphism's effect on plasma total Hcy levels was based on a recent meta-analysis of genome-wide association studies comprising 44, 147 individuals. We conducted a meta-analysis to assess the risk estimate for the association of *MTHFR* C677T polymorphism with HIP in Chinese. All analyses were conducted using the STATA software. Results: Sixteen case-control studies, including 1,077 HIP cases and 1,111 controls were included in this meta-analysis to calculatethe risk estimate for the association of *MTHFR* C677T polymorphism with HIP in Chinese. Overall, the results indicated that the T allele of *MTHFR* C677T polymorphism was significantly associated with increased HIP risk in all genetic models (T vs. C: OR = 1.66, 95% CI 1.46-1.89, *P*<0.001; TT vs. CC: OR = 2.45, 95% CI 1.87-3.22, *P*<0.001; CT vs. CC: OR = 1.78, 95% CI 1.28-2.47, *P* = 0.001; TT/CT vs. CC: OR = 1.96, 95% CI 1.50-2.55, *P*<0.001; TT vs. CT/CC: OR = 1.87, 95% CI 1.49-2.34, *P*<0.001). The combined MR estimate showed a significant effect of the plasma total Hcy on HIP risk, representing an OR of 3.21 (95% CI = 2.36-4.07; $P = 7.4\times10^{-4}$) for HIP per 1-SD increase in the natural log-transformed plasma total Hcy levels. Conclusions: The present meta-analysis suggested that the T allele of *MTHFR* C677T polymorphism probably increased HIP risk in Chinese, and the MR study provided strong evidence of a causal relationship between the plasma total Hcy and HIP. The result was more advantageous for understanding the pathophysiological mechanism and treatment of HIP.

Keywords: Homocysteine, MTHFR, hypertension in pregnancy, mendelian randomization analysis

Introduction

Hypertension in pregnancy (HIP) is the most common pregnancy related complications affecting approximately 10% of pregnancies [1]. HIP is a major risk factor for perinatal mortality in the offspring of mothers and the cardiovascular disease of women worldwide [2, 3]. The pathophysiology of HIP characterized by blood flow changed is complex and multifactorial, and has not yet been clarified [4]. Homocysteine (Hcy), a key substance in the methionine cycle, has been reported to induce arteriolar constriction, increased sodium reabsorption, increase arterial stiffness and oxidative stress [5]. Recent studies have observed that plasma Hcy levels significantly increased in HIP and it was a risk factor for HIP [6-8]. However, due to confounding and reverse causation problems, the observational studies have difficult to distinguish between causal and spurious associations between Hcy and HIP.

5, 10 methylenetetrahydrofolatereductase (MTHFR) is the key rate-limiting enzyme that catalyzes the reduction of 5, 10 methylenetetrahydrofolate to 5-methytetrahydrololate, which is the essential carbon donor for the remethylation of Hcy to methionine [9]. The *MTHFR* gene is located on chromosome 1 at 1p36.6, and the C677T polymorphism is the well characterized genetic variant of this gene [10, 11]. Resear-

chers have demonstrated that the C677T of the *MTHFR* gene could result in alanine to valine substitution and thus reduce the enzyme activity, decrease folate levels, and subsequently cause heper-Hcy [9]. Recent a published meta-analysis of genome-wide association studies hasconfirmed the association between this variant and plasma total Hcy [12].

There are numerous epidemiological case-control studies have examined the association between *MTHFR* C677T polymorphism and HIP risk in Chinese [13-28]. Though, the findings were inconclusive or even contradictory which may be attributed to possible selection bias or small size from individual studies. In order to provide a more comprehensive assessment of the association between the C677T polymorphism and HIP risk in Chinese, we carried out a meta-analysis of all eligible studies. Furthermore, we conducted the Mendelian randomization (MR) analysis [29] based on *MTHFR* C677T polymorphism as an instrumental variablevia the result of above meta-analysis to evaluate the causal relationship between plasma total Hcy and HIP.

Method

Data on gene associations with HIP risk

To estimate the association of the *MTHFR* C677T polymorphism with HIP risk, we performed a meta-analysis of case-control studies. Eligible studies were identified using PubMed, Excerpta Medica Database (EMBASE), Chinese Biomedical Literature (CBM) and Chinese National Knowledge Infrastructure (CNKI) web databases with the combination of following terms "Methylenetetrahydrofolate reductase or *MTHFR*" and "gene polymorphism or allele or variation" and "hypertension in pregnancy or pregnancy induced hypertension or preeclampsia or eclampsia or gestational hypertension" updated until October 17, 2015. Reference lists of relevant articles were reviewed manually to look foradditional studies.

Studies included for further meta-analysis had to meet all of the following criteria: a) must investigated the association between *MTHFR* gene polymorphism and HIP risk, b) used a case-control study design, c) have available detail genotype frequencies in case and control groups. The major exclusion criteria were: a) overlapping data, b) abstract, review, comment and editorial, c) case-only studies, d) family or sibling pairs based studies, e) genotype frequencies or numbers of the subjects were unavailable, even contacting the corresponding author of the relevant articles. If there was more than one study published using the same patients population, only the complete design and larger sample size study would be selected in the meta-analysis.

Two reviewers independently extracted the following information from each qualified articleaccording to a fixed protocol: first author's surname, year of publication, region, source of control, mean age of cases and controls, diagnosis standard, genotyping method, matching criteria, sample size of genotyped cases and controls, genotype frequencies in case and control groups. Any disagreements on the data from the collected studies were fully debated with investigators toreachthe final consensus.

Data on gene associations with plasma total homocysteine

A pooled estimateper-T allele standardized β coefficient (0.158) of the effect of the *MTHFR* C677T polymorphism on the natural log-transformed plasmatotal Hcy levels was based on the findingsof a recent meta-analysis. The meta-analysis included 44,147 individualsderived from 10 genome-wide association studies.

Statistical analysis

Hardy-Weinberg equilibrium (HWE) of genotypes distribution in control groups weremeasuredvia Chi-square test and a P-value<0.05 was considered as significant disequilibrium [30]. Studies with controls not in HWE were subjectedto a sensitivity analysis. The pooled odds ratios (ORs) with their 95% confidence intervals (95% CIs) were calculated to evaluate the strength of the association between *MTHFR* C677T polymorphism and HIP risk based on different genetic models: allele model (T vs. C), homozygous model (TT vs. CC), heterozygous model (CT vs. CC), dominant model (TT/CT vs. CC), and recessive model (TT vs. CT/CC). The chi-square based Cochran's Q test and I^2 were carried out to assess the heterogeneity between studies across the eligible comparison [31]. Heterogeneity was considered to be

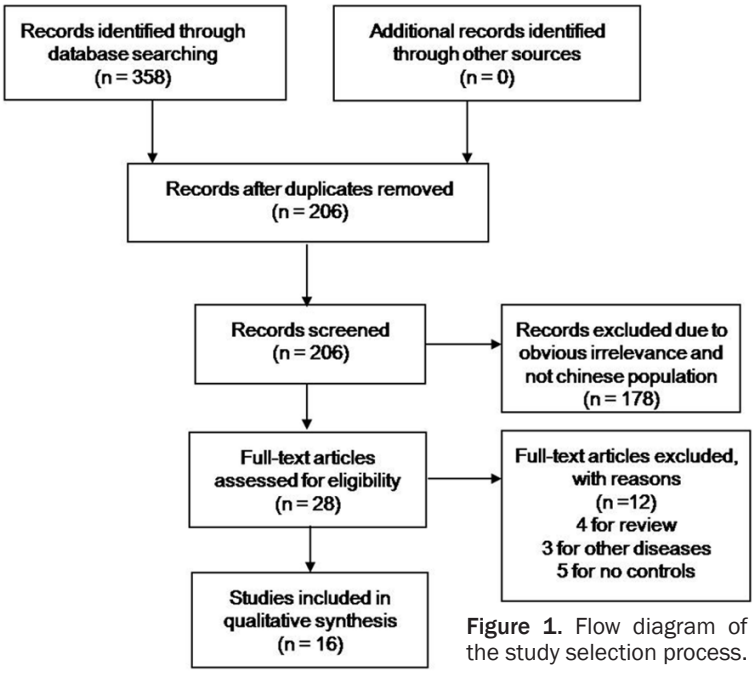

Figure 1. Flow diagram of the study selection process.

significant at *P*-value<0.10 and I^2 exceeding 50%, then the random-effects model (DerSimoniane-Laird method) was applied to calculate the pooled ORs; otherwise, the fixed-effects model (Mantel-Haenszel method) was used [32]. In order to evaluate the influence of each study on the overall estimate, we carried out sensitivity analysis by sequentially removing studies not in HWE. Finally, Egger's linear regression test was conducted to measure the funnel plot asymmetry, and *P*-value<0.05 was considered to be statistically significant publication bias [33].

We calculated a MR estimate ofthe effect of the plasma total Hcy levels on therisk of HIP ($OR_{HIP/hcy}$) as log $OR_{HIP/hcy}$ = (log$OR_{HIP/per\ T\text{-}allele}$)/ $beta_{hcy/per\ T\text{-}allele}$, as in previousstudies [34, 35]. Log $OR_{HIP/hcy}$ is the (log) increase of HIP risk by 1-standard deviation (SD) increasein the natural log-transformed plasma total Hcy (MR estimate, plasma total Hcy-HIP association). Log $OR_{HIP/per\ T\text{-}allele}$ is the (log) increase in HIP risk perT allele of the *MTHFR* C677T polymorphism (gene-HIP association). Beta $_{hcy/per\ T\text{-}allele}$ is thenumber of SD differences in the natural log-transformedplasma total Hcy levels per T allele (SD/allele) (gene-plasma total Hcy association). The standard error (SE) of the MR estimate was derivedusing the Delta method. The MR estimate is presented interms of OR, by exponentiating thelog $OR_{HIP/hcy}$. All *P* values were two sided, and all statistical analyses were conducted by using STATA statistical software (version 12.0; Stata Corp, College Station, Texas USA).

Results

Characteristics of eligible studies

358 relevant articles were identified after initial screening based on our search strategy. The flow chart in **Figure 1** illustrated the study selection procedures for *MTHFR* C677T polymorphism and HIP risk. After careful selection, finally there were a total of 16 case-control studies with 1,077 cases and 1,111 controls included in our meta-analysis. The main characteristics of each study were summarized in **Table 1**. Among the 16 applicable studies, 6 studies were carried out in south China and 10 in north China (demarcated by Qinling Mountains and Huaihe River). In view of control source, all of them were hospital-based. The genotyping method in all studies was polymerase chain reaction-restriction fragment length polymorphism. Distributions of genotypes in the controls and cases have been given in **Table 2**. As shown in **Table 2**, the genotype distributions in the controls in 11 studies were in agreement with HWE, and 5 studies were not in HWE.

Association of MTHFR C677T and HIPrisk

Overall, when all include studies were pooled into the meta-analysis, we found that the *MTHFR* C677T polymorphism was significantly associated with an increased HIP risk in all genetic models (T vs. C: OR = 1.66, 95% CI 1.46-1.89, *P*<0.001; TT vs. CC: OR = 2.45, 95% CI 1.87-3.22, *P*<0.001; CT vs. CC: OR = 1.78, 95% CI 1.28-2.47, *P* = 0.001; TT/CT vs. CC: OR = 1.96, 95% CI 1.50-2.55, *P*<0.001; TT vs. CT/CC: OR = 1.87, 95% CI 1.49-2.34, *P*<0.001). In the subgroup analysis stratified by region, the *MTHFR* C677T polymorphism was associated with HIP in both south and north populations (**Table 3**).

Table 1. Characteristics of the eligible studies in the meta-analysis

Author	Year	Region	Controls	Mean age of	Diagnosis	Genotyping	Matching criteria
			Source	Cases/controls	Standard	Method	Criteria
Li et al.	2000	Heilongjiang	HB	NR/NR	140/90	PCR-RFLP	NR
Wei et al.	2001	Heilongjiang	HB	26.8/25.7	140/90	PCR-RFLP	NR
Fu et al.	2003	Jiangxi	HB	26.3/25.7	140/90	PCR-RFLP	NR
Zhang et al.	2003	Tianjin	HB	28.5/25.4	140/90	PCR-RFLP	NR
Li et al.	2004	Heilongjiang	HB	28.7/27.8	140/90	PCR-RFLP	Gestational age
Niu et al.	2004	Tianjin	HB	29/25	140/90	PCR-RFLP	NR
Wang et al.	2004	Guangdong	HB	28.0/28.0	140/90	PCR-RFLP	Age, area
Tian et al.	2005	Guangdong	HB	NR/NR	140/90	PCR-RFLP	Ethnicity, age, area
Wang et al.	2006	Jiangsu	HB	27.9/27.4	140/90	PCR-RFLP	Ethnicity, area
Zhang et al.	2007	Shandong	HB	29.2/28.7	NR	PCR-RFLP	NR
Sun	2007	Liaoning	HB	27.5/25.4	NR	PCR-RFLP	NR
Ding et al.	2008	Shandong	HB	NR/NR	140/90	PCR-RFLP	NR
Wang et al.	2008	Shandong	HB	30.0/29.0	140/90	PCR-RFLP	Ethnicity, area
Zhang et al.	2008	Tianjin	HB	27.5/25.4	140/90	PCR-RFLP	Ethnicity, gestational age
Shen et al.	2009	Zhejiang	HB	27.9/29.7	140/90	PCR-RFLP	NR
Zhong et al.	2010	Zhejiang	HB	24.2/23.8	140/90	PCR-RFLP	Age, area

Abbreviation: HB, hospital based; PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism; NR, data not reported.

Table 2. Genotypic distribution of MTHFR C677T polymorphism

Author	Year	Sample size (case/control)	Genotype distribution						HWE
			Case			Control			
			CC	CT	TT	CC	CT	TT	
Li et al.	2000	57/120	9	30	18	44	58	18	0.876
Wei et al.	2001	42/36	23	13	6	20	11	5	0.118
Fu et al.	2003	102/100	24	53	25	46	40	14	0.277
Zhang et al.	2003	73/74	6	45	22	12	43	19	0.137
Li et al.	2004	82/90	28	36	18	46	32	12	0.106
Niu et al.	2004	73/74	6	45	22	12	43	19	0.137
Wang et al.	2004	99/54	53	31	15	25	24	5	0.824
Tian et al.	2005	61/56	34	20	7	40	12	4	0.044
Wang et al.	2006	54/125	26	22	6	89	27	9	0.003
Zhang et al.	2007	53/49	12	21	20	10	30	9	0.115
Sun	2007	50/40	22	21	7	29	8	3	0.052
Ding et al.	2008	92/89	20	37	35	18	55	16	0.026
Wang et al.	2008	42/64	6	19	17	13	40	11	0.044
Zhang et al.	2008	50/40	22	21	7	29	8	3	0.052
Shen et al.	2009	80/60	20	42	18	30	21	9	0.117
Zhong et al.	2010	67/40	24	32	11	26	8	6	0.003

Abbreviation: HWE: Hardy-Weinberg equilibrium.

Heterogeneity analysis

In order to assess the heterogeneity among the included studies, Q-test and I^2 were carried out. There was no significant heterogeneity observed in allelic comparison (T vs. C), homozygote model (TT vs. CC) and recessive model (TT vs. CT/CC) for overall analysis (P_Q>0.10 for all), and fixed effects model was applied to synthesize the data. However, significant heterogeneity was observed in heterozygote model (CT vs. CC) and dominant model (TT/CT vs. CC) for overall analysis (both P_Q<0.10). Thus we conducted subgroup analyses to explore the source of heterogeneity. Although the results of subgroup analyses showed the heterogeneity was still significant in south populations (CT vs. CC: P_Q = 0.08; TT/CT vs. CC: P_Q = 0.026), the heterogeneity decreased obviously in north populations (TT/CT vs. CC: P_Q = 0.158) (**Table 3**).

Sensitivity analysis and publication bias

As there were six studies included in our meta-analysis not in agreement with HWE, we performed the sensitivity analysis by omittingthese studies for all genetic models to assess the

Table 3. Meta-analysis of DICER rs1057035 polymorphism and cancer risk

Comparisons		N[b]	Test of association			Test of heterogeneity	
			OR (95% CI)	*P* value	Model	*P* value	I^2 (%)
Overall	T vs. C	16	1.66 (1.46-1.89)	<0.001	F	0.403	4.4
	TT vs. CC	16	2.45 (1.87-3.22)	<0.001	F	0.972	0.0
	CT vs. CC	16	1.78 (1.28-2.47)	0.001	R	0.002	57.6
	TT/CT vs. CC	16	1.96 (1.50-2.55)	<0.001	R	0.033	43.5
	TT vs. CT/CC	16	1.87 (1.49-2.34)	<0.001	F	0.898	0.0
Overall for HWE[a]							
	T vs. C	11	1.63 (1.40-1.89)	<0.001	F	0.178	28.0
	TT vs. CC	11	2.56 (1.85-3.54)	<0.001	F	0.847	0.0
	CT vs. CC	11	1.81 (1.23-2.65)	0.002	R	0.016	54.1
	TT/CT vs. CC	11	1.98 (1.42-2.78)	<0.001	R	0.034	48.8
	TT vs. CT/CC	11	1.75 (1.34-2.28)	<0.001	F	0.921	0.0
Region							
South	T vs. C	6	1.75 (1.42-2.15)	<0.001	F	0.259	23.3
	TT vs. CC	6	2.42 (1.58-3.71)	<0.001	F	0.857	0.0
	CT vs. CC	6	2.14 (1.24-3.71)	0.007	R	0.008	67.8
	TT/CT vs. CC	6	2.17 (1.37-3.42)	0.001	R	0.026	60.7
	TT vs. CT/CC	6	1.67 (1.12-2.48)	0.011	F	0.978	0.0
North	T vs. C	10	1.61 (1.37-1.89)	<0.001	F	0.460	0.0
	TT vs. CC	10	2.48 (1.75-3.51)	<0.001	F	0.879	0.0
	CT vs. CC	10	1.56 (1.03-2.36)	0.036	R	0.036	49.8
	TT/CT vs. CC	10	1.83 (1.40-2.38)	<0.001	F	0.158	31.3
	TT vs. CT/CC	10	1.97 (1.50-2.59)	<0.001	F	0.603	0.0

[a]Data after excluding those studies' controls not in Hardy-Weinberg equilibrium; [b]number of sutdies; R: random-effects model; F: fixed-effects model.

Table 4. Egger's linear regression test to measure the funnel plot asymmetric

Comparisons	Egger's regression analysis		
	Intercept	95% CI	*P*-value
T vs. C	0.81	-2.41-4.03	0.597
TT vs. CC	-1.04	-3.06-0.97	0.285
CT vs.CC	0.14	-4.68-4.96	0.952
TT/CT vs. CC	-0.12	-4.27-4.02	0.950
TT vs. CT/CC	-0.57	-2.36-1.21	0.499

stability of the current analysis. As shown in **Table 3**, after removal of the studies not in HWE, the pooled ORs were similar in all genetic models (**Table 3**). The sensitivity analysis suggested that the results of the meta-analysis were reliable enough. Funnel plot and Egger's linear regression test were performed to assess the potential publication bias among the included articles. The shape of the funnel plots were seemed symmetrical in all genetic models of this study (not shown). And the results of Egger's linear regression test also showed no publication bias (**Table 4**).

Mendelian randomization analysis for the association of Hcy with HIP risk

Under the principles of MR, we combined two pooled estimates, $OR_{HIP/per\ T\text{-}allele}$ from our current meta-analysis and $beta_{hcy/per\ T\text{-}allele}$ from the meta-analysis of genome-wide association studies by van Meurs and colleagues. The results indicated that a significant effect of the plasma total Hcy on HIP risk in the MR analysis, representing an $OR_{HIP/hcy}$ of 3.21 (95% CI = 2.36-4.07; $P = 7.4\times10^{-4}$) for HIP per 1-SD increase in the natural log-transformed plasma total Hcy levels (**Figure 2**).

Discussion

Extending the finding of a recent meta-analysis of genome-wide association studies by van Meurs and colleagues [12], we firstly performed a meta-analysis enrolled sixteen case-control studies, including 1,077 HIP cases and 1,111 controls to explore the association between the *MTHFR* C677T polymorphism and HIP risk in Chinese, and the result demonstrated that *MTHFR* C677T polymorphism T allele conferred a increased HIP risk. Moreover, the most noteworthy work was that we conducted a MR analysis using *MTHFR* gene C677T polymorphism as an instrument variable, and the findings supported that elevated plasma Hcy was a causal risk factor for the development of HIP in Chinese. To the authors' knowledge, this study is to date the first to address the causal relevance between plasma Hcy and HIP risk in Chinese.

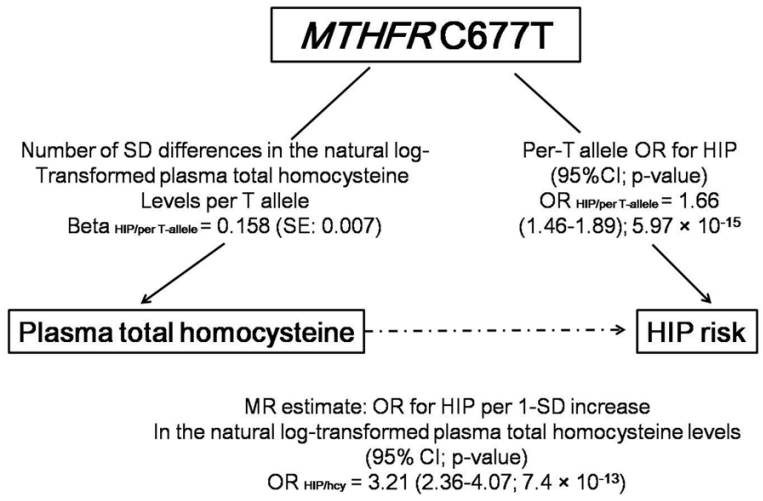

Figure 2. Graphical representation of the Mendelian randomization approach.

Although two meta-analysis were conducted by Niu et al. [36] and Yang et al. [37] to assess the association of *MTHFR* C677T polymorphism with HIP risk. That seemed our meta-analysis study was overrepresented. However, there were a number of advantages of our research over that in Niu et al.'s and Yang et al.'s study. First, our meta-analysis included a total of sixteen Chinese populations with 2188 samples, which enrolled more Chinese studies and subjects than the aforementioned studies. Second, there were studies not in agreement with HWE and we additionally applied sensitivity analysis by omitting these studies for all genetic models to assess the stability of the current analysis. However, that was not performed in Niu et al.'s study and Yang et al.'s study. Third, compared with Yang et al.'s study, our meta-analysis focused on Chinese population and which could avoid possible genetic background noise arising from different ethnic populations. Therefore, our meta-analysis could provide additional precision to characterize the association of the *MTHFR* C677T polymorphism with HIP in Chinese. In overall our meta-analysis, the T allele of MTHFR C677T polymorphism was significantly associated with increased HIP risk in Chinese. The T allele of MTHFR C677T polymorphism could result in alanine to valine substitution and thus reduce the enzyme activity, decrease folate levels, and subsequently cause heper-Hcy, and which contributed to increase the risk of HIP.

Researchers have confirmed that HIP was a major risk factor for women to develop cardiovascular disease later in life [38]. Theresult may be contributed to the hyper-Hcy. Numerous studies have observed that plasma Hcy levels significantly increased in HIP [6-8]. Several observational and clinical studies also have demonstrated a positive association between plasma Hcy and cardiovascular disease [39-41]. However, an update of Cochrane Review found no evidence to suggest homocysteine-reducing strategies in the form of supplements of vitamins B6, B9 or B12 should be used for preventing cardiovascular events [42]. The discordant results from observational studies and randomized trials might be caused by confounding and reverse causation problems. Fortunately, MR analysis could overcome limitations of observational studies and obtain robust causal estimates [43]. As a published meta-analysis of genome-wide association studies has revealed that the presence of T allele of *MTHFR* C677T polymorphism was associated with significantly increased plasma total Hcy [12], we selected the *MTHFR* C677T polymorphism as a surrogate marker to perform the MR analysis. Our results confirmed that elevated plasma Hcy was a causal risk factor for the development of HIP in Chinese. The biological rationale for implicating hyper-Hcy in HIP is based on the fact that hyper-Hcy could induce arteriolar constriction, increased sodium reabsorption, increase arterial stiffness and oxidative stress, further resulting in HIP [5].

Despite the significant findings from our current analysis, some limitations should also be acknowledged. First, although we included sixteen studies with 2188 subjects to investigate the association of *MTHFR* C677T polymorphism with HIP, the sample sizes were still not huge enough to provide adequate statistical power. Second, there were other important genetic polymorphisms could affect the plasma total Hcy levels according a recent meta-analysis of genome wide association studies. How-

ever, we justly used the *MTHFR* C677T polymorphism as the instrumental variable. Therefore, multiple genetic variants involved in Hcy regulation should be selected as the multiple instruments to increase the precision of the instrumental variable estimates. Third, our MR analysis could not avoid a pleiotropic effect [44], as the MTHFR C677T polymorphism may directly influence more than one post-transcriptional process. Four, we estimated the gene-HIP association using the Chinese population. However, the gene-plasma total Hcy association was assessed in mixed populations.

In conclusion, our study provided evidence that the T allele of *MTHFR* C677T contributed to HIP risk, and supported that increased plasma total Hcy was a causal risk factor for the development of HIP in Chinese. However, our findings have to be interpreted with caution because of the above limitations.

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Disclosure of conflict of interest

None.

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