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Review Article

Genetic variants of *miR-146a* and *miR-499* and risk of ischemic stroke in the Chinese population: a meta-analysis and trial sequential analysis

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Abstract: Objective: Genetic variants in miRNA sequences may alter miRNA expression and/or maturation, resulting in diverse functional consequences. The aim of the present meta-analysis was to investigate the association between *miR-146a* rs2910164 and *miR-499* rs3746444 genetic polymorphisms and risk of ischemic strokes (IS) in the Chinese population. Methods: A literature search was conducted using PubMed, EMbase, Web of Science, and Chinese National Knowledge Infrastructure (CNKI) databases up to October 2017. Pooled effects (odds ratio [OR] together with 95% confidence intervals [CI]) were calculated. Subgroup analyses were carried out by status of Hardy-Weinberg equilibrium, sample size, genotyping methods, and subtypes of IS. Trial sequential analysis (TSA) was used to reduce the risk of type I errors and determine whether the evidence was firm. Results: A total of 10 eligible studies, consisting of 4,251 patients with IS and 5,812 controls, were finally included. Results showed that pooled OR for IS of the rs2910164 G allele was 1.23 (95% CI: 1.03-1.46, $P = 0.022$), compared to wild-type C allele under a recessive model, with high heterogeneity ($I^2 = 56.2\%$, $P = 0.015$). No significant association was found between the rs3746444 variant and IS under different genetic models. TSA showed a solid conclusion for association between the rs2910164 variant and IS risk. Conclusion: Current evidence suggests a weak association between *miR-146a* rs2910164 variant and risk of IS, whereas *miR-499* rs3746444 variant might not be associated with elevated risk of IS in a Chinese population.

Keywords: MicroRNA, polymorphism, ischemic stroke, Chinese population

Introduction

Stroked are one of the leading causes of adult chronic disability and death, worldwide [1, 2]. In China, the annual stroke mortality rate is approximately 157 per 100,000, which has had a significant impact on Chinese society [3]. Ischemic stroke (IS) is the most common type of stroke, accounting for 85% to 90% of all strokes [4]. Multiple factors, including hypertension, diabetes mellitus, dyslipidemia, smoking, and genetic variants, contribute to the risk of ischemic stroke [5-7].

MicroRNAs (miRNAs, miRs) are a group of small non-coding RNAs that play key roles of post-transcriptional gene silencing in the pathophysiology of ischemic strokes. It has been suggested that miR-497 induces neuronal death and miR-15a contributes to the pathogenesis of ischemic vascular injuries [8, 9]. miR-21 and miR-126 have been found to be involved in the pathologic atherosclerosis of ischemic strokes [10, 11].

Genetic variants located in miRNA genes may affect pre-miRNA maturation or target selection. Many studies have investigated the association of two miRNA polymorphisms in pre-miRNA sequences (*miR-146a* C > G rs2910164 and *miR-499* A > G rs3746444) with IS. Li et al. [12] first reported that individuals

with rs2910164 GG genotype have a higher risk of ischemic stroke, in accord with recent findings [13, 14]. The rs3746444 variant was also found to be associated with susceptibility to IS [15]. However, these results have not been replicated in other studies [15, 16]. Hence, this meta-analysis of previous publication studies was conducted. To date, four meta-analyses were carried out to assess the relationship of these two genetic variants and IS [17-20]. Although subgroup analysis by ethnicity was performed in these studies, findings were still controversial, partly be due to the limited number of studies and underpowered studies. As previous meta-analyses have not comprehensively investigated the association between *miR-146a* rs2910164 and *miR-499* rs3746444 polymorphisms and risk of ischemic strokes in a Chinese population, the present analysis aimed to provide insight on this issue.

Materials and methods

Search strategy

This study systematically searched PubMed, EMbase, Web of Science, and Chinese National Knowledge Infrastructure (CNKI) databases for studies reported before October 2017, using the terms "miR-146a" or "miR-499" paired with "polymorphism'', "genetic variant", "ischemic stroke", "cerebral infarction", "ischemic cerebrovascular disease", "Chinese", and "China", respectively. No language restrictions were applied. Review articles and bibliographies of relevant studies were manually scanned to identify eligible studies.

Studies were selected according to the following criteria: (1) Case-control designed; (2) Regarding *miR-146a* (rs2910164) or *miR-499* (rs3746444) variants and IS risk; (3) Studies with complete data about genotype and allele frequencies or providing related information; and (4) Chinese origin. Exclusion criteria included: (1) Studies without raw data; (2) Family-based studies of pedigree design; and (3) Case reports, letters, commentaries, meeting records, or review articles.

Data extraction and quality assessment

The following information was extracted from each study: first author, years of publication, study design, total number of cases and controls, mean age of cases and controls, percentage of males in patients and controls, genotype frequencies, genotyping method, and Hardy-Weinberg equilibrium (HWE) in controls. Data extraction was performed independently by two authors (Wang and Wang). Any disagreements were resolved by consensus with a third author (Li).

Statistical analysis

Stata software (version 12.0, Stata Corporation, College Station, TX, USA) was used in this meta-analysis. Strength of association was estimated as odds ratio (OR) and 95% confidence intervals (CIs). For both polymorphisms, statistical analysis was performed under the allelic (rs2910164, G vs. C; rs3746444, G vs. A), dominant (rs2910164, GG+GC vs. CC; rs3746444, GG+GA vs. AA), and recessive (rs2910164, GG vs. GC+CC; rs3746444, GG vs. GA+AA) genetic models. Significance of the pooled OR was determined by Z test. A test of heterogeneity was conducted using Cochran's Q test (heterogeneity was considered statistically significant with a corresponding p-value < 0.10) and Higgins I^2 statistic (I^2 > 50% indicates significant heterogeneity among studies). A random-effects model (DerSimonian and Laird method) was applied if heterogeneity was observed. Otherwise, a fixed-effects model (Mantel-Haenszel method) was used. Subgroup analyses were performed to investigate the probable source of heterogeneity, according to status of HWE (yes or no), sample size (≥ 500 or < 500 cases), genotyping methods (restriction fragment length polymorphism [RFLP], Taqman, or others), and subtypes of IS (large artery atherosclerosis [LAA] or small vessel disease [SVD]). Publication bias was assessed using Begg's and Egger's tests and by visual inspection of corresponding funnel plots. P-values < 0.05 indicate statistical significance regarding publication bias. Sensitivity analysis was performed to assess the effects of an individual study on pooled results and the stability of results. In trial sequential analysis (TSA), two-sided tests were used. Type I error was set at 5% and power was set at 80%. Required information size was calculated based on a 15% relative risk reduction. Trials ignored in the interim appeared to be due to low use of information (< 1%). TSA was carried out with the use of TSA software (version 0.9.5.5; Copenhagen Trial Unit, Copenhagen, Denmark, 2011).

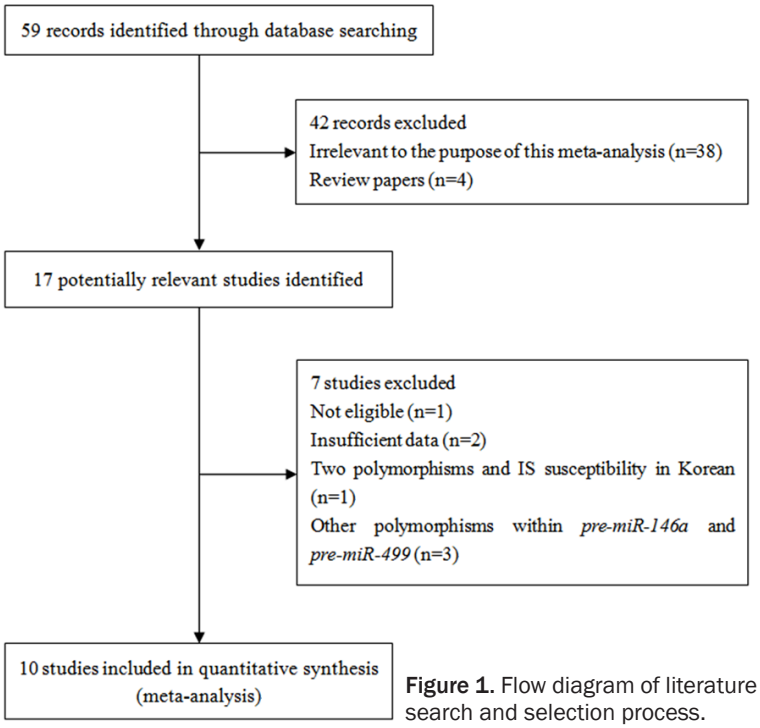

Figure 1. Flow diagram of literature search and selection process.

Results

Study characteristics

As shown in **Figure 1**, a total of 10 eligible original reports, consisting of 10,063 individuals for *miR-146a* and *miR-499,* were included according to inclusion criteria. For *miR-146a* rs2910164 polymorphism, 10 studies [12-16, 21-25] were finally enrolled, including a total of 4,251 patients with IS and 5,812 controls. For *miR-499* rs3746444 polymorphism, 5 studies [14-16, 23, 25] were finally enrolled, including a total of 1,899 patients with IS and 1,981 controls. Enrolled studies and main characteristics are shown in **Table 1**. The genotype distribution among control subjects of most included studies did not deviate from the HWE, except for two studies performed by Li et al. [12] and Qu et al. [24], respectively.

Association between miR-146a rs2910164 variant and IS

Under the recessive genetic model, the rs2910164 GG genotype was weakly associated with IS risk, compared with the wild-type C allele using a random effects model. Pooled OR was 1.23 (95% CI: 1.03-1.46, P = 0.022) in the Chinese population. Significant heterogeneity was found among 10 studies (I^2 = 56.2%, P = 0.015) (**Figure 2A**) and no significant publication bias was observed (Begg's test P = 0.474, Egger's test P = 0.357) (Supplementary Figure 1A). However, no significant association was found between the rs2910164 variant and IS risk under the dominant genetic model. Pooled OR was 1.10 (95% CI: 0.96-1.26, P = 0.177), with high heterogeneity (I^2 = 58.5%, P = 0.010) (**Figure 2B**). Under the allelic model, pooled OR was 1.10 (95% CI: 0.98-1.23, P = 0.119), with high heterogeneity (I^2 = 72.9%, P < 0.001) (**Figure 2C**). No significant publication bias was detected under either model (Begg's test P = 0.592 and 0.592, Egger's test P = 0.357 and 0.412, respectively) (Funnel plots are presented in Supplementary Figure 1B, 1C).

Subgroup analyses were further performed examining the effects of rs2910164 polymorphism on risk of IS (**Table 2**). Under the recessive model, when studies were restricted to those within the Hardy-Weinberg's equilibrium, for the risk of IS, the pooled OR of the GG genotype of rs2910164 compared with the GC+CC genotype was 1.23 (95% CI: 1.00-1.51, P = 0.006), with high heterogeneity (I^2 = 50.3%, P = 0.050). Heterogeneity within groups disappeared when analyses were restricted to the SVD subtype of IS (I^2 = 0.0%, P = 0.611). However, the OR for IS decreased to 1.04 with a 95% CI of 0.84-1.28. Results became statistically insignificant (P = 0.740).

Association between miR-499 rs3746444 variant and IS

No significant association was detected between *miR-499* rs3746444 and IS under the recessive model (OR = 1.20, 95% CI: 0.81-1.79, P = 0.366), without heterogeneity (I^2 = 11.7%, P = 0.334) (**Figure 3A**). No significant publication bias was detected (Begg's test P = 0.308, Egger's test P = 0.469, Supplementary Figure 1D). Pooled ORs were similar under the other

Table 1. Main characteristics of studies included in meta-analysis

First Author [Ref.]	Year	Disease (Subtypes)	Subjects, n (Cases/Controls)	Age (years), Mean ± SD (Cases/Controls)	Gender Component in Case/Control (% male)	Genotyping Method	Genotype Distribution (Cases/Controls)			HWE of Control
10 Studies for rs2910164 Polymorphism of *pre-miR-146a*							CC	CG	GG	
Li [12]	2010	IS (LAA)	1278, 268/1010	64±11/45±12*	67.2/57.3	PCR-RFLP	79/345	110/455	79/210	0.009
Sun [21]	2011	IS (LAA, SVD)	1031, 381/650	63±12/62±13	61.9/53.4	PCR-RFLP	146/228	170/304	65/118	0.345
Zhu [22]	2014	IS (LAA, SVD)	749, 368/381	61.62±0.99/62.05±0.98	68.8/68.5	PCR-LDR	145/132	173/185	50/64	0.952
Liu [15]	2014	IS	687, 296/391	67.52±10.29/66.34±11.07	60.8/58.1	PCR-RFLP	85/116	159/198	52/77	0.650
Hu [13]	2014	IS	401, 196/205	64±11.7/63±10.5	48.0/46.3	PCR-RFLP	75/97	87/82	34/26	0.193
Huang [14]	2015	IS	1062, 531/531	63 (54, 70)/61 (54, 68)†	61.6/61.6	TaqMan	189/219	261/257	81/55	0.106
Zhu [23]	2016	IS (LAA, SVD)	774, 396/378	63.74±4.49/63.31±4.84	54.3/53.4	PCR-RFLP	131/154	194/179	71/45	0.521
Lv [16]	2016	IS	756, 378/378	58±11.9/58±11.9	55.6/55.6	TaqMan	119/153	198/187	61/38	0.079
Qu [24]	2016	IS (LAA, SVD)	2724, 1139/1585	61.30±9.40/59.50±8.50*	63.0/57.0	PCR-LDR	355/483	618/869	166/233	< 0.001
Luo [25]	2017	IS	601, 298/303	60.70±12.33/60.17±10.32	65.8/59.7	NaPshot	129/119	130/139	39/45	0.672
5 Studies for rs3746444 Polymorphism of *pre-miR-499*							AA	AG	GG	
Liu [15]	2014	IS	687, 296/391	67.52±10.29/66.34±11.07	60.8/58.1	PCR-RFLP	181/278	96/99	19/14	0.170
Huang [14]	2015	IS	1062, 531/531	63 (54, 70)/61 (54, 68)†	61.6/61.6	TaqMan	398/403	133/128	0/0	0.002
Zhu [23]	2016	IS (LAA, SVD)	774, 396/378	63.74±4.49/63.31±4.84	54.3/53.4	PCR-RFLP	255/249	123/116	18/13	0.910
Lv [16]	2016	IS	756, 378/378	58±11.9/58±11.9	55.6/55.6	TaqMan	257/250	110/113	11/15	0.621
Luo [25]	2017	IS	601, 298/303	60.70±12.33/60.17±10.32	65.8/59.7	SNaPshot	215/244	78/53	5/6	0.131

*, $P < 0.05$; †, Data are expressed as median (25th, 75th quartiles); IS, ischemic stroke; LAA, large artery atherosclerosis; SVD, small vessel disease; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphisms; LDR, ligation detection reaction; HWE, Hardy-Weinberg equilibrium.

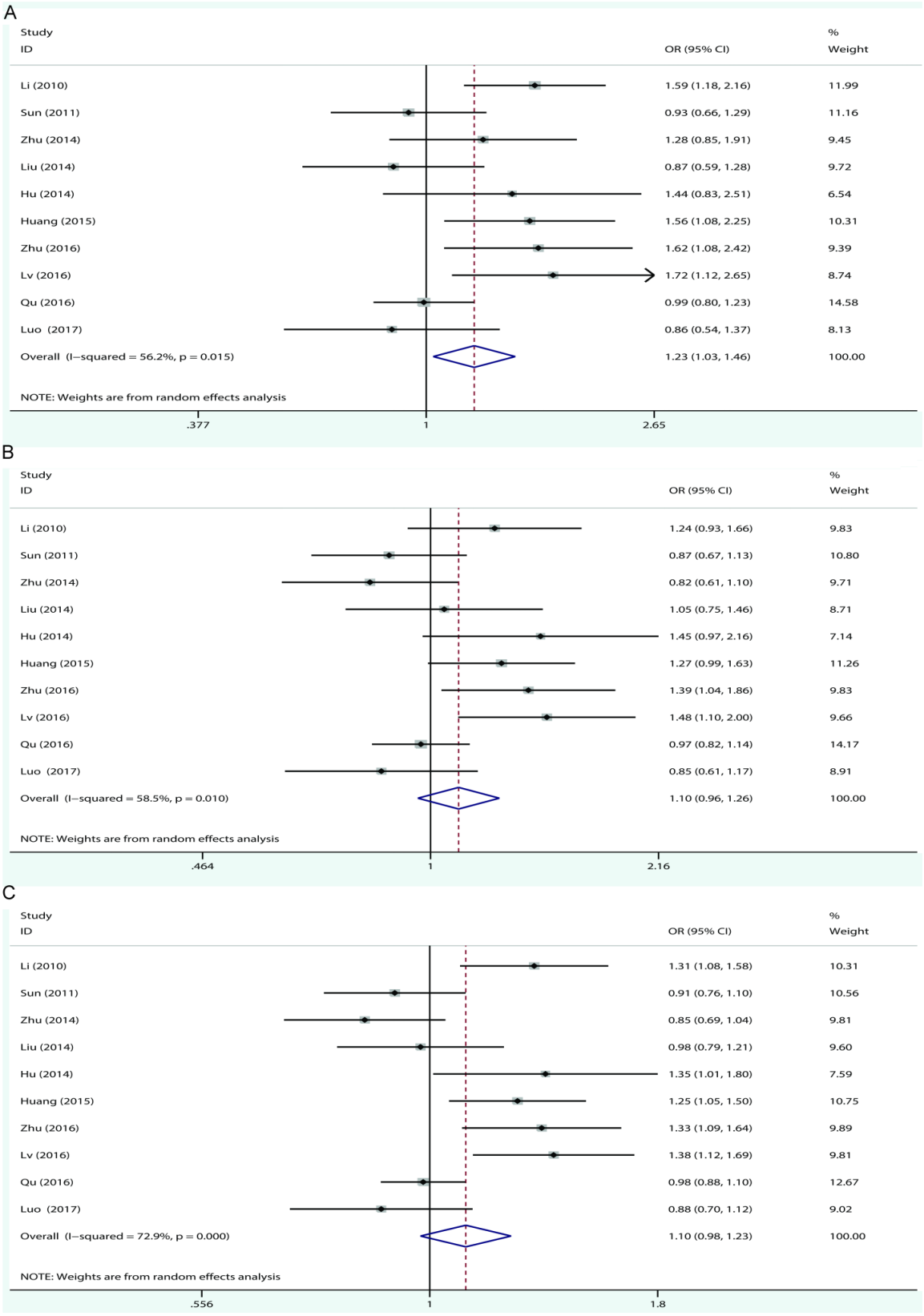

Figure 2. Forest plots of *miR-146a* rs2910164 variant and ischemic stroke. A. Association between the *miR-146a* rs2910164 and ischemic stroke using the recessive model. B. Association between the *miR-146a* rs2910164 and ischemic stroke using the dominant model. C. Association between the *miR-146a* rs2910164 and ischemic stroke using the allelic model.

Table 2. Stratified meta-analysis of the effects of rs2910164 polymorphism on risk for IS

Variables	No. of studies	No. of subjects		Per allele risk		Test for heterogeneity within group	
		Cases	Controls	OR (95% CI)	*P*	I^2	*P*
Total	10	4251	5812	1.23 [1.03, 1.46]	0.022	56.2%	0.015
Hardy-Weinberg equilibrium							
Yes	8	2844	3217	1.23 [1.00, 1.51]	0.040	50.3%	0.050
No	2	1407	2595	1.24 [0.78, 1.97]	0.365	84.1%	0.012
Sample size for patients							
< 500 cases	8	2581	3696	1.24 [1.01, 1.53]	0.043	54.2%	0.033
≥ 500 cases	2	1670	2116	1.21 [0.78, 1.88]	0.395	77.2%	0.036
Genotyping method							
PCR-RFLP	5	1537	2634	1.24 [0.93, 1.64]	0.139	62.6%	0.030
Taqman	2	909	909	1.62 [1.23, 2.15]	0.001	0.0%	0.729
Others	3	1805	2269	1.02 [0.85, 1.21]	0.834	0.0%	0.413
Subtypes of IS							
LAA	5	1648	4004	1.15 [0.85, 1.55]	0.370	70.1%	0.010
SVD	4	844	2994	1.04 [0.84, 1.28]	0.740	0.0%	0.611

PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphisms; IS, ischemic stroke; LAA, large artery atherosclerosis; SVD, small vessel disease.

two genetic models. For the dominant model, the OR was 1.19 (95% CI: 0.96-1.47, $P = 0.108$) with high heterogeneity ($I^2 = 55.8\%$, $P = 0.060$) (**Figure 3B**). For the allelic model, the OR was 1.16 (95% CI: 0.96-1.40, $P = 0.120$) with heterogeneity ($I^2 = 57.6\%$, $P = 0.051$) (**Figure 3C**). No significant publication bias was detected under either model (Begg's test $P = 0.462$ and 0.806, Egger's test $P = 0.155$ and 0.322, respectively; Funnel plots are presented in Supplementary Table 1E, 1F).

Sensitivity analysis

Omitting one study each time, regarding association between *miR-146a* rs2910164 and risk of IS under the recessive genetic model, the significance of pooled ORs disappeared after multiple studies were excluded. This suggests the high probability of the presence of false-positive results. Remaining results remained similar for the two variants under different genetic models (Supplementary Figure 2).

Trial sequential analysis

For overall analysis of the polymorphism *miR-146a* rs2910164, the total number of cases and controls were more than required. It was found that the cumulative Z-curve exceeded monitoring boundaries before reaching the required information size, indicating that cumulative evidence is adequate and further trials were unnecessary (**Figure 4A**). On the other hand, TSA did not allow this study to draw any solid conclusions regarding association between *miR-499* rs3746444 polymorphism and IS risk. Further trials are warranted (**Figure 4B**).

Discussion

Strokes are a complex multifactorial disease. China has approximately 2.5 million new stroke cases each year. Strokes have become the leading cause of death in the country [26]. IS is the most common type of stroke, but its precise pathophysiology remains unclear. Underlying mechanisms of IS have been shown to comprise both genetic and environmental factors. Numerous genetic studies have been conducted to investigate the influence of gene polymorphisms, including the miRNA variants, on occurrence of IS. To date, growing evidence has suggested that miRNAs, including miR-146a and miR-499, are involved in thrombosis and inflammation pathways in the circulation system [27-29]. However, opinions concerning their roles in IS are controversial and far from conclusive.

As previously reported, miR-146a negatively regulates interleukin-1 receptor-associated kinase 1 (IRAK1) and tumor necrosis factor

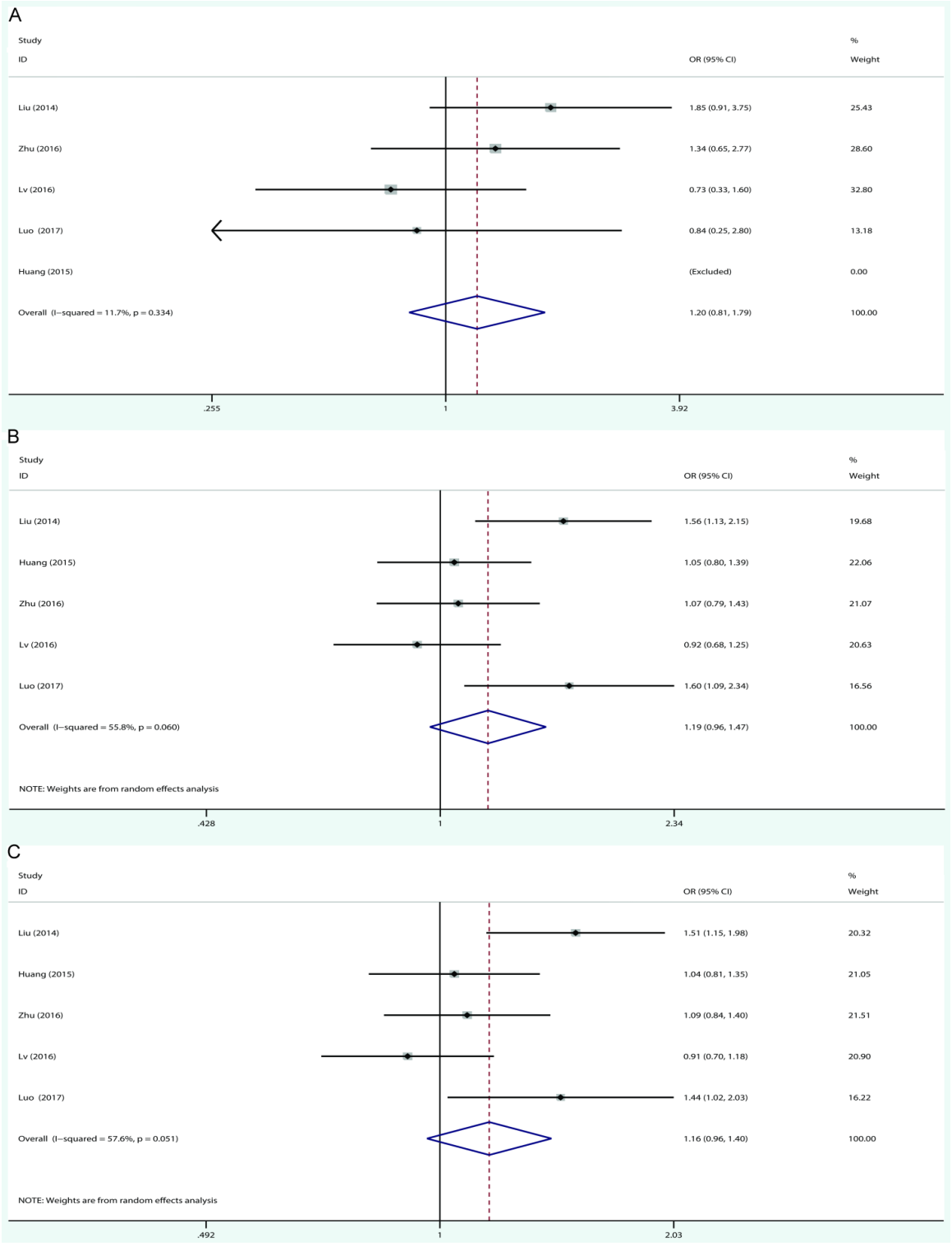

Figure 3. Forest plots of *miR-499* rs3746444 variant and ischemic stroke. A. Association between the *miR-499* rs3746444 and ischemic stroke using the recessive model. B. Association between the *miR-499* rs3746444 and ischemic stroke using the dominant model. C. Association between the *miR-499* rs3746444 and ischemic stroke using the allelic model.

receptor-associated factor 6 (TRAF6), which play essential roles in the inflammation process [30]. Upregulation of miR-146a might attenuate pro-inflammatory effects via inhibit-

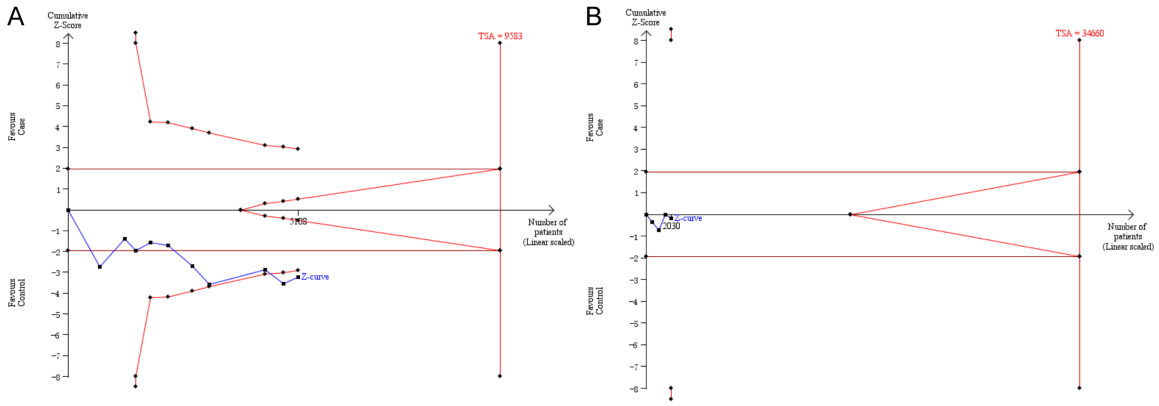

Figure 4. Trial sequential analysis for overall analysis of the two variants. A. Trial sequential analysis for overall analysis of *miR-146a* rs2910164. B. Trial sequential analysis for overall analysis of *miR-499* rs3746444.

ing TRAF6 and IRAK-1 expression, causing decreased levels of pro-inflammatory cytokines, such as interleukin (IL)-1, IL-6, IL-8, and tumor necrosis factor-a (TNF-a) [31]. Moreover, miR-146a represses the pro-inflammatory NF-κB pathway as well as the MAP kinase pathway [32], which are important regulators in the pathology process of IS. On the other hand, miR-499 can regulate C-reactive protein [33], which is a sensitive indicator of inflammation associated with the risk of IS [34]. Although several epidemiological studies have assessed the relationship between *miR-146a* rs2910164 and *miR-499* rs3746444 variants and risk of IS, the composite of these studies has failed to provide a consensus [17-20]. Few studies have focused on this issue in the Chinese population.

To the best of our knowledge, this is the first meta-analysis that comprehensively assesses association between the two well-known miRNA polymorphisms and susceptibility of IS in the Chinese population. The present analysis found a weak association between *miR-146a* rs2910164 polymorphism and IS in the Chinese population, with high heterogeneity. However, this association became non-significant under sub-group studies. For example, when restricted to the SVD subtype of IS or larger sample size studies, results indicated the high probability that the association was falsely positive. This may have been caused by shortcomings in the design and conduct of selected studies, "winner's curse" phenomenon [35], or chance. On the other hand, *miR-499* rs3746444 variant yielded no significant overall association with the risk of IS, with evidence of significant heterogeneity across studies.

Meta-analyses aim to increase the power and precision of the estimated effects of genetic variants on disease. However, results of the present meta-analysis might be prone to systematic or random errors due to repeated significance testing of accumulated data. Thus, TSA was conducted to decrease the risk of type I errors and confirm more statistical reliability of the data by estimation of required information size. This was accomplished by adjusting the threshold of significance levels with the use of Alpha-spending boundaries. In the current meta-analysis, TSA results indicated that the cumulative evidence might be adequate for the analysis of *miR-146a* rs2910164, whereas more trials are warranted for that of *miR-499* rs3746444. Although TSA results of *miR-146a* rs2910164 showed that the cumulative Z-curve reached the perpendicular line (required information size), considering that high heterogeneity was detected among studies and weak significance of pooled results was present, more well conducted studies with uniform methodology are necessary.

Currently, no data is available focusing on the relationship between *miR-146a* rs2910164 polymorphism and susceptibility to IS for more nationalities. There is only one article demonstrating that the G allele of the *miR-146a* polymorphism is associated with an increased risk of IS in the Korean population [36]. However, the present meta-analysis only suggests a weak association between rs2910164 poly-

morphism and IS risk in the Chinese population. Notably, differential allele frequencies of *miR-146a* polymorphisms exerted disproportionate levels of influence on stroke risks in different populations. It should be noted that the frequency of the G allele in East Asians was significantly lower than that in Caucasians [37] and Indians [38]. Prevalence of the G allele was 35% in the Chinese population [14], which is similar to that found in the Korean population [36]. Such a discrepancy may be caused by distinct ethnic specificity, which might affect levels of mature *miR-146a* production.

Results of the present meta-analysis should be interpreted carefully because of the following potential limitations. First, the present study was mainly based on unadjusted estimates. Potential covariates, including age, drinking status, obesity, cigarette consumption, or other lifestyle factors, may have caused confounding bias. Second, differences in the clinical classification of IS patients and subtypes, as well as the enrolled controls among the selected studies, might have affected overall results. Third, studies involved in this meta-analysis were small or medium-sized, with insufficient statistical power. Fourth, potential weakness of genetic association studies, such as genotyping errors, gene-gene, and gene-environment interactions, might have also distorted outcomes.

In summary, based on current studies, the present analysis showed a weak association between *miR-146a* rs2910164 GG genotype and increased risk of IS in the Chinese population, whereas *miR-499*-rs3746444 might not be associated with elevated risk of IS in the Chinese population. Well-conducted studies with larger sample sizes and improved methodologies are required to verify the present findings. Moreover, analyses concerning IS subtypes and gene-gene and gene-environment interactions are necessary regarding the heterogeneity of this disease.

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Disclosure of conflict of interest

None.

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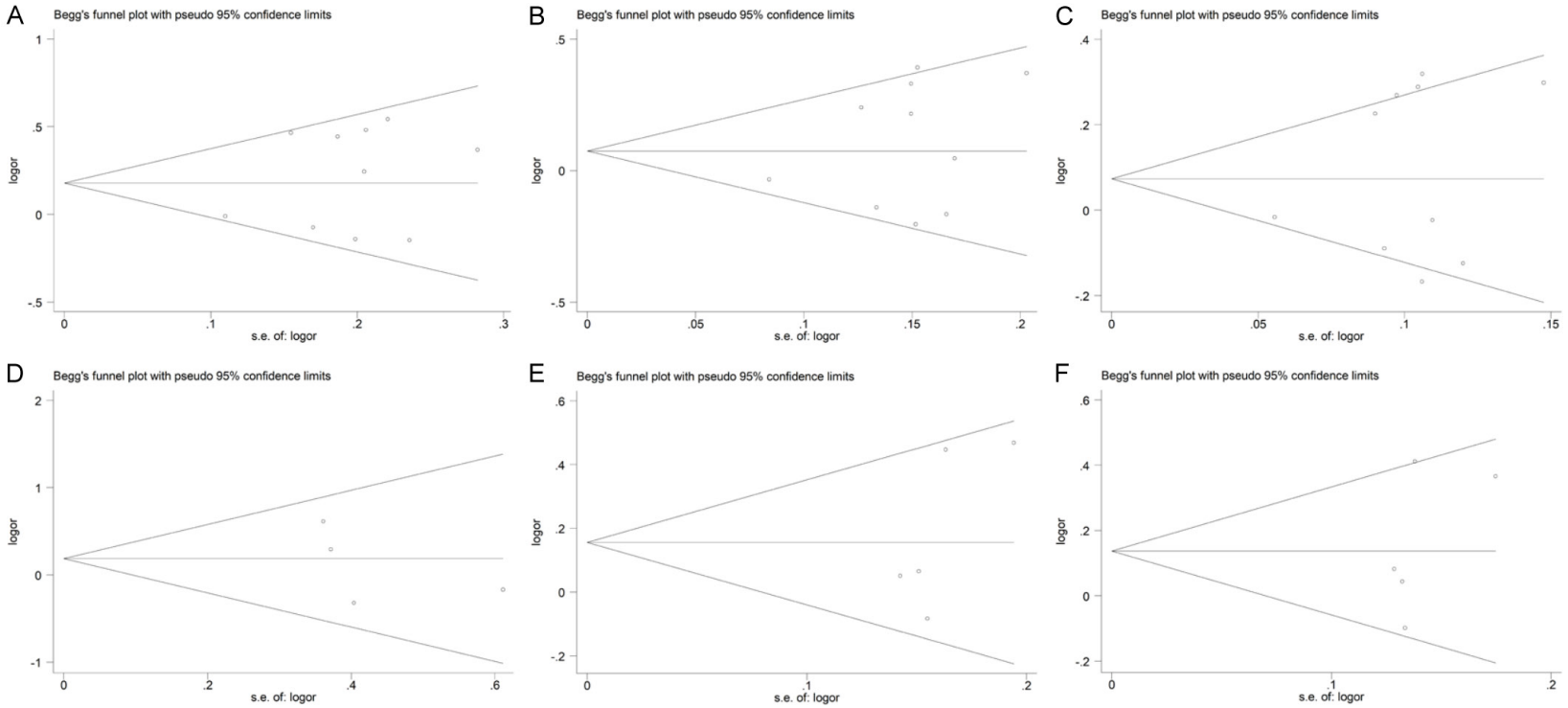


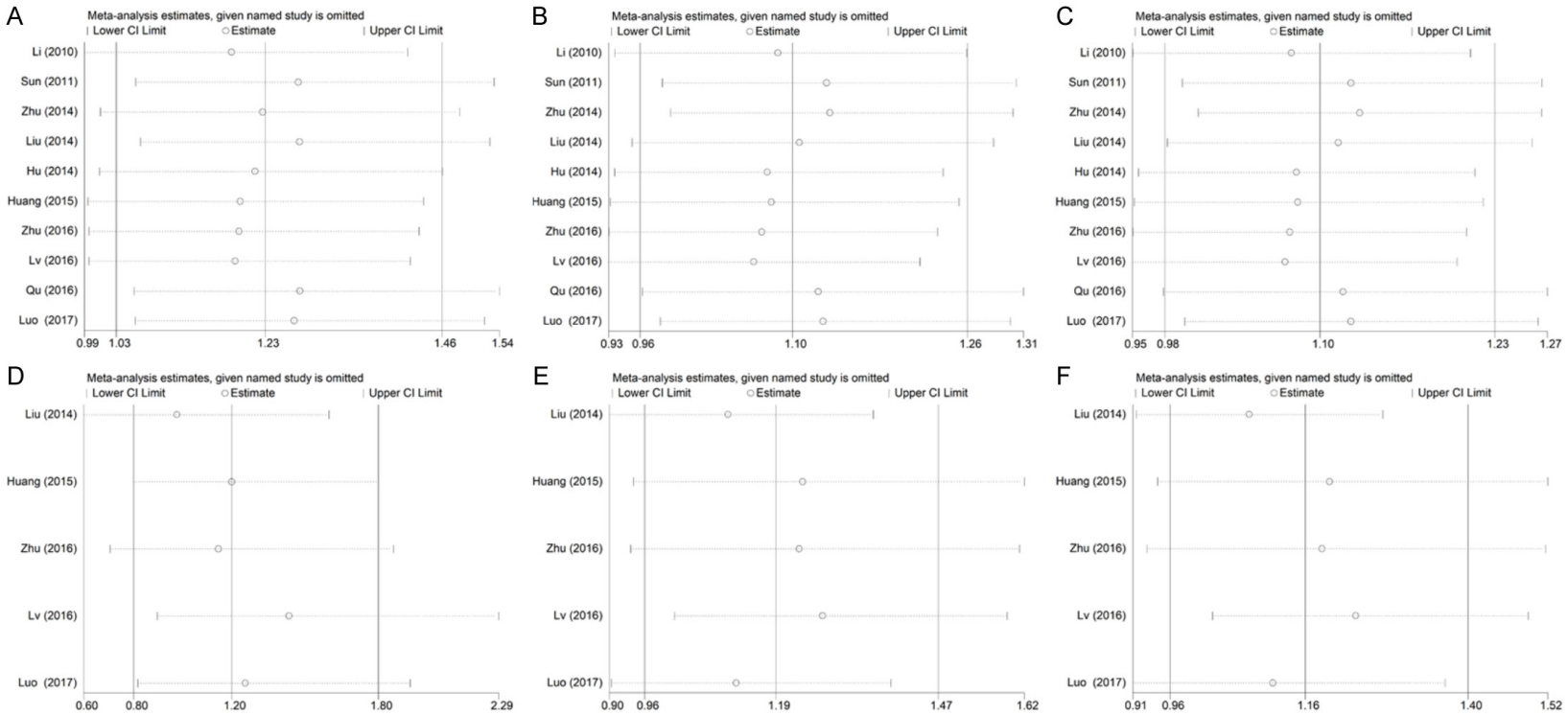
Supplementary Figure 1. Corresponding funnel plots for analyses of the association between *miR-146a* rs2910164, *miR-499* rs3746444 variants and ischemic stroke. A. Funnel plot for *miR-146a* rs2910164 using the recessive model. B. Funnel plot for *miR-146a* rs2910164 using the dominant model. C. Funnel plot for *miR-146a* rs2910164 using the allelic model. D. Funnel plot for *miR-499* rs3746444 using the recessive model. E. Funnel plot for *miR-499* rs3746444 using the dominant model. F. Funnel plot for *miR-499* rs3746444 using the allelic model.

Supplementary Table 1E. Begg's test and Egger's test for funnel plot asymmetries of miR-146a

miRNA (minor allele)	Genetic Model	Models of test	
		Begg's test	Egger'test
miR-146a-rs2910164 (G)	Allelic	0.592	0.412
	Dominant	0.592	0.357
	Recessive	0.474	0.357

Supplementary Table 1F. Begg's test and Egger's test for funnel plot asymmetries of miR-499

miRNA (minor allele)	Genetic Model	Models of test	
		Begg's test	Egger'test
miR-499-rs3746444 (G)	Allelic	0.806	0.322
	Dominant	0.462	0.155
	Recessive	0.308	0.469



Supplementary Figure 2. Sensitivity analyses for pooled results of the association between *miR-146a* rs2910164, *miR-499* rs3746444 variants and ischemic stroke. A. Sensitivity analysis for *miR-146a* rs2910164 using the recessive model. B. Sensitivity analysis for *miR-146a* rs2910164 using the dominant model. C. Sensitivity analysis for *miR-146a* rs2910164 using the allelic model. D. Sensitivity analysis for *miR-499* rs3746444 using the recessive model. E. Sensitivity analysis for *miR-499* rs3746444 using the dominant model. F. Sensitivity analysis for *miR-499* rs3746444 using the allelic model.