

Original Article

The vascular endothelial growth factor (VEGF) gene rs2010963 and rs3025039 polymorphisms and risk of osteosarcoma in Chinese population: evidence from a case-control study and a meta-analysis

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Abstract: The gene encoding vascular endothelial growth factor (VEGF) is recognized as promising candidate for the development of osteosarcoma. Two genetic polymorphisms (rs2010963 and rs3025039) in VEGF have been widely evaluated in association with osteosarcoma in Chinese population, and the results are often inconsistent. We therefore conducted a case-control study and a comprehensive meta-analysis to shed some light on this issue. Although our case-control association study failed to find the association between VEGF gene rs3025039 polymorphism with osteosarcoma occurrence, after a comprehensive meta-analysis over 3700 subjects, we provided evidence that VEGF rs3025039T allele was associated with a significantly increased risk (%) to osteosarcoma (95% confidence interval (95% CI) for odds ratio (OR) = 1.25, 95% CI: 1.12-1.39), in Chinese Han population. The tendency was similar to the homozygous comparison (OR = 1.66, 95% CI: 1.29-2.14), dominant model (OR = 1.22, 95% CI: 1.07-1.40), and recessive model (OR = 1.58, 95% CI: 1.24-2.02). As to rs2010963, this study along with the meta-analysis including 7 studies indicated that rs2010963G allele was associated with a significantly increased risk osteosarcoma occurrence in Chinese population, in the allelic comparison (OR = 1.25, 95% CI: 1.09-1.43) as well as the homozygous comparison (OR = 1.57, 95% CI: 1.21-2.04). We conclude that the VEGF gene rs2010963 and rs3025039 polymorphisms were associated with the risk for osteosarcoma in Chinese population.

Keywords: Vascular endothelial growth factor, polymorphism, osteosarcoma, meta-analysis, association study

Introduction

Human osteosarcoma, arising from mesenchymal tissues, is one of the most frequent primary aggressive bone neoplasm in children and young adults, and is a leading cause of cancer-related death in this age group [1, 2]. Although multimodal therapy including neoadjuvant and adjuvant chemotherapy with aggressive surgical resection has improved clinical outcomes, the prognosis in a large number of patients is still unsatisfactory. It is reported that the overall five-year survival rates remains 60-70% [2], and only less than 20% for patients with metastases [3]. The main mechanism of the exact etiology and development of osteosarcoma is complicated, multistep and multifactorial, that is still not fully explained. However, genetic and environmental factors play important roles in

the carcinogenesis process [4, 5]. The former in particular has been revealed by previous molecular biology studies to contribute to osteosarcoma pathogenesis significantly [6, 7]. A large panel of genes including vascular endothelial growth factor (VEGF) gene has been proposed as susceptible candidates for osteosarcoma [8-11].

Tumor angiogenesis is a prerequisite for osteosarcoma growth and a fundamental step in tumor development and expansion [12, 13]. The gene encoding VEGF on chromosome 6p21.3, which functions in the regulation of endothelial cell growth, has been regarded as one of the most important angiogenesis-promoting factors [14, 15]. Basic research indicated that VEGF silencing could suppress cells proliferation, promote cells apoptosis and reduce

osteosarcoma angiogenesis *in vitro* [16]. Recently, two common polymorphisms in VEGF gene at nucleotide -634G/C (rs2010963) and +936C/T (rs3025039) have drawn wide attention as their mutations were linked with associated with alteration of VEGF protein production. Moreover, the two polymorphisms have been widely accessed in the morbidity of osteosarcoma with an altered risk [8, 10, 11, 17-20], possibly because of the insufficient sample sizes, genetic backgrounds, and selection of study populations.

In this study, we first tried to evaluate the association of rs2010963 and rs3025039 polymorphisms of VEGF gene with osteosarcoma risk in a large Han Chinese population. Then, given the accumulating data and to shed some light on recent conflicting or inconclusive claims, we sought to conduct a comprehensive meta-analysis of this association from both English and Chinese published literature.

Methods study population

This was a hospital-based case-control study with a total of subjects consecutively recruiting from Shanghai Songjiang District Central Hospital, China from January 2009 to December 2015. The study population included 322 unrelated cases with histopathologically confirmed osteosarcoma and 343 cancer-free controls, and all subjects were local residents of Han descent. This study was approved by the Ethics Committee of Shanghai Songjiang District Central Hospital, and was conducted according to the Declaration of Helsinki Principles. All subjects signed the written informed consent.

Genotyping

Blood samples (1 mL) were collected, and genomic DNA was extracted from white blood cells using the TIANamp Blood DNA Kit (Tiangen Biotect [Beijing] Co., LTD). The rs2010963 and rs3025039 polymorphisms of the VEGF gene were determined using polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) methods. The probes and primers for rs2010963 and rs3025039 were designed using the Primer 5.0 software (PREMIER Biosoft, Palo Alto, CA, USA). The forward and reverse primers for VEGF rs2010963 were 5'-GTAGCAAGAGCTCCAGAGAGAA-GT-3' and 5'-TGGACGAAAAGTTTCAGTGCGACG-

3', respectively. The primers for VEGF rs3025039 were 5'-CTCGGTGATTTAGCAGCAAG-3' and 5'-CTCGGTGATTTAGCAGCAAG-3', respectively. PCRs were carried out in a Perkin-Elmer 9700 thermocycler (Waltham, MA, USA) with an initial denaturation step of 8 min at 94°C, followed by 30 cycles at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min. The resulting DNA fragments were electrophoresed on a 3.5% agarose gel and visualized under UV light after ethidium bromide staining. The accuracy of the PCR-RFLP method was tested in 133 (20%) randomly selected DNA samples, which were genotyped again for quality control and found to have complete concordance.

Statistical analysis

Comparisons between osteosarcoma patients and controls were conducted by unpaired t-test for continuous variables and by χ^2 test for categorical variables. To avoid gross genotyping error, rs2010963 and rs3025039 polymorphisms were checked for consistency with Hardy-Weinberg equilibrium (HWE) by χ^2 test. Genotypes were compared by conditional logistic regression analysis under assumptions of additive, dominant and recessive models of inheritance, respectively. Statistical significance was accepted as $P < 0.05$.

Meta analysis

Search strategy for identification of studies: PubMed and EMBASE databases were screened for articles published before May 12, 2016 using the Boolean combinations of subjects terms (vascular endothelial growth factor OR VEGF OR rs2010963 OR -634G/C OR rs3025039 OR +936C/T) AND (osteosarcoma OR OS) AND (polymorphism OR allele OR genotype OR variant OR variation). Articles were restricted to English or Chinese-language and human studies in Chinese population. The full text of the retrieved articles was scrutinized to decide whether information on the topic of interest was included. Reference lists of these retrieved articles and systematic reviews were also checked to determine whether citations of articles that were not initially identified.

Articles were included in this meta-analysis if they examined the hypothesis that VEGF gene rs2010963/rs3025039 polymorphism was associated with osteosarcoma in Chinese popula-

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Table 1. Baseline characteristics of study population

Variable	Patients	%	Controls	%	P value
Age (years)					
< 20	217	67.4	213	62.1	0.1678
≥ 20	105	32.6	130	37.9	
Gender					
Females	91	28.3	101	29.4	0.7973
Males	231	71.7	242	70.6	
Family history of cancer					
Negative	298	92.5	316	92.1	0.8847
Positive	24	7.5	27	7.9	
Tumor location					
Long tubular bones	226	70.2			
Axial skeleton	96	29.8			
Tumor metastasis					
Negative	242	75.9			
Positive	80	24.8			

Table 2. The genotype distributions and allele frequencies of VEGF rs2010963 and rs3025039 polymorphisms between patients and controls, and their prediction for osteosarcoma

Polymorphism		Patients (n = 322)	Controls (n = 343)	P_{χ^2}	OR; 95% CI; P^*
rs2010963	CC	93	123		1.32; 1.06-1.63; 0.013
Genotype (n)	CG	156	165	0.04299	1.38; 0.99-1.91; 0.055
	GG	73	55		1.54; 1.04-2.26; 0.031
Allele (%)	G	46.89	40.09	0.01231	
rs3025039	CC	188	213		1.18; 0.93-1.50; 0.163
Genotype (n)	CT	103	108	0.2802	1.17; 0.86-1.59; 0.328
	TT	31	22		1.55; 0.88-2.75; 0.129
Allele (%)	T	25.62	22.16	0.1384	

Abbreviations: OR, odds ratio; 95% CI: 95% confidence interval; P values were calculated under the additive (the upper), dominant (the middle), and recessive (the lower) models of inheritance.

tion, if they followed a case-control or cross-sectional study design, and if they provided sufficient information on rs2010963/rs3025039 genotype counts between osteosarcoma patients and controls for determining an estimate of odds ratio (OR) and its corresponding 95% confidence interval (95% CI).

In this meta-analysis, we assessed the association between rs2010963/rs3025039 polymorphism and osteosarcoma risk with respect to allelic model, as well as the homozygous comparison, the dominant genetic model and the recessive genetic model. Irrespective of between-study heterogeneity, the random-effects model using the DerSimonian & Laird

method was implemented to bring the individual effect-size estimates together, and the estimate of heterogeneity was taken from the Mantel-Haenszel model [21]. Unadjusted OR and 95% CI were used to compare genetic contrasts between patients and controls. Satisfaction of rs2010963/rs3025039 genotypes with Hardy-Weinberg proportions was performed using the χ^2 test or Fisher's exact test in control groups.

Between-study heterogeneity was assessed by the inconsistency index I^2 statistic (ranging from 0 to 100%), which was documented for the percentage of the observed between-study variability due to heterogeneity rather than chance, with higher values suggesting the existence of heterogeneity [22].

The funnel plots and Egger regression asymmetry test were used to assess publication bias. Egger's test can detect funnel plot asymmetry by determining whether the intercept deviates significantly from zero in a regression of the standardized effect estimates against their precision.

Probability less than 0.05 was judged significant with the exception of the I^2 statistic and Egger's test, where a significance level of less than 0.1 was chosen. Data management and statistical analyses were conducted using STATA version 11.0 for Windows.

Results

Results

Baseline characteristics

We studied 322 osteosarcoma cases (231 male and 91 female) with a mean age of

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Table 3. The baseline characteristics of all eligible studies for meta-analysis

First author	Year	City	Study design	Genotyping method	rs2010963			rs3025039		
					CC (Ca./Co.)	CG (Ca./Co.)	GG (Ca./Co.)	CC (Ca./Co.)	CT (Ca./Co.)	TT (Ca./Co.)
Tie Z. et al.	2014	Inn Mongolia	HB	PCR-RFLP	42/120	80/151	43/59	111/232	39/74	15/24
Li-lian Z. et al.	2015	Beijing	HB	PCR-RFLP	61/67	85/81	30/28	85/92	75/71	16/13
Wang Z. et al.	2014	Ningxia	PB	PCR-RFLP	50/58	165/166	115/118	185/207	116/123	29/12
Zhang G. et al.	2015	Chongqing and Inn Mongolia	HB	PCR-RFLP	48/138	90/170	42/53	66/148	92/175	22/37
Hu GL. et al.	2015	Wuhan	HB	PCR-RFLP	20/18	68/65	42/46	67/79	47/44	16/7
Liu JQ. et al.	2015	Jinan	HB	PCR-RFLP	50/69	91/86	45/31	125/134	46/42	16/10
Zhang HF. et al.	2015	Weifang	HB	PCR-RFLP	/	/	/	128/138	35/32	19/12
Lei C. et al. (the present study)	2016	Shanghai	HB	PCR-RFLP	93/123	156/165	73/55	188/213	103/108	31/22

Abbreviations: HB = hospital-based design; PB = population-based design; Ca. = Case; Co. = Control.

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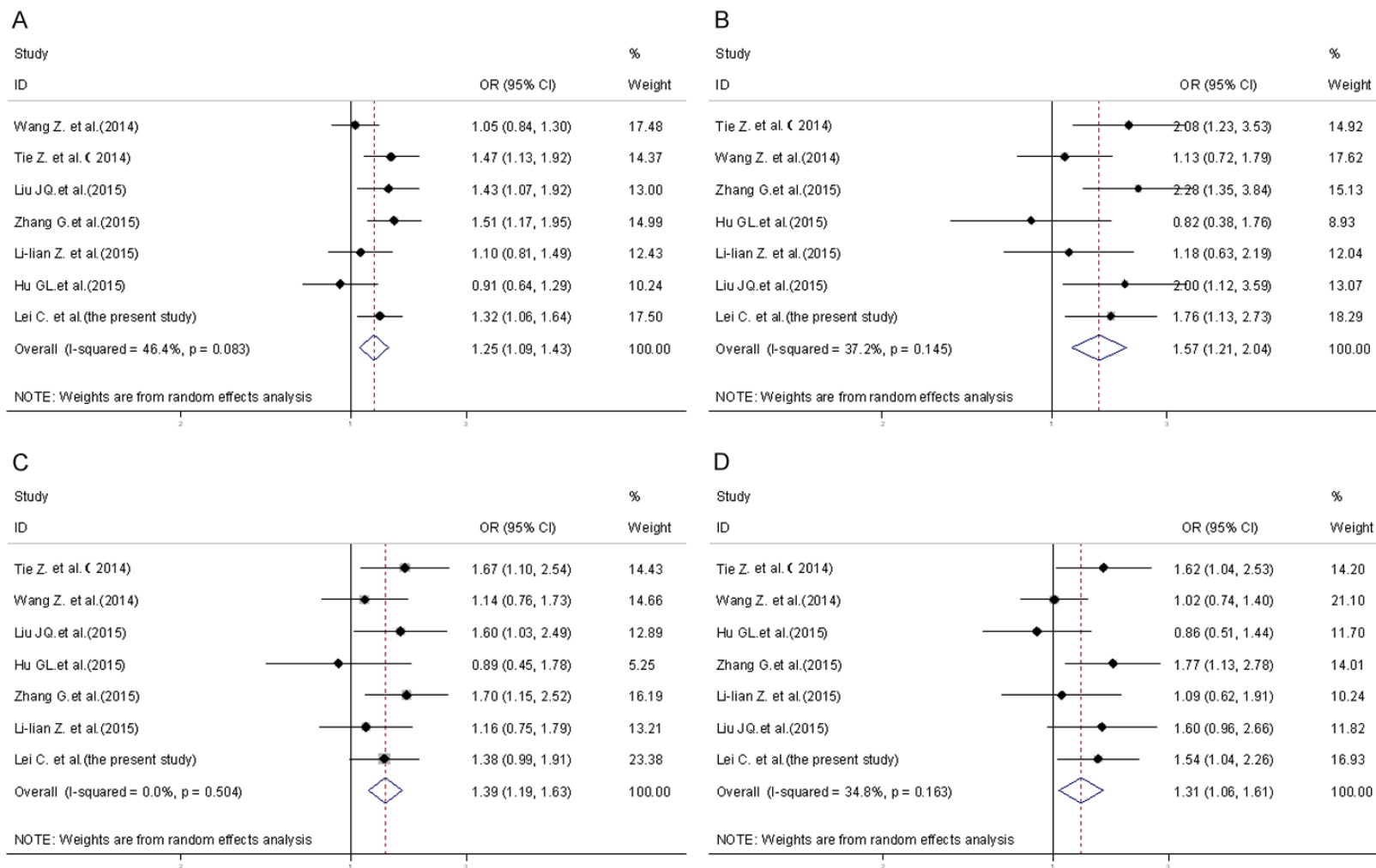


Figure 1. Association of rs2010963 polymorphism with osteosarcoma. (A. Allele comparison; B. Homozygote model; C. Dominant model; D. Recessive model).

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18.1±10.2 years, and 343 healthy controls (242 male and 101 female) with a mean age of 19.3±10.3 years. The demographics in the study population are summarized in **Table 1**. No significant difference was found between osteosarcoma patients and controls in age ($P = 0.1678$) and gender ($P = 0.7973$).

Single-locus analysis

The genotype distributions and allele frequencies of the two polymorphisms from osteosarcoma patients and healthy controls are compared in **Table 2**. The genotype distributions of these two examined polymorphisms respected Hardy-Weinberg equilibrium in both cases and controls ($P > 0.05$).

A significant difference was observed between patients and controls' genotype ($P = 0.04$) and allele ($P = 0.01$) distributions of variant rs2010963; with its mutant G allele was overrepresented in patients relative to controls (46.89% versus 40.09%). The statistical analysis revealed a significant association between rs2010963 polymorphism and the risk of osteosarcoma under the additive model (OR: 1.32 95% CI: 1.06-1.63; $P = 0.013$) and recessive model (OR: 1.54 95% CI: 1.04-2.26; $P = 0.031$).

However, there was no significant difference in the genotype and allele distributions of rs3025039 polymorphism between osteosarcoma cases and controls, and this non-significance was also mirrored under assumptions of the additive (OR = 1.18; 95% CI: 0.93-1.50; $P = 0.163$), dominant (OR = 1.17; 95% CI: 0.86-1.59; $P = 0.328$) and recessive (OR = 1.55; 95% CI: 0.88-2.75; $P = 0.129$) models (**Table 2**).

Meta-analysis

According to the searching rules, the primary screening produced 43 potentially relevant articles. Further application of our inclusion/exclusion criteria left 7 articles qualified. There are 7 studies [8, 10, 11, 17-20] for rs3025039 and 6 studies [8, 10, 11, 18-20] for rs2010963, respectively. Therefore, seven separate studies plus the present study encompassing a total of 1672 patients with osteosarcoma and 2049 controls were finally meta-analyzed for polymorphism rs3025039, and 1489 cases and

1867 controls for rs2010963, respectively. The baseline characteristics of qualified studies are presented in **Table 3**. Taking into account only the controls, genotype distributions were in Hardy-Weinberg equilibrium for all qualified studies.

After combining all qualified studies, we found a significant association between rs2010963 polymorphism and osteosarcoma risk in allelic comparison (OR = 1.25, 95% CI: 1.09-1.43), while accompanying moderate evidence of between-study heterogeneity ($I^2 = 46.4\%$; $P = 0.083$) (**Figure 1**). Besides the suggestive symmetry of funnel plot (**Figure 2**), Egger's test indicated no publication bias for allelic association ($P = 0.681$). The magnitude of OR in allele comparison was similar to the dominant models (OR = 1.39, 95% CI: 1.19-1.63) as well as the recessive model (OR = 1.31, 95% CI: 1.06-1.61). Further, this association was potentially strengthened in the homozygous comparison with OR doubled to 1.57 (95% CI: 1.21-2.04).

As to rs3025039, the pooled OR from all included studies indicated a significant association for osteosarcoma risk in allelic comparison (OR = 1.25, 95% CI: 1.12-1.39), without evidence of heterogeneity ($I^2 = 0.0\%$ $P = 0.941$) and publication bias ($P_{\text{Egger}} = 0.195$). The significant association was also observed in homozygous comparison (OR = 1.66, 95% CI: 1.29-2.14), dominant model (OR = 1.22, 95% CI: 1.07-1.40), and recessive model (OR = 1.58, 95% CI: 1.24-2.02) (**Figure 3**). In addition, there was no publication bias for all models as reflected by funnel plots (**Figure 4**).

Discussion

VEGF coding the key regulator of angiogenesis, is the crucial gene involved in the development and progression of solid tumors including osteosarcoma. Recently researches indicated that VEGF protein has been shown to be amplified in osteosarcoma with poor prognosis [23]. Compare to those with a VEGF-negative tumors, patients with a VEGF-positive osteosarcoma were associated with the worse overall survival rate and lower disease-free survival rate [24, 25]. Furthermore, elevated VEGF secreted by osteosarcoma cells elicits angiogenesis, which critically contributes to the development of pulmonary metastasis of

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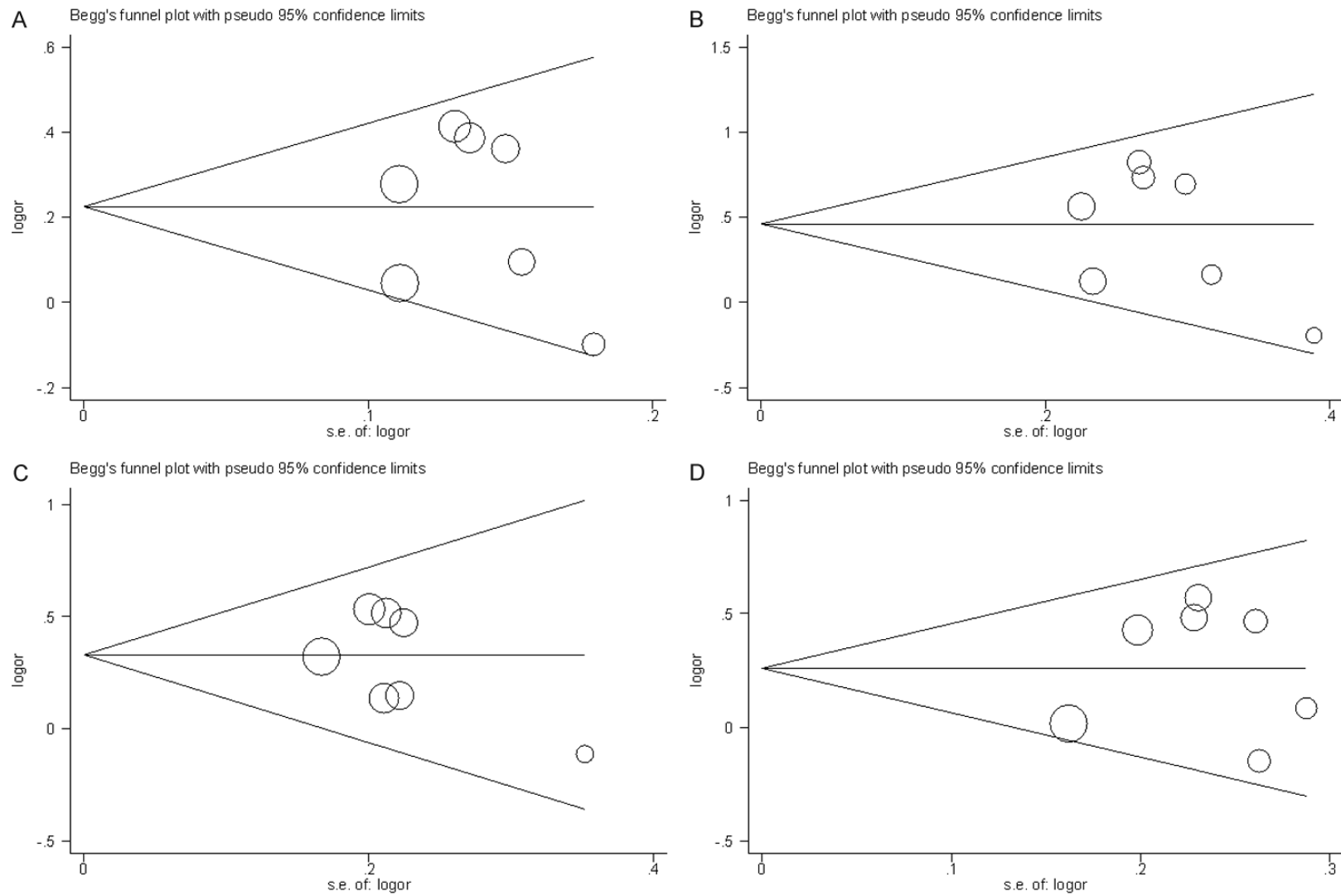


Figure 2. Begg's funnel plots of publication bias test for rs2010963 polymorphism with osteosarcoma. (A. Allele comparison; B. Homozygote model; C. Dominant model; D. Recessive model).

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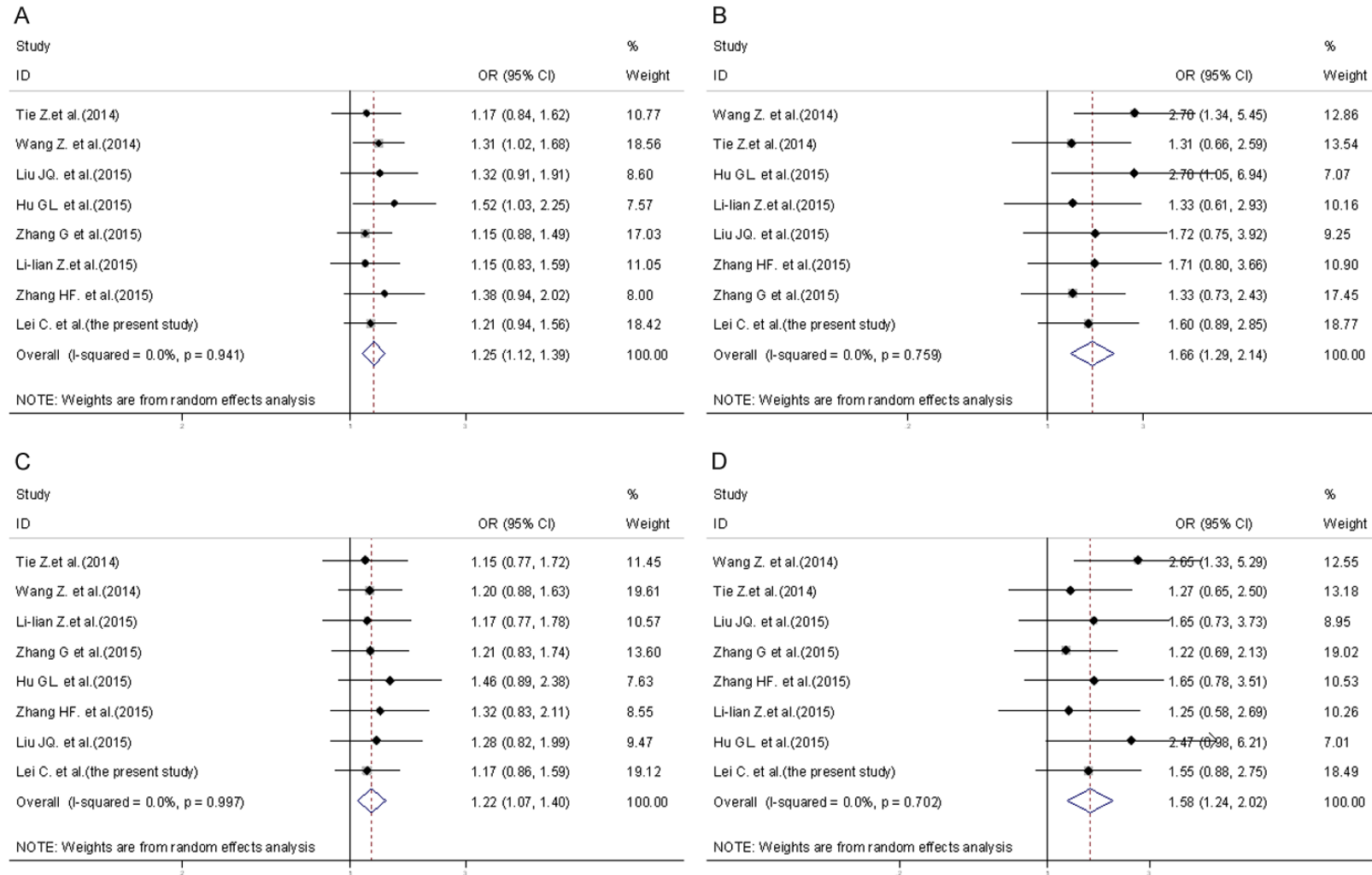


Figure 3. Association of rs3025039 polymorphism with osteosarcoma. (A. Allele comparison; B. Homozygote model; C. Dominant model; D. Recessive model).

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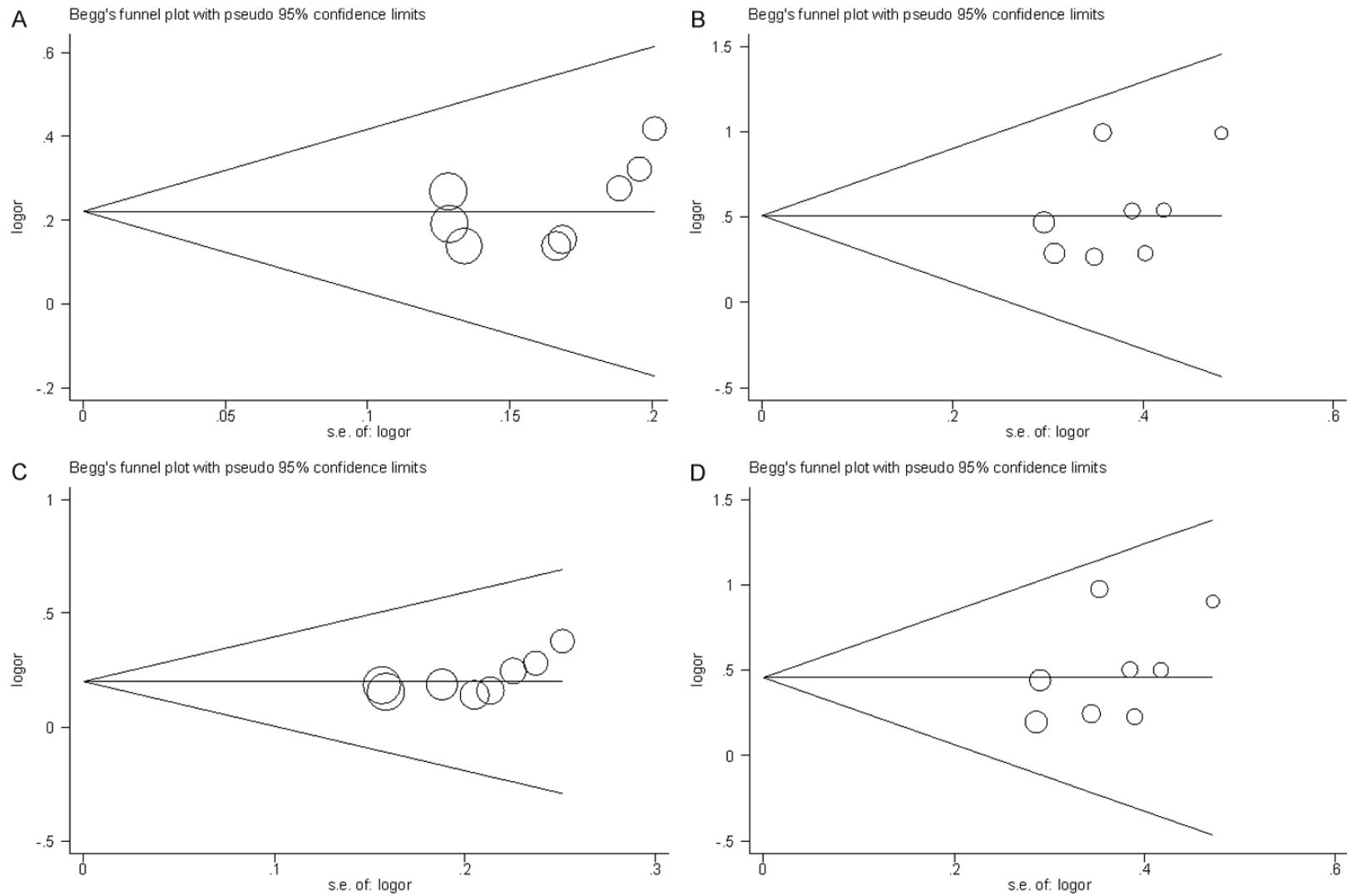


Figure 4. Begg's funnel plots of publication bias test for rs3025039 polymorphism with osteosarcoma. (A. Allele comparison; B. Homozygote model; C. Dominant model; D. Recessive model).

osteosarcoma [25]. Contrarily, suppression the expression of VEGF could not only inhibit the osteosarcoma cell proliferation capability and promote apoptosis *in vitro*, but also suppress osteosarcoma tumor growth and reduce osteosarcoma angiogenesis in the Wistar rats model *in vivo* [16]. With respect to the important roles of VEGF in tumor angiogenesis regulation, it is biologically plausible that VEGF genetic polymorphisms, may modulate the risk of various cancers and prognosis. Kim *et al.* found that the rs3025039 CC/CT genotype strongly correlated with favourable leukaemia-free survival at 2 years (51.3%) versus with rs3025039 CC genotype (33.6%, $P = 0.03$) [26]. Wu *et al.* [27] reported that rs3025035 is associated with recurrence of hepatocellular carcinoma after transplantation, patients with rs3025035 CT heterozygous was independently associated with a shortened recurrence-free survival (OR: 3.3; 95% CI: 1.8-6.0; $P < 0.001$). Meanwhile, in a retrospective multicentre study (ALICE-1 study), rs2010963 polymorphsim was identified as one of independent factors influencing PFS and overall survival in sorafenib-treated hepatocellular carcinoma patients [28].

In this study, our case-control study in Han Chinese population along with the meta-analysis including 7 studies indicated that rs2010963G allele was associated with a significantly increased risk osteosarcoma occurrence. In addition, although this case-control study in Han Chinese failed to find the association between VEGF gene rs3025039 polymorphism with osteosarcoma occurrence, after a comprehensive meta-analysis over 3700 subjects, we provided evidence that VEGF rs3025039T allele was associated with a significantly increased risk to osteosarcoma, in Chinese Han population. To the authors' knowledge, this is the most comprehensive meta-analysis investigating the genetic susceptibility of VEGF gene rs2010963 and rs3025039 polymorphisms to osteosarcoma in Chinese population. Furthermore, the relatively large samples examined and low probability of publication bias as reflected by visual inspection of the funnel plots along with Egger's tests indicated the robustness of our results. This finding is biologically plausible, especially in light of the putative function of these polymorphisms [29-32]. Rs3025039 polymorphism located at the promoter region of VEGF and play a role in influence the VEGF protein translation efficiency,

circulating plasma concerntrations and expression of VEGF in tumor tissues, while the variant C allele of rs2010963 polymorphism has also been associated with lower VEGF production [29, 33]. Numerous studies have reported that these two functional polymorphisms were associated with several types of cancers. For example, Li *et al.* [34] conducted a hospital-based case-control study to identify that rs2010963 [OR = 1.29; 95% CI = 1.04-1.58; GC/CC vs. GG] was associated with increased risk for glioma. A meta-analysis of six case-control studies suggested that the VEGF rs3025039 T allele was associated with an increased risk of oral cancer, especially among Caucasian populations [35].

It should be noted, however, that the exact functions of the rs2010963 and rs3025039 polymorphisms are still debated, partly because different genetic backgrounds may cause this discrepancy or different populations may have different linkage disequilibrium patterns. The situation is not rare. Amongst the few studies in ovarian cancers, rs3025039 was observed to be associated as a risk factor in the Brazilian [36] and Indian populations [37], whereas was associated with better prognosis in another USA study [38]. As for the VEGF rs2010963 polymorphism, Lose *et al.* [39] found that ovarian cancer patients homozygous for the functional rs2010963 displayed significantly shortened overall survival in the Australian population (HR 2.09, 95% CI 1.16-3.78). However, another study including rs2010963, rs1570360 and rs699947 indicated no significant association of the polymorphisms with any clinico-pathological parameters and no association with progression free survival or prognosis in Denmark population [40]. Sáenz-López *et al.* [41] failed to find any VEGF polymorphisms to exert a significant influence on renal cell carcinoma progression or prognosis in Spanish population. However, Qin *et al.* [42] conducted a large case-control study to find the rs2010963 was associated with risk of clear cell renal cell carcinoma in Chinese population.

Despite the clear strengths of our study including large sample sizes, no deviation from Hardy-Weinberg equilibrium for all studies and low possibility of publication bias across all genetic models, interpretation of our current study, should be viewed in light of several technical

limitations. All of the studies in this meta-analysis were case-control studies, which are susceptible to selection bias by including only non-fatal cases. In addition, because only published studies were retrieved in this meta-analysis and the “grey” literature (articles in languages other than English or Chinese) was not included, publication bias might be possible, even though our funnel plots and statistical tests did not show it. Moreover, the single-locus-based nature of meta-analysis precluded the possibility of gene-gene and gene-environment interactions, as well as haplotype-based effects, suggesting that additional studies assessing these aspects will be necessary. Furthermore, we only centered on two functional (rs2010963 and rs3025039) polymorphisms of *VEGF* gene, and did not cover other candidate genes or polymorphisms. So whether these two polymorphisms integrated with other risk factors will enhance the prediction requires additional research.

In summary, we performed a case-control study and a meta-analysis to expand previous single studies on osteosarcoma by suggesting that *VEGF* gene rs2010963 and rs3025039 polymorphisms might contribute to the occurrence of osteosarcoma in Chinese population. Moreover, considering the fact of divergent genetic profiles across different ethnic groups, further large, well-designed studies in different ethnic populations are needed. Nonetheless, for practical reasons, we hope that this study will not remain just another endpoint of research instead of a beginning to establish the background data for further investigation on pathophysiological mechanisms of *VEGF* gene on osteosarcoma.

Disclosure of conflict of interest

None.

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