Original Article

Plasma homocysteine levels and risk of vascular dementia: a Mendelian randomization study

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Abstract: Observational studies have demonstrated an association between elevated homocysteine (Hcy) level and risk of vascular dementia (VaD); however, it remains unresolved whether this relationship is causal. We carried out a Mendelian randomization (MR) study to evaluate whether genetically increased Hcy level influences the risk of VaD. We used the methylene tetrahydrofolate reductase (MTHFR) C677T polymorphism as an instrumental variable, which affects the plasma Hcy levels. Estimate of its effect on plasma Hcy was based on a recent genome-wide meta-analysis of 44147 individuals, while estimate of its effect on VaD risk was obtained through meta-analysis of case-control studies with 722 cases and 1158 controls. By combining these two estimates, we found that per 1 standard-deviation (SD) increased in natural log-transformed plasma Hcy levels conferred a 4.29-fold increase in risk for VaD (95% CI: 1.11-16.57, P = 0.034). Our study suggests that elevated Hcy levels are causally associated with an increased risk of developing VaD. Whether Hcy-lowering therapy can prevent VaD merits further investigation in long-term randomized controlled trials.

Keywords: Homocysteine, vascular dementia, mendelian randomization, methylene tetrahydrofolate reductase

Introduction

Vascular dementia (VaD) is the second leading cause of dementia after Alzheimer’s disease by accounting for 15-20 percent of all dementia cases in the world [1]. Vascular dementia is thought to be irreversible and it is caused by cerebrovascular diseases including stroke, diabetes, and hypertension which result in the impairment of specific brain regions involving memory and cognitive function [2]. The incidence of VaD increases with age rapidly, rising from 1.5% at age 70-75 years to 15% at age 80 years and above, which lead to largely irreversible deterioration in patients’ quality of life and increased economic burden of their families [3]. Because treatments for VaD are limited, the best approach to reduce mortality and morbidity is primary prevention through modification of acquired risk factors.

Homocysteine (Hcy) is a key metabolite in one-carbon metabolism. Hcy levels could influence several cellular processes including DNA methylation and synthesis of nucleic acids and proteins [4]. A common functional polymorphism, C677T (rs1801133), in the gene encoding methylene tetrahydrofolate reductase (MTHFR), an enzyme involved in homocysteine metabolism, has been associated with differences in homocysteine concentration [5-7]. The association of this variant with the plasma homocysteine was confirmed by a recent meta-analysis of genome-wide association studies [8].

Data from case-control studies showed a trend for higher Hcy levels in VaD patients as compared to healthy controls and AD patients [9, 10]. However, meta-analysis of four prospective studies, with a total of 2631 participants, did not support a causal relationship between high Hcy level and risk of developing dementia [11]. The discordant results might be caused by the limited period of the trials, confounding factors, or reverse causation.

In the absence of evidence from high-quality randomized controlled trials (RCTs), the principles of Mendelian randomization (MR) can be
utilized to strengthen or refute the causality of biomarkers in disease etiology [12]. Mendelian randomization (MR) is a study design in which genetic variants are used as instrumental variables for estimating the unconfounded effect of an exposure (for example, Hcy) on a disease (for example, stroke) [13]. This approach is based on the principle that genetic variants are assigned randomly when passed from parents to offspring during meiosis, and consequently these genetic variants are independent of many factors that bias observational studies, such as confounding and reverse causation. MR methods have been used previously to investigate the influence of Type 2 Diabetes (T2D) and fasting glucose (FG) on CHD risk, which demonstrate a causal relationship between T2D and CHD [14]. MR approach may be of particular significance for understanding the etiology of VaD since the date of disease onset is often poorly discerned clinically and MR studies assess the effect of lifelong exposures.

MR analyses using MTHFR C677T polymorphism as an instrument variable have been carried out in the past. The researchers provided evidence from MR that plasma Hcy level is causally related to stroke, T2D, schizophrenia and offspring birthweight [13, 15-17]. In the present study, we used this polymorphism as an instrumental variable to obtain MR estimate of the effect of Hcy on VaD.

Materials and methods

Data on gene association with VaD risk

To estimate the association of the MTHFR C677T polymorphism with VaD risk, we performed a meta-analysis of case-control studies. Eligible studies were identified using PubMed, Embase and China Biological Medicine electronic databases before May 1, 2016. The search terms and keywords used were as follows: “methylene tetra hydro folate reductase” or “MTHFR”, “vascular dementia” or “VaD” or “VD”, and “polymorphism” or “variant” or “mutation” or “genotype” or “allele” or “SNP”, without any restriction on the language. Reference lists of relevant articles were reviewed manually to look for additional studies. For inclusion, studies had to meet the following criteria: (1) evaluation for the association between MTHFR C677T polymorphism and VaD; (2) studies were designed as the case-control type; (3) genotype frequencies for both cases and controls were available. Studies were excluded if: (1) no detailed genotype frequency; and (2) case reports, family-based studies, abstracts, editorials and review articles. When multiple publications reported the same population, only the most recent one with the largest sample sets was selected for this meta-analysis. Two authors selected the articles independently according to the above criteria. Any uncertainty regarding the eligibility was adjudged by further joint inspection of the publications.

The following data were independently extracted by two investigators from each eligible article according to a fixed protocol: first author’s name, publication year, country and ethnicity of population, genotyping methods, source of control, matching status, number of cases and controls, genotype distributions in cases and controls and the Hardy-Weinberg Equilibrium (HWE) in controls (P value). If these were not possible, the authors of the publications were contacted via E-mail for more detailed data.

The methodological quality of the included studies was assessed by two authors respectively according to the Newcastle Ottawa Scale (NOS) (www.ohri.ca/programs/clinical_epidemiology/oxford.asp) [18]. The NOS criteria consist of three aspects: selection, comparability and exposure. Scores ranged from 0 stars (worst) to 9 stars (best) and a score ≥ 7 indicated that a study was of high quality.

Data on gene association with Hcy

Estimate of the effect sizes of the MTHFR C677T polymorphism on the plasma Hcy levels was based on the findings of a recent GWAS meta-analysis [8]. The meta-analysis included data from a total of 44,147 white individuals of European ancestry derived from 10 GWAS on Hcy levels.

Statistical analysis

Hardy-Weinberg equilibrium (HWE) of genotype distribution in the control group was checked by the x²-test and P < 0.05 was considered as significant disequilibrium. Studies with controls not in HWE were subjected to a sensitivity analysis. The pooled odds ratios (ORs) with their 95% confidence intervals (95%
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Figure 1. Flow chart of the search strategy and study selection. The terms “N” in the boxes represent the number of corresponding studies.

CIs) were calculated to evaluate the strength of the association between MTHFR C677T polymorphism and VaD 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). Statistical heterogeneity between eligible studies was evaluated by using the Cochran’s Q statistic and I² test [19]. P < 0.1 and I² exceeding 50% indicated substantial heterogeneity across studies, then a random-effects model was chosen to perform meta-analysis, otherwise, the fixed-effects model was selected. Subgroup analyses were performed according to ethnicity (Asian and Caucasian), source of control (population-based and hospital-based), quality score (low quality: score < 7; high quality: score ≥ 7) and matched status. A power calculation was performed using Power and Sample Size Calculation version 3.1.2 (http://biostat.mc.vanderbilt.edu/twiki/bin/view/Main/PowerSampleSize). Begg’s funnel plot and Egger’s regression test were used to search for publication bias and a P value > 0.05 suggests no significant publication bias have been detected [20]. The fail-safe number (Nfs) at a significance of 0.05 was also calculated to inspect publication bias, according to the formula 

\[ N_{fs,0.05} = (\sum Z/1.64)^2 - k \]

where k is the number of studies included. If the Nfs was less than the number of observed studies for a polymorphism, we deemed that there exists a significant publication bias.

We calculated a MR estimate of the effect of the plasma Hcy levels on the risk of VaD (OR\textsubscript{VaD/Hcy}) as

\[ \text{Log OR}_{\text{VaD/Hcy}} = \left( \log \text{OR}_{\text{VaD/per T-allele}} / \beta_{\text{Hcy/per T-allele}} \right) \]

as in previous studies [21, 22]. Log OR\textsubscript{VaD/Hcy} is the (log) increase of VaD risk per SD unit increase in the natural log-transformed plasma Hcy (MR estimate). Log OR\textsubscript{Hcy/per T-allele} is the (log) increase in VaD risk per allele (gene-VaD association). Beta\textsubscript{Hcy/per T-allele} is the number of SD differences in the natural log-transformed plasma Hcy levels per allele (SD/allele) (gene-Hcy association). The standard error of the MR estimate was derived using the Delta method [23]. The MR estimate is presented in terms of OR, by exponentiating the Log OR\textsubscript{VaD/Hcy}. All P values were two sided. All above statistical analyses were performed using STATA software version 12.0 (STATA Corporation, College Station, TX, USA).

**Results**

**Summary statistics**

The process of literature retrieval and exclusion was shown in Figure 1. The initial comprehensive search generated a total of 67 potentially relevant articles, 20 articles were excluded for duplication, and 23 additional articles were excluded for their unmatched titles or abstracts. After reading the full text of the remaining 24 articles, 12 articles were removed due to review, meeting abstract, study with insufficient data and case only study. Finally, 12 stud-
ies with a total of 722 cases and 1158 controls, were included in our meta-analysis [24-35]. Detailed characteristics and genotype distributions of included studies were summarized in Table 1. The distribution of the genotypes in the control group was consistent with HWE. The number of cases among all selected studies varied from 6 to 143, while the numbers of controls varied from 33 to 217. All the studies included met quality criteria ranging from 6 to 8.

Association of MTHFR C677T polymorphism with VaD risk

The main results of meta-analysis and heterogeneity test were summarized in Table 2. Overall, the pooled results showed a significant association between MTHFR C677T polymorphism and the risk of VaD under allele model (T vs C: OR = 1.26, 95% CI = 1.02-1.56), homozygous model (TT vs CC: OR = 1.48, 95% CI = 1.08-2.02) and recessive model (TT vs CT + CC: OR = 1.41, 95% CI = 1.06-1.87) (Figure 2). If we set α = 0.05, based on the data set for 677T allele, we have a 90.6% power to detect an OR of 1.26. When stratified by ethnicity, a significant association was also found in Asian population (T vs C: OR = 1.40, 95% CI = 1.15-1.70; TT vs CC: OR = 2.03, 95% CI = 1.33-3.11; TT vs CT + CC: OR = 1.93, 95% CI = 1.31-2.82; TT + CT vs CC: OR = 1.37, 95% CI = 1.05-1.79), while a null result was noted in the Caucasian population under all genetic models. In the subgroup analysis stratified by control source and matched status, similar trends with overall results were observed in HB and no matched subgroups. With regard to quality score, there were no significant findings observed under any genetic models in low-quality studies (quality score < 7) or in high-quality studies (quality score ≥ 7). Moderate heterogeneity was observed under allele, heterozygous and dominant models (I² = 47.4%, I² = 47.5%, I² = 48.1%, respectively). In the subgroup analysis, heterogeneity vanished in HB subgroup as well as dramatically decreased in Asian subgroup, high-quality studies and matched subgroup.

Mendelian randomization analysis for the association of Hcy with VaD risk

Under the principles of Mendelian randomization, we observed that each 1-SD increase in the natural log-transformed plasma Hcy level was significantly associated with a 4.29-fold increased risk of VaD (95% CI: 1.11-16.57, P = 0.034) (Figure 3). Considering that the null hypothesis value of unity was not covered by derived 95% CIs for predicted estimate, it is safe to reject the null hypothesis of none causal relationship between plasma Hcy level and VaD.

Sensitivity analysis and publication bias

The leave-one-out sensitivity analysis showed that no single study qualitatively changed the summary ORs (data not shown). Begg’s funnel plot and Egger’s test were performed to evaluate the potential publication bias of literatures.

<table>
<thead>
<tr>
<th>First Author</th>
<th>Year</th>
<th>Country</th>
<th>Ethnicity</th>
<th>Genotyping Methods</th>
<th>Source of control</th>
<th>Matched Variables</th>
<th>Sample Size (Case/Control)</th>
<th>HWE (P value)</th>
<th>Quality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bottiglieri</td>
<td>2001</td>
<td>Italy</td>
<td>Caucasian</td>
<td>PCR-SSCP</td>
<td>HB</td>
<td>NA</td>
<td>6/36</td>
<td>0.769</td>
<td>6</td>
</tr>
<tr>
<td>Chapman</td>
<td>1998</td>
<td>Israel</td>
<td>Caucasian</td>
<td>PCR-SSCP</td>
<td>Age</td>
<td>41/40</td>
<td>7 20 14 9 16 15</td>
<td>0.251</td>
<td>8</td>
</tr>
<tr>
<td>Mclroy</td>
<td>2002</td>
<td>Ireland</td>
<td>Caucasian</td>
<td>PCR</td>
<td>PB</td>
<td>NA</td>
<td>76/71</td>
<td>0.904</td>
<td>6</td>
</tr>
<tr>
<td>Pollak</td>
<td>2000</td>
<td>Israel</td>
<td>Caucasian</td>
<td>PCR-SSCP</td>
<td>PB</td>
<td>Ethnicity</td>
<td>85/82</td>
<td>0.501</td>
<td>8</td>
</tr>
<tr>
<td>Wehr</td>
<td>2006</td>
<td>Poland</td>
<td>Caucasian</td>
<td>PCR-SSCP</td>
<td>PB</td>
<td>NA</td>
<td>65/141</td>
<td>0.360</td>
<td>6</td>
</tr>
<tr>
<td>Zuliani</td>
<td>2001</td>
<td>Italy</td>
<td>Caucasian</td>
<td>PCR-SSCP</td>
<td>PB</td>
<td>NA</td>
<td>60/54</td>
<td>0.627</td>
<td>6</td>
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<td>Nishiyama</td>
<td>2000</td>
<td>Japan</td>
<td>Asian</td>
<td>PCR-SSCP</td>
<td>PB</td>
<td>NA</td>
<td>35/33</td>
<td>0.845</td>
<td>7</td>
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<tr>
<td>Pandey</td>
<td>2009</td>
<td>India</td>
<td>Asian</td>
<td>PCR-SSCP</td>
<td>Age</td>
<td>80/170</td>
<td>2 29 49 7 45 118</td>
<td>0.315</td>
<td>8</td>
</tr>
<tr>
<td>Sun</td>
<td>2014</td>
<td>China</td>
<td>Asian</td>
<td>PCR-RFLP</td>
<td>PB</td>
<td>NA</td>
<td>52/56</td>
<td>0.254</td>
<td>6</td>
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<tr>
<td>Wu</td>
<td>2006</td>
<td>China</td>
<td>Asian</td>
<td>PCR-RFLP</td>
<td>PB</td>
<td>NA</td>
<td>29/138</td>
<td>0.383</td>
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<td>Yoo</td>
<td>2000</td>
<td>Korea</td>
<td>Asian</td>
<td>PCR-RFLP</td>
<td>Age, gender</td>
<td>143/217</td>
<td>36 58 49 26 114 77</td>
<td>0.099</td>
<td>8</td>
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<tr>
<td>Mansoori</td>
<td>2012</td>
<td>India</td>
<td>Asian</td>
<td>PCR-SSCP</td>
<td>HB</td>
<td>Age, gender</td>
<td>50/120</td>
<td>0.836</td>
<td>8</td>
</tr>
</tbody>
</table>

NA = not available, PCR = polymerase chain reaction, PCR-RFLP = polymerase chain reaction-restriction fragment length polymorphism, PCR-SSCP = polymerase chain reaction-single strand conformation polymorphism, HWE = Hardy-Weinberg equilibrium, PB = population-based, HB = hospital-based.
## Table 2. Meta-analysis of the association between MTHFR C677T polymorphism and VaD risk

<table>
<thead>
<tr>
<th>Groups</th>
<th>N</th>
<th>Allele model OR (95% CI)</th>
<th>P\text{het}</th>
<th>Homozygous model OR (95% CI)</th>
<th>P\text{het}</th>
<th>Heterozygous model OR (95% CI)</th>
<th>P\text{het}</th>
<th>Recessive model OR (95% CI)</th>
<th>P\text{het}</th>
<th>Dominant model OR (95% CI)</th>
<th>P\text{het}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall</td>
<td>12</td>
<td>1.26 (1.02-1.56)</td>
<td>0.034</td>
<td>1.48 (1.08-2.02)</td>
<td>0.160</td>
<td>1.27 (0.93-1.74)</td>
<td>0.034</td>
<td>1.41 (1.06-1.87)</td>
<td>0.179</td>
<td>1.32 (0.98-1.78)</td>
<td>0.031</td>
</tr>
<tr>
<td>Ethnicity</td>
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</tr>
<tr>
<td>Caucasian</td>
<td>6</td>
<td>1.12 (0.75-1.70)</td>
<td>0.009</td>
<td>1.01 (0.63-1.62)</td>
<td>0.169</td>
<td>1.22 (0.73-2.02)</td>
<td>0.058</td>
<td>0.95 (0.62-1.47)</td>
<td>0.353</td>
<td>1.20 (0.70-2.08)</td>
<td>0.017</td>
</tr>
<tr>
<td>Asian</td>
<td>6</td>
<td>1.40 (1.15-1.70)</td>
<td>0.826</td>
<td>2.03 (1.33-3.11)</td>
<td>0.769</td>
<td>1.33 (0.86-2.05)</td>
<td>0.069</td>
<td>1.93 (1.31-2.82)</td>
<td>0.643</td>
<td>1.37 (1.05-1.79)</td>
<td>0.249</td>
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<tr>
<td>Control source</td>
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</tr>
<tr>
<td>HB</td>
<td>3</td>
<td>1.33 (1.02-1.74)</td>
<td>0.891</td>
<td>2.14 (1.20-3.82)</td>
<td>0.898</td>
<td>0.93 (0.62-1.38)</td>
<td>0.528</td>
<td>2.33 (1.39-3.91)</td>
<td>0.811</td>
<td>1.12 (0.77-1.63)</td>
<td>0.788</td>
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<tr>
<td>PB</td>
<td>9</td>
<td>1.25 (0.94-1.66)</td>
<td>0.009</td>
<td>1.26 (0.87-1.84)</td>
<td>0.111</td>
<td>1.38 (0.94-2.02)</td>
<td>0.028</td>
<td>1.13 (0.80-1.60)</td>
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<td>1.38 (0.93-2.05)</td>
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<td>Quality score</td>
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<tr>
<td>High</td>
<td>7</td>
<td>1.18 (0.99-1.42)</td>
<td>0.430</td>
<td>1.38 (0.94-2.03)</td>
<td>0.196</td>
<td>1.08 (0.83-1.41)</td>
<td>0.635</td>
<td>1.27 (0.72-2.23)</td>
<td>0.057</td>
<td>1.15 (0.89-1.47)</td>
<td>0.836</td>
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<td>Low</td>
<td>5</td>
<td>1.44 (0.87-2.39)</td>
<td>0.007</td>
<td>1.67 (0.97-2.88)</td>
<td>0.151</td>
<td>1.64 (0.76-3.51)</td>
<td>0.005</td>
<td>1.36 (0.82-2.24)</td>
<td>0.597</td>
<td>1.70 (0.78-3.67)</td>
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</tr>
<tr>
<td>Yes</td>
<td>5</td>
<td>1.13 (0.93-1.38)</td>
<td>0.330</td>
<td>1.23 (0.80-1.90)</td>
<td>0.124</td>
<td>1.07 (0.81-1.42)</td>
<td>0.477</td>
<td>0.99 (0.44-2.22)</td>
<td>0.026</td>
<td>1.11 (0.85-1.45)</td>
<td>0.764</td>
</tr>
<tr>
<td>NR</td>
<td>7</td>
<td>1.45 (1.02-2.06)</td>
<td>0.025</td>
<td>1.80 (1.14-2.84)</td>
<td>0.309</td>
<td>1.48 (0.84-2.61)</td>
<td>0.014</td>
<td>1.54 (1.02-2.33)</td>
<td>0.710</td>
<td>1.60 (0.92-2.80)</td>
<td>0.008</td>
</tr>
</tbody>
</table>

N = Number of studies; P\text{het} = P value for heterogeneity test. The OR values with statistical significance were shown in bold.
The shape of the funnel plot showed no evidence of obvious asymmetry (Figure 4). The Egger’s test result did not support the existence of publication bias (allele model, $P = 0.565$). The $N_{0.05}$ value was 68, which is greater than the number of studies included in this meta-analysis, implying a low probability of publication bias.

**Discussion**

Using summary-level data for VaD and Hcy levels, our study demonstrated that a genetic increase in natural log-transformed plasma Hcy by 1 SD was associated with a 4.29-fold increased risk of VaD, providing strong evidence in support of a causal role of Hcy in VaD susceptibility. Since genetic effects on Hcy levels represent differences that generally persist throughout adult life, the estimate of our MR study reflects an effect of Hcy over the course of a lifetime. These findings are consistent with evidence from observational studies that have shown that high levels of plasma Hcy influence risk of VaD [9, 10]. To the authors’ knowledge, this report is the first to provide evidence for putative causal nature of the association between plasma Hcy and VaD.
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In the present study, risk estimates of \textit{MTHFR} C677T variant with VaD were heterozygous between Asians and Caucasians. Considering the multifactorial nature of VaD, divergent genetic backgrounds or linkage disequilibrium patterns might be the most likely explanation for such divergence. The finding suggests a potential implication for genotyping \textit{MTHFR} C677T variant in VaD risk appraisal among Asians.

A reasonable first step to understanding the role of Hcy therapy in delaying the onset or severity of VaD would be to treat high Hcy level in those most at risk of developing VaD. VaD is the type of dementia caused by problems in the supply of blood to the brain, typically by a series of stroke [36]. The symptoms of VaD may progress gradually or step-wise after each stroke, thereby providing a therapeutic window and rationale for intervening with Hcy reduction. Future RCTs are required to validate the therapeutic approach (for example, supplementation with folic acid and vitamin B12) to prevent VaD, and may therefore provide needed insights into the role of Hcy reduction. An important difference between MR studies and RCTs is that MR studies describe the effect of lifetime exposure to Hcy-increasing allele in the general population, whereas RCTs provide insights from intervention for shorter periods (generally less than a decade) in individuals at risk. Thus, it is possible that RCTs designed to test Hcy lowering may need considerably long-term follow-up to adequately assess the effect of these interventions on VaD.

Our analysis has several strengths. First, because of the random allocation of genotype in advance of disease development, these results indicate that the relation between Hcy concentration and VaD is not subject to potential confounding or reverse causality bias. Second, using data from the largest GWAS meta-analysis for Hcy level (n = 44147), and from the current meta-analysis for VaD risk (up to 722 cases and 1158 controls) have enabled us to more precisely examine our study hypothesis than if we had employed individual-level data from a small study. Thirdly, the findings from this study represent the relationship of a lifetime exposure to elevated Hcy levels with VaD.
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References


A few limitations of our study merit consideration. Firstly, MR estimates which utilize instrumental variables accounting for little variance in a trait tend to be biased towards the null [37]. In this study, we used only one genetic variant as the instrumental variable that influences the plasma Hcy levels. However, such bias does not seem to have affected either the direction or significance of the results of this study since our MR analysis suggests a positive association between Hcy and VaD. Secondly, canalization, the process by which compensatory feedback mechanisms attenuate the phenotypic consequences of genetic variation, has been extensively investigated in the context of MR [12, 38, 39]. Although compensatory feedback interactions tend to bias results towards the null, the presence of this mechanism would not alter the statistical significance or direction of the effects we identify through MR. Thirdly, it seems impractical for us to exclude the pleiotropy of MTHFR C677T polymorphism since data on other clinical parameters across C677T genotypes are rarely provided from most qualified literatures, requiring further confirmation. Finally, considering the differences in minor allele frequencies between populations and other demographic characteristics in the included studies, it is hard to ignore an impact of population stratification, but significant results in the meta-analysis argue against stratification.

In summary, our MR study suggests that genetically increased Hcy level is causally associated with an increased risk of developing VaD. These findings provide rationale for further exploring the potential therapeutic benefits of Hcy-lowering in preventing the onset and progression of VaD.

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Disclosure of conflict of interest

None.
Plasma homocysteine levels and risk of vascular dementia


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