Association of *CDKN2B-AS1* rs1333049 with Brain Diseases: A Case-control Study and a Meta-analysis

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Objective: *CDKN2B–AS1* polymorphisms were shown to associate with the risk of stroke in European. The goal of this study was to evaluate the contribution of *CDKN2B–AS1* rs1333049 to the risk of hemorrhagic stroke (HS) and brain tumor (BT) in Han Chinese.

Methods: A total of 142 HSs, 115 BTs, and 494 controls were included in the current association study. The genotyping test was performed using the melting temperature shift method.

Results: We failed to validate the association of *CDKN2B–AS1* rs1333049 with the risk of brain disease. Significantly higher levels of low–density lipoprotein cholesterol (LDL–C) (p=0.027), high–density lipoprotein cholesterol (HDL–C) ($p<0.001$) and total cholesterol (TC) ($p<0.001$) were found in HSs in the genotype GG/GC carriers, but not the genotype CC carriers ($p>0.05$). The meta–analysis of 10 studies among 133,993 individuals concluded that rs1333049 of *CDKN2B–AS1* gene was likely to increase a 16% incidence rate of cerebrovascular disease (CD) among various populations (odds ratio 1.16, 95% confidence interval 1.08–1.25; $p<0.0001$, random–effect method).

Conclusion: Our case–control study identified rs1333049 genotypes showed different association with the concentration of the LDL–C, HDL–C and TC in the HS patients. Meta–analysis supported the association between rs1333049 and CD risk in various populations, although we were unable to observe association between rs1333049 and the risk of HSs in Han Chinese.

KEY WORDS: Hemorrhagic stroke; Cerebrovascular disease; *CDKN2BAS1*; rs1333049; Lipid concentration.

INTRODUCTION

Hemorrhagic stroke (HS) accounts for approximately 30% of all strokes, and it tends to affect young adults and carries a high morbidity and mortality in the worldwide.[1] Stroke, particularly HS, is becoming the most frequent and important vascular disorder in China.[2] The stroke incidence is threefold higher in northern than that in southern Chinese cities, suggesting important environmental or genetic influences.[3] Current studies have affirmed that inherited factor may play a key role in the pathogenesis of HS.[4] Yet the mechanisms of brain diseases are not completely understood.

CDKN2B anti-sense RNA (*CDKN2B-AS1*) spans 126.3 kb, overlaps at its 5' end with *CDKN2B* (*p15*), and includes 20 exons subjected to alternative splicing.[5] *CDKN2B-AS1*, nearby the *CDKN2A* and *CDKN2B* genes, has been found to associate with the risk of multiple diseases including coronary heart disease (CHD),[6] myocardial infarction (MI),[7] hypertension,[8] and stroke.[9] *CDKN2B-AS1* polymorphisms have been extensively identified as predictors of cardiovascular disease,[10] cerebrovascular disease (CD),[11] and also brain tumors (BTs).[12] *CDKN2B-AS1* is differentially expressed in a variety of tissues such as vascular endothelial cells and smooth coronary muscle cells.[5] As a large antisense non-coding RNA, the *CDKN2B-AS1* function is still unclear, but the *CDKN2B-AS1* transcript level shows significant association with the severity of vascular diseases[13] and cancers.[14] *CDKN2B-AS1* variants are showed to connect with CDKN2BAS expression in cardiovascular diseases.[15] The single nucleotide polymorphism (SNP) rs1333049 is located in the 3'-untranslated region of the *CDKN2B-AS1* gene. And this polymorphism may play a pivotal role in

the development of cardio- or cerebra-vascular disease by altering the dynamics of vascular cell proliferation.[16] *CDKN2B-AS1* rs1333049 has been studied in different diseases such as CHD,[17] atherosclerosis,[14] MI,[15] metabolic disease[18] and Alzheimer's disease.[19] There are some published studies[20-22] that focus on the association between rs1333049 and stroke, notwithstanding, no published study on Han Chinese population.

In this work, we recruited 257 patients with brain diseases (including 142 HSs and 115 BTs) and 494 controls, and performed a case-control test to validate the contribution of *CDKN2B-AS1* rs1333049 to the risk of brain diseases in Han Chinese. In addition, we performed a meta-analysis of the available case-control studies between *CDKN2B-AS1* rs1333049 and CD.

METHODS

Sample Collection

With the informed consent of all patients and approval of the Ethical Committee of Ningbo First Hospital (2013-002), a total of 257 unrelated patients and 494 controls (274 males and 220 females) were collected from the Department of Neurosurgery of the Ningbo First Hospital between March 2013 and March 2014. Of these, 142 HS patients (89 males and 53 females) diagnosed by digital subtraction angiography and 115 BT patients (60 males and 55 females) diagnosed by way of brain magnetic resonance imaging or computed tomography. Control participants were without symptoms or history of diseases, including stroke, autoimmune diseases, and severe liver or kidney disease. Five milliliters of venous blood sample was collected from each subject, saved into 3.2% citrate sodium-treated tubes and stored at -20℃ till use. The levels of triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) were determined by standard enzymatic methods.[23]

Single−nucleotide Polymorphism Genotyping

DNA extraction was performed though the magnetic bead isolation method.[24] Temperature shift genotyping methods were used for the *CDKN2B-AS1* rs1333049 genotyping test.[25] The sequences of the primers of *CDKN2B-AS1* rs1333049 are as follows: short primer 5'-gattaccg CCTCATACTAACCATATGATCAACAGTTG-3'; long primer 5'-gcgggcagggcggcCCTCATACTAACCATATG ATCAACAGTTC-3'; and one common primer 5'-GCTT ACCTCTGCGAGTGGCTGCT-3'. The polymerase chain

reaction (PCR) amplification for genotyping was performed on the Roche Light Cycler® 480 (Roche, Mannheim, Germany) according to the manufacturer's instructions. The PCR conditions included an initial denaturation at 95℃ for 30 seconds, followed by 40 cycles of 95℃ for 30 seconds, 59℃ for 30 seconds, 72℃ for 30 seconds, and a final extension for 30 seconds at 72℃. The SNP types were determined by the melting curve analysis using the analysis software from the quantitative PCR instrument (Fig. 1).

Statistical Analysis

Statistical analysis for the case-control study was performed using SPSS software ver. 16.0 (SPSS Inc., Chicago, IL, USA). The continuous variables were presented as means±standard deviation (SD). Student's *t* test was performed to test the degree of difference between data from cases and controls. Genotype frequencies were tested for departure from Hardy-Weinberg equilibrium using the Arlequin program version 3.5 (University of Bern, Bern, Switzerland).[26] The genotype distribution between case and control groups were analyzed by CLUMP22.[27] The odds ratio (OR) and 95% confidence interval (CI) were calculated for the risk analysis. Power analysis was made by Power and Sample Size Calculation software version 3.0.43 (Nashville, TN, USA). The meta-analysis was performed using the Stata statistical software version 11.0 (Stata Corporation, College Station, TX, USA). The publication bias was evaluated by the egger test. Z test was used to conclude the pooled OR. A *p* value ＜0.05 was considered to be significant.
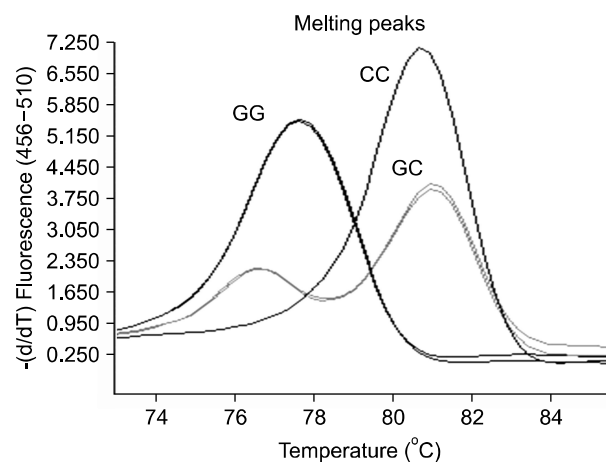

Fig. 1. Genotyping results for the rs1333049 using the melting temperature shift polymerase chain reaction.

RESULTS

Distribution of rs1333049 in Neurosurgical Patients and Controls

The genotype and allele frequencies of rs1333049 in cases and controls were shown in Table 1. The rs1333049-C allele frequencies were 0.494 in all patients, 0.487 in BT cases, 0.500 in HS participants and 0.466 in healthy controls. There were no significant differences in the genotype and allele distribution between controls and each of the three case groups (allele model, dominant model and recessive model, $p > 0.05$; Table 1). A breakdown analysis

Table 1. The distribution of genotypic and allelic frequencies in rs1333049 between cases and controls

Variable	Group (n)	Genotype (n), GG/GC/CC	Allele (n), G/C	Allele model	Dominant model	Recessive model
All	Controls (494)	134/260/100	528/460			
	All patients (257)	60/140/57	260/254	1.12 (0.91-1.39)	1.12 (0.78-1.62)	1.22 (0.86-1.73)
	BT (115)	25/68/22	118/112	1.09 (0.82-1.45)	0.93 (0.56-1.56)	1.34 (0.84-2.19)
	HS (142)	35/72/35	142/142	1.15 (0.88-1.50)	1.29 (0.83-2.00)	1.14 (0.74-1.75)
Gender						
Male	Controls (274)	76/134/64	286/262			
	All patients (149)	34/80/148	148/150	1.11 (0.83-1.47)	1.01 (0.63-1.61)	1.30 (0.82-2.07)
	BT (60)	13/36/11	62/58	1.02 (0.69-1.52)	0.74 (0.36-1.50)	1.39 (0.71-2.71)
	HS (89)	21/44/24	86/92	1.17 (0.83-1.64)	1.21 (0.70-2.09)	1.24 (0.71-2.17)
Female	Controls (220)	58/126/36	242/198			
	All patients (108)	26/60/22	112/104	1.13 (0.92-1.57)	1.31 (0.73-2.36)	1.13 (0.66-1.93)
	BT (55)	12/32/11	56/54	1.18 (0.78-1.79)	1.28 (0.60-2.71)	1.28 (0.63-2.60)
	HS (53)	14/28/11	56/50	1.09 (0.71-1.67)	1.34 (0.63-2.84)	1.00 (0.51-1.97)
Age (yr)						
<65	Controls (373)	101/202/70	404/342			
	All patients (196)	44/106/46	194/198	1.21 (0.94-1.54)	1.33 (0.87-2.02)	1.28 (0.85-1.92)
>65	Controls (121)	33/58/30	124/118			
	All patients (61)	16/34/11	66/56	0.89 (0.58-1.38)	0.67 (0.31-1.44)	1.05 (0.53-2.12)

Values are presented as number only or odds ratio (95% confidence interval).
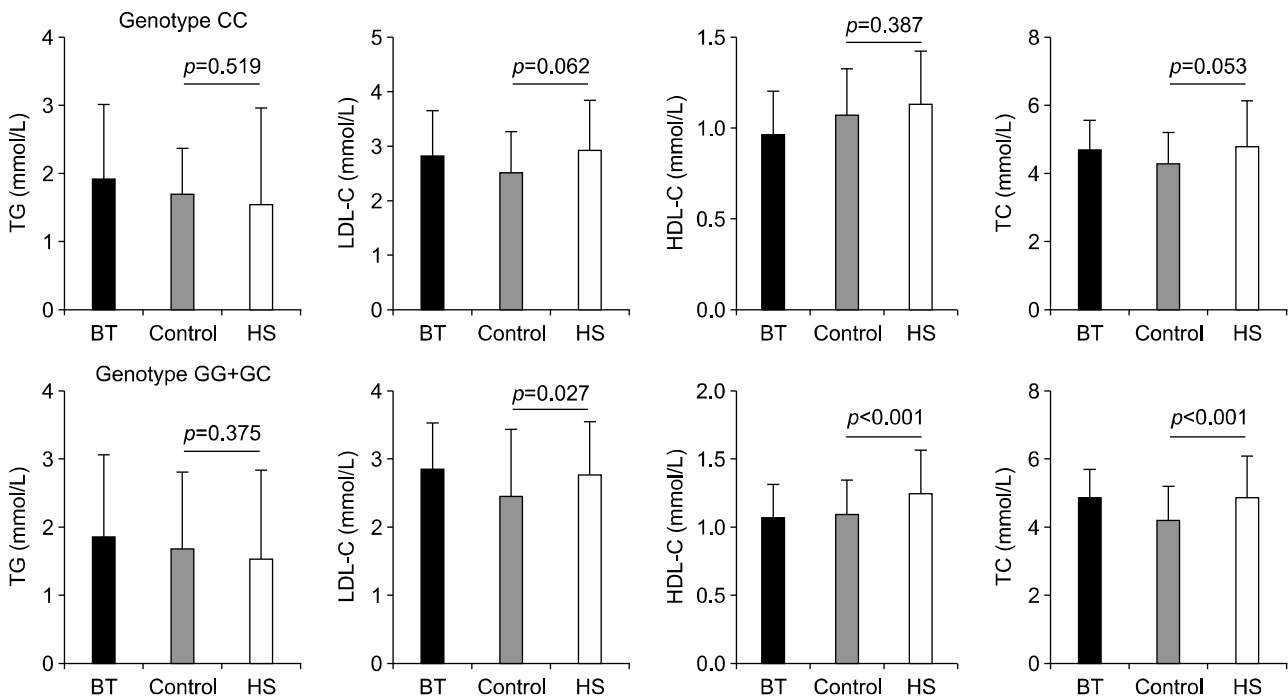BT, brain tumor; HS, hemorrhagic stroke.

Fig. 2. The comparison of characteristics between cases and controls in different genotypes.
BT, brain tumor; HS, hemorrhagic stroke; TG, triglyceride; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TC, total cholesterol.
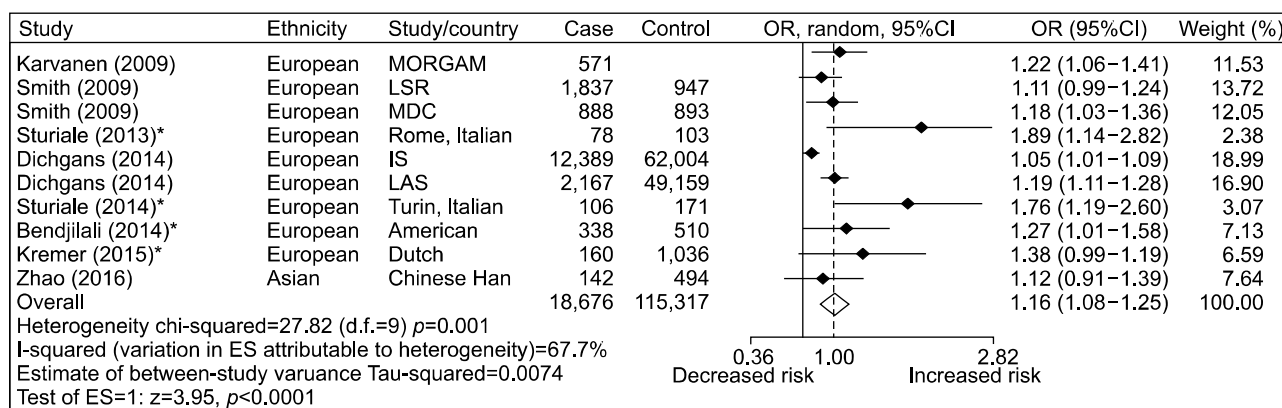
Study	Ethnicity	Study/country	Case	Control	OR, random, 95%CI	OR (95%CI)	Weight (%)
Karvanen (2009)	European	MORGAM	571			1.22 (1.06−1.41)	11.53
Smith (2009)	European	LSR	1,837	947		1.11 (0.99−1.24)	13.72
Smith (2009)	European	MDC	888	893		1.18 (1.03−1.36)	12.05
Sturiale (2013)*	European	Rome, Italian	78	103		1.89 (1.14−2.82)	2.38
Dichgans (2014)	European	IS	12,389	62,004		1.05 (1.01−1.09)	18.99
Dichgans (2014)	European	LAS	2,167	49,159		1.19 (1.11−1.28)	16.90
Sturiale (2014)*	European	Turin, Italian	106	171		1.76 (1.19−2.60)	3.07
Bendjilali (2014)*	European	American	338	510		1.27 (1.01−1.58)	7.13
Kremer (2015)*	European	Dutch	160	1,036		1.38 (0.99−1.19)	6.59
Zhao (2016)	Asian	Chinese Han	142	494		1.12 (0.91−1.39)	7.64
Overall			18,676	115,317		1.16 (1.08−1.25)	100.00

Heterogeneity chi-squared=27.82 (d.f.=9) *p*=0.001
I-squared (variation in ES attributable to heterogeneity)=67.7%
Estimate of between-study varuance Tau-squared=0.0074
Test of ES=1: z=3.95, *p*<0.0001

0.36   1.00   2.82
Decreased risk   Increased risk

Fig. 3. Meta-analysis of the studies between rs1333049 and cerebrovascular disease risk.
MORGAM, an international pooling of cardiovascular cohorts; LSR: the Lund Stroke Register; MDC, the Malmo Diet and Cancer Study; IS, ischemic stroke; LAS, large artery stroke; OR, odds ratio; CI, confidence interval; d.f., degree of freedom; ES, effect size.
*rs1333049 showed high linkage disequilibrium with rs1333040 $(0.81 \leq r^2 \leq 0.97)$ in the previous studies.[36,37]

by gender demonstrated a negative association of rs1333049 with brain diseases under any genetic models ($p > 0.05$; Table 1). In addition, our age-stratified association test was unable to detect any relationship of rs1333049 with neurosurgical patients ($p > 0.05$; Table 1).

Previous study showed that rs1333049 genotypes might play a key pharmacogenomics role in hypercholesterolemia. Therefore, we further stratified the data analysis in the different lipids by rs1333049 genotypes. As shown in Figure 2, there were significantly higher levels of LDL-C (p=0.027), HDL-C ($p < 0.001$) and TC ($p < 0.001$) in HSs than in controls in the genotype GG/GC carriers, but not the genotype CC carriers ($p > 0.05$).

Meta−analysis of the Association between rs1333049 and the Risk of CD

A total of 10 study stages[11,20-22,28-30] (including 18,676 cases and 115,317 controls) were selected into the meta-analysis for the association of rs1333049 of *CDKN2B-AS1* gene with CD. Since substantial heterogeneity were observed among the overall studies (p=0.001, I^2=67.7%), random-effect method was applied for the meta-analysis. As shown in Figure 3, the data showed that *CDKN2B-AS1* rs1333049 was a risk factor of CD (overall OR=1.16, 95% CI=1.08-1.25, $p < 0.0001$, random-effect method). The publication bias of studies was analyzed by the Egger test. As shown in Figure 4, the results did not show any evidence of publication bias for the analysis ($p_{(Egger)}$=0.056).

DISCUSSION

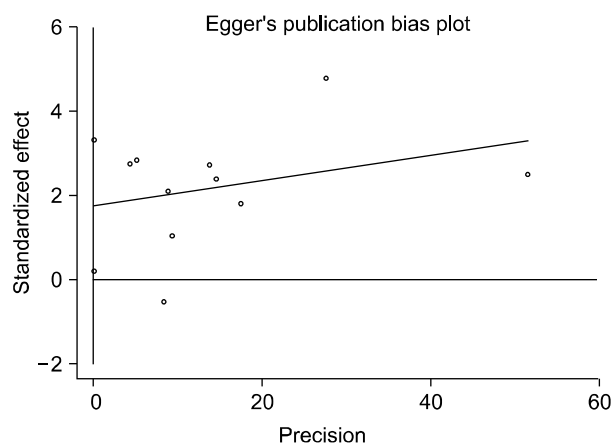In the present study, we aimed to explore the significant

Fig. 4. Egger test for the publication bias of the enrolled studies.

association between the *CDKN2B-AS1* rs1333049 with the risk of brain disease through the case-control study and meta-analysis. Our case-control study couldn't find obvious relationship with the risk of BTs or HSs. But, the results showed that rs1333049 genotypes were strongly connected with the lipid concentration in HS patients. Our meta-analysis enrolled 133,993 subjects concluded that *CDKN2B-AS1* rs1333049 contributed to the risk of CD, although substantial heterogeneity was shown in the involved studies.

Epidemiologic studies had demonstrated that blood lipid lipoprotein abnormalities were connected with the clinical manifestations in different diseases.[31,32] And genetic variants could influence the circulating lipid levels in patients.[33] Ahmed *et al.*[34] found that the *CDKN2B-AS1* rs1333049 genotype could influence the statin therapy in

the hyperlipidemia families. The patients on statin therapy with the rs1333049-CC genotype had significantly lower LDL-C and TC as compared to the rs1333049-CG/GG carriers.[34] In current study, our results showed that the genotype GG/GC carriers had significantly higher levels of LDL-C (p=0.027), HDL-C ($p<0.001$) and TC ($p<0.001$) in HSs than in controls, but not the genotype CC carriers ($p>0.05$). We suspected that this phenomenon was related to increased plasma levels of pharmacologically active metabolites.

SNP rs1333049 in *CDKN2B-AS1* was located on chromosome 9p21.3, which was considered as the most widely and consistently replicated risk locus for cardiovascular and CDs.[29] Previous study showed that *CDKN2B-AS1* polymorphism significantly associated with the risk of glioma by affecting the gliomagenesis.[35] The *CDKN2B-AS1* polymorphism rs1333040 was located on the risk locus chromosome 9p21.3, and showed high linkage disequilibrium with rs1333049 ($0.81 \leq r^2 \leq 0.97$) in previous studies.[36,37] Sturiale *et al.*[28] suggested that the distribution of the rs1333040 genotypes was statistically different between sporadic brain arteriovenous malformations and controls. And the rs1333040 was significantly associated with BAVMs in all the three genetic models.[29] *CDKN2B-AS1* rs1333049-C allele was certified to increase the risk of stroke in the MORGAM Project.[20] In the large-scale genetic association study, Smith *et al.*[21] detected that rs1333049 was a common genetic determinant for ischemic stroke on chromosome 9p21. In the following study, Dichgans *et al.*[22] demonstrated that rs1333049 was associated with a significant excess risk for ischemic stroke and particularly for the large artery stroke subtype with CHD. The meta-analysis of 10 studies (including our study) among 133,993 individuals concluded that rs1333049 of *CDKN2B-AS1* gene was likely to increase a 16% incidence rate of CD among various populations. Power analyses showed that our meta-analysis of rs1333049 had a perfect power (power=98%) to detect a susceptibility locus at the nominal type1 error rate of 0.05. In the case-control study, the results had only less than 30% power in the association tests. Thus, our results could not find any significant association between rs1333049 and BTs or HSs in Han Chinese. The lack of association may be due to the genetic heterogeneity of this locus or a lack of power in our sample for this minor-effect genetic marker in Chinese.

In present study, we failed to validate the association of *CDKN2B-AS1* rs1333049 with the risk of brain disease in Han Chinese. The sample size of the case-control study

was comparatively small, and the age and gender were not well matched. Therefore, we could not exclude a chance of random positive finding of the genotype association. Further studies utilizing a well-matched group with a larger sample size are warranted to increase the confidence of our findings.

In conclusion, our case-control study identified rs1333049 genotypes showed different association with the concentration of the LDL-C, HDL-C and TC in the HS patients. Meta-analysis supported the association between rs1333049 and CD risk in various populations. However, this study was designed as a pilot study and further investigations are needed to confirm our results and to elucidate unresolved questions.

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