

Clinicopathological significance and potential drug target of *RUNX3* in non-small cell lung cancer: a meta-analysis

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Background: Emerging evidence indicates that *RUNX3* is a candidate tumor suppressor in several types of human tumors, including non-small cell lung cancer (NSCLC). However, the correlation between *RUNX3* hypermethylation and clinicopathological characteristics of NSCLC remains unclear. Here, we conducted a systematic review and meta-analysis to quantitatively evaluate the effects of *RUNX3* hypermethylation on the incidence of NSCLC and clinicopathological characteristics.

Methods: A detailed literature search was made using Medline, Embase and Web of Science for related research publications written in English. The methodological quality of the studies was evaluated. The data were extracted and assessed independently by two reviewers. Analysis of pooled data was performed. The odds ratio (OR) and hazard ratio were calculated and summarized.

Results: Final analysis of 911 NSCLC patients from 13 eligible studies was performed. We observed that *RUNX3* hypermethylation was significantly higher in NSCLC than in normal lung tissue; the pooled OR from seven studies including 361 NSCLC and 345 normal lung tissue (OR 7.08, confidence interval 4.12–12.17, $P < 0.00001$). *RUNX3* hypermethylation may also be associated with pathological types. The pooled OR was obtained from eleven studies including 271 squamous cell carcinoma and 389 adenocarcinoma (OR 0.41, confidence interval 0.19–0.89, $P = 0.02$), which indicated that *RUNX3* hypermethylation is significantly higher in adenocarcinoma than in squamous cell carcinoma. We did not find that *RUNX3* hypermethylation was correlated with clinical stage or differentiated status. However, NSCLC patients with *RUNX3* hypermethylation had a lower survival rate than those without *RUNX3* hypermethylation.

Conclusion: The results of this meta-analysis suggest that *RUNX3* hypermethylation is associated with an increased risk and worse survival in NSCLC. *RUNX3* hypermethylation, which induces inactivation of the *RUNX3* gene, plays an important role in lung carcinogenesis and clinical outcome.

Keywords: *RUNX3*, methylation, lung cancer, tumor suppressor gene, meta-analysis, odds ratio, hazard ratio

Introduction

Non-small cell lung cancer (NSCLC) includes several histological types, including adenocarcinoma, squamous cell carcinoma, large cell carcinoma, and mixed histologies, and accounts for approximately 85% of all lung cancers, making NSCLC the leading cause of cancer-related deaths worldwide.^{1,2} Despite advances in early detection, radical curative surgery, and multimodal therapeutic modalities, about 80% of NSCLC cases are in an advanced stage at diagnosis; systemic chemotherapy remains the standard care but provides marginal improvement in survival.³ Therefore, further investigation on the mechanism of initiation, progression, and identification of prognostic markers

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is still needed and will help to select patients with a higher risk of disease recurrence and provide a better prognosis and individualized treatment.

The runt-domain-related (*RUNX*) family genes, which comprise *RUNX1*, *RUNX2*, and *RUNX3*, have come to prominence recently because of their roles as essential regulators of cell fate in development and their paradoxical effects in cancer, as well as in the regulation of p53-dependent DNA damage response and/or tumorigenesis.⁴⁻⁶ The *RUNX3* gene is one of the most important members of the runt-domain family, and plays a critical role in regulation of cell proliferation and cell death by apoptosis, and in angiogenesis, cell adhesion, and invasion.^{7,8} The *RUNX3* gene, localized on chromosome 1p36, a region that exhibits frequent loss of heterozygosity events in breast, colon, gastric, and ovarian cancers, is considered to be a tumor suppressor gene involved in the transforming growth factor-beta signaling pathway.⁹ *RUNX3* and the ubiquitous p53 protein are both principal responders of the p14(ARF)-MDM2 cell surveillance pathway that prevents pathologic consequences of abnormal oncogene activation.¹⁰ Its precise function has been intensively studied in several tumors, and it is associated with upregulation of inducing cell cycle arrest, apoptosis, and downregulation of cyclin D1 expression.¹¹⁻¹⁵ Lack of protein expression of *RUNX3* by promoter methylation (hypermethylation) has been found to play an important role in regulation of lung alveolar differentiation and epithelial tumorigenesis.¹⁶⁻²⁰ However, its role in NSCLC and its clinical significance have not been thoroughly investigated. In this study, we review and update the published clinical investigations regarding the effect of *RUNX3* in patients with NSCLC.

Materials and methods

Search strategy and selection criteria

We searched PubMed, Embase, and ISI Web of Knowledge to identify studies from January 1, 2000 to October, 2014 using the following MeSH terms and keywords: “lung” and “cancer or tumor or neoplasm or carcinoma”, “methylation”, and “*RUNX3*”. We also manually searched the reference lists of the retrieved articles and reviews for additional articles. Although our search did not have language limits initially, we only included studies published in the English language for full-text reading and final evaluation. Conference abstracts were not selected because of insufficient data reported. After exclusion of non-relevant and/or redundant publications from the databases, the remaining papers were evaluated in the full-text version for inclusion and exclusion and for relevant articles in the reference lists. All searched data were retrieved.

Authors' bibliographies and references of selected studies were also searched for additional relevant studies. The most complete study was chosen to avoid duplication if the same patient population was reported in several publications.

Criteria that an eligible study has to meet were as follows: *RUNX3* methylation evaluated in the primary NSCLC tissues; researchers revealed the relationship between *RUNX3* methylation, NSCLC clinicopathological parameters, and prognosis; *RUNX3* methylation examined by polymerase chain reaction; studies that provide a hazard ratio (HR) and 95% confidence interval (CI); and studies that provided sufficient information to estimate HR for overall survival and 95% CI. Letters, reviews, case reports, conference abstracts, editorials, expert opinion, and all publications regarding in vitro/ex vivo studies, cell lines, and human xenografts were excluded.

Data extraction and methodological assessment

Two authors (LX, HL) independently reviewed and extracted the data from eligible studies. Disagreements were resolved by discussion and consensus. Two authors (YS, JL) reviewed all articles that fitted the inclusion and exclusion criteria. The following information was recorded for each study: the first author's name, year of publication, sample source, number of cases, clinicopathological parameters, cancer TNM (tumor node metastasis) stage, methylation detection method, methylation rate and/or expression, and follow-up. Data for study characteristics and clinical responses were summarized and the data were converted into table format. Heterogeneity of investigation was evaluated to determine whether the data from the various studies could be subjected to meta-analysis.

For the methodological evaluation of the studies, three investigators (LX, HL, XZ) read through each publication independently, and assessed and scored them according to the REMARK (REporting recommendations for tumor MARKer prognostic studies) guidelines and European Lung Cancer Working Party quality scale.^{21,22} The three readers provided quality scores and compared them, and then reach a consensus value for each item.

Statistical analysis

The statistical analysis was conducted using Stata version 12.0 (Stata Corporation, College Station, TX, USA) and Review Manager 5.2 (Cochrane Collaboration, Oxford, UK). The pooled frequency of *RUNX3* hypermethylation and 95% CI were estimated. The frequency of *RUNX3* hypermethylation

was compared against different tumor characteristics. Heterogeneity among the studies was evaluated using the Cochran's Q test²³ and the I^2 statistic.^{24,25} When heterogeneity was not an issue (I^2 values $<50\%$), a fixed-effect model was used to calculate parameters. If there was substantial heterogeneity (I^2 values $\geq 50\%$), a random-effects model was used to pool data and an attempt was made to identify potential sources of heterogeneity based on subgroup analyses. The pooled odds ratio (OR) was estimated for the association between *RUNX3* hypermethylation and clinicopathological features. P -values tailed less than 0.05 were considered to be statistically significant.

Publication bias was assessed using a method reported by Egger et al.²⁶ We also explored reasons for statistical heterogeneity using meta-regression, subgroup analysis, and sensitivity analysis. The analysis of meta-regression and publication bias was performed using Stata version 10.0.

Results

Identification of relevant studies

Forty-seven publications were identified by the search method described above. Thirty-four papers were excluded

for being laboratory studies, non-original articles (reviews), or studies irrelevant to the current analysis. Eventually, 13 studies were included in the final meta-analysis,²⁷⁻³⁹ as shown in Figure 1. Of the 13 studies, eleven scored eight points, five scored seven points, and one scored six points. Hence, the studies were of relatively high quality (Table 1).

Study characteristics

Thirteen studies published from 2003 to 2012 were eligible for meta-analysis. A total of 911 NSCLC patients from the People's Republic of China, Japan, Singapore, South Korea, Spain, and the USA were enrolled. Their basic characteristics are summarized in Table 2.

Correlation of *RUNX3* hypermethylation with clinicopathological features

Inactivation of *RUNX3* through hypermethylation in NSCLC

The loss of *RUNX3* mRNA and/or protein expression is strongly correlated with promoter hypermethylation in several types of cancer, including NSCLC.^{28,34,40-45} *RUNX3* hypermethylation is significantly higher in NSCLC than

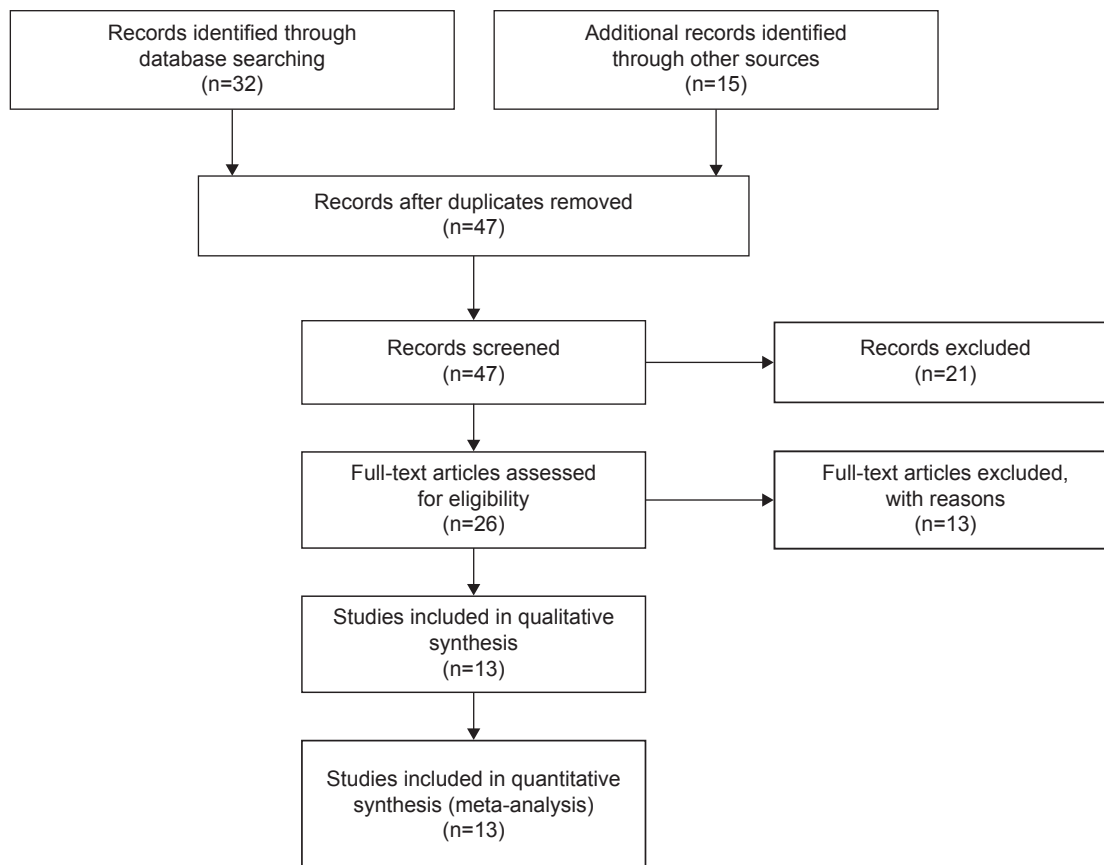


Figure 1 Flow chart of study selection.

Table 1 Quality assessment of the included studies according to the Newcastle–Ottawa scale

Study	Selection	Comparability	Exposure	Total score
Yu et al ²⁷	3	2	2	7
Omar et al ²⁸	3	2	3	8
Tang et al ²⁹	3	2	2	7
Yanagawa et al ³⁰	3	2	3	8
Castro et al ³¹	2	2	3	7
Yoshino et al ³²	3	2	3	8
Yanagawa et al ³³	3	2	3	8
Sato et al ³⁴	3	2	2	7
Suzuki et al ³⁵	3	2	3	8
Li et al ³⁶	3	2	2	7
Yanagawa et al ³⁷	3	2	3	8
Hawes et al ³⁸	3	2	3	8
Feng et al ³⁹	3	1	2	6

in normal lung tissue. The pooled OR from seven studies including 361 NSCLC and 345 normal lung tissue is shown in Figure 2 (OR 6.17, CI 3.66–10.41, $P < 0.00001$), indicating that *RUNX3* inactivation through hypermethylation plays an important role in the pathogenesis of NSCLC.

Relationship between frequency of *RUNX3* hypermethylation and smoking status

Sato et al reported that the *RUNX3* hypermethylation rate in NSCLC patients without a smoking history was significantly higher than that in patients with a smoking history.³⁴ The

Table 2 Basic characteristics of the included studies

Study	Country	Patients (n)	Methods	Primary aim	Methylation site	<i>RUNX3</i> expression
Yu et al ²⁷	People’s Republic of China	58	MSP	Determine methylation status of the <i>RUNX3</i> gene and correlate results with clinicopathological characteristics	Promoter, CpG islands	–
Omar et al ²⁸	Singapore	9	MSP	Determine frequency of <i>RUNX3</i> promoter hypermethylation	Promoter, CpG islands	+
Tang et al ²⁹	People’s Republic of China	80	MSP	Determine methylation status of the <i>RUNX3</i> gene and correlate results with clinicopathological characteristics	Promoter, CpG islands	–
Yanagawa et al ³⁰	Japan	62	MSP	Determine methylation status of ten tumor suppressors in NSCLC	Promoter, CpG islands	–
Castro et al ³¹	Spain	54	MS-MLPA	Determine clinical significance of several tumor suppressors in NSCLC	Promoter, CpG islands	–
Yoshino et al ³²	Japan	44	MSP	Determine methylation status of 14 tumor suppressors in NSCLC	Promoter, CpG islands	–
Yanagawa et al ³³	Japan	101	MSP	Determine whether methylation status of several genes is correlated with clinicopathological features	Promoter, CpG islands	–
Sato et al ³⁴	Japan	120	MSP, RT-PCR	Determine role of <i>RUNX3</i> in pathogenesis of NSCLC	Promoter, CpG islands	+
Suzuki et al ³⁵	Japan	117	MSP	Determine role of TGF-β-related genes in many human malignancies	Promoter, CpG islands	–
Li et al ³⁶	South Korea	25	MSP, RT-PCR	Determine role of <i>RUNX3</i> in pathogenesis of NSCLC	Promoter, CpG islands	+
Yanagawa et al ³⁷	Japan	75	MSP	Analysis of clinicopathological significance of gene promoter methylation in NSCLC	Promoter, CpG islands	–
Hawes et al ³⁸	USA	117	MethyLight	Detection of methylation profiles for 27 genes in NSCLC	Promoter, CpG islands	–
Feng et al ³⁹	USA	49	MethyLight	Analyze DNA methylation status of several genes in NSCLC patients	Promoter, CpG islands	–

Abbreviations: NSCLC, non-small cell lung cancer; MS-MLPA, methylation-specific multiplex ligation-dependent probe amplification; MSP, methylation-specific polymerase chain reaction; TGF-β, transforming growth factor-beta; RT-PCR, reverse transcription polymerase chain reaction; +, positive; –, negative.

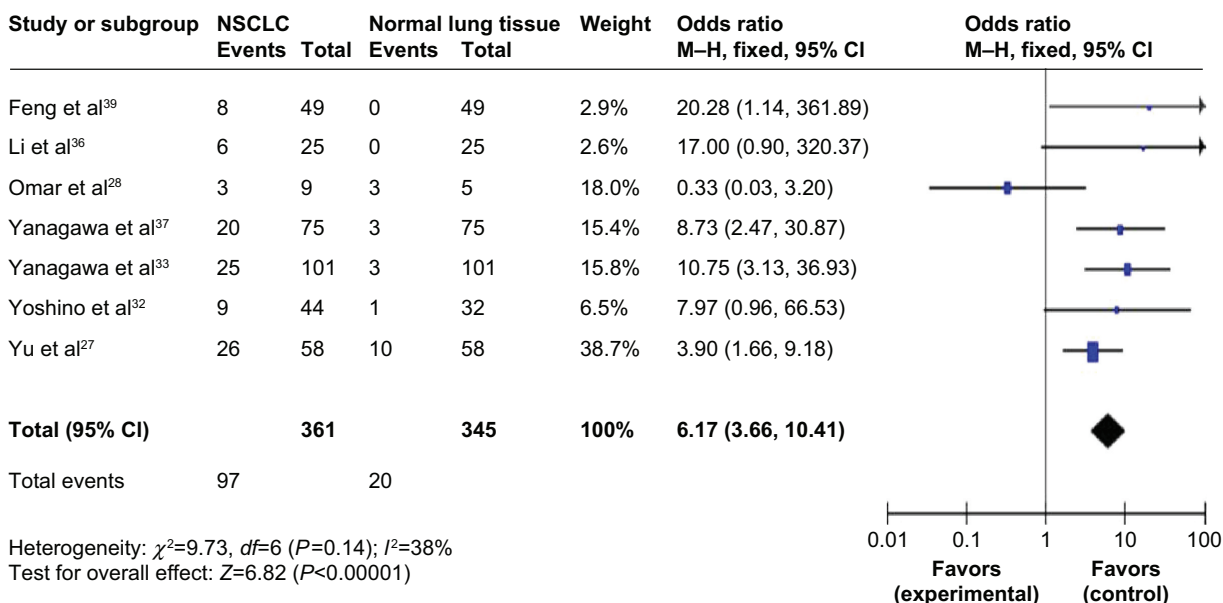


Figure 2 Pooled OR from seven studies including 361 NSCLC and 345 normal lung tissue (OR 6.17, CI 3.66–10.41, $P<0.00001$).
Abbreviations: CI, confidence interval; OR, odds ratio; M-H, Mantel-Haenszel; NSCLC, non-small cell lung cancer.

pooled OR from eight studies including 455 and 202 NSCLC with or without a smoking history is shown in Figure 3A (OR 0.51, CI 0.24–1.08, $P=0.08$), which indicates that *RUNX3* hypermethylation is not strongly associated with smoking status in NSCLC patients.

Relationship between frequency of *RUNX3* hypermethylation and pathological types

Previously, Omar et al reported that there was an obvious difference between the activity of *RUNX3* in adenocarcinoma and squamous cell carcinoma, indicating that the absence of active *RUNX3* in adenocarcinoma and the aberrant downregulation of *RUNX3* through hypermethylation may be associated with the development of these tumors.²⁸ The pooled OR from eleven studies including 271 squamous cell carcinomas and 389 adenocarcinomas is shown in Figure 3B (OR 0.41, CI 0.19–0.89, $P=0.02$), indicating that *RUNX3* hypermethylation plays a more important role in the pathogenesis of adenocarcinoma.

Role of *RUNX3* hypermethylation in progression of NSCLC

We analyzed 634 NSCLC patients pooled from eight studies to assess whether or not the aberrant *RUNX3* hypermethylation in NSCLC was associated with more advanced stage. As shown in Figure 4A, aberrant *RUNX3* hypermethylation is not significantly higher in advanced NSCLC (III and IV) than in early stage NSCLC (I and II; OR 1.21, CI 0.61–2.42, $P=0.59$). In addition, as shown in Figure 4B, aberrant *RUNX3* hypermethylation is not significantly higher in poorly differentiated

NSCLC than in moderately and highly differentiated NSCLC (OR 0.65, CI 0.14–2.99, $P=0.58$). These results suggest that epigenetic silencing of *RUNX3* gene expression by promoter hypermethylation may not play an important role in the progression and development of NSCLC.

RUNX3 hypermethylation as a prognostic factor for NSCLC

Only three studies evaluated the relationship between *RUNX3* hypermethylation and overall survival in NSCLC. The pooled HR for overall survival showed that *RUNX3* hypermethylation was associated with worse survival in NSCLC patients, as shown in Figure 4C (HR 3.12, 95% CI 1.47–6.62, $P=0.003$).

Subgroup analysis and meta-regression

For the group in which heterogeneity is strong, we performed subgroup analysis and meta-regression to analyze the sources of the heterogeneity. In the case of the frequency of *RUNX3* hypermethylation and smoking status, the pooled OR for two studies from the People's Republic of China for patients with or without a smoking history is shown in Figure 5A ($I^2=47\%$, OR 0.68, CI 0.30–1.50, $P=0.33$), indicating that heterogeneity was not strong, suggesting that *RUNX3* hypermethylation is not strongly associated with smoking status in NSCLC patients. The pooled OR for six studies from Japan for patients with or without smoking history is shown in Figure 5B ($I^2=76\%$, OR 0.46, CI 0.17–1.22, $P=0.12$), indicating that the heterogeneity is still strong, and that *RUNX3* hypermethylation was not significantly

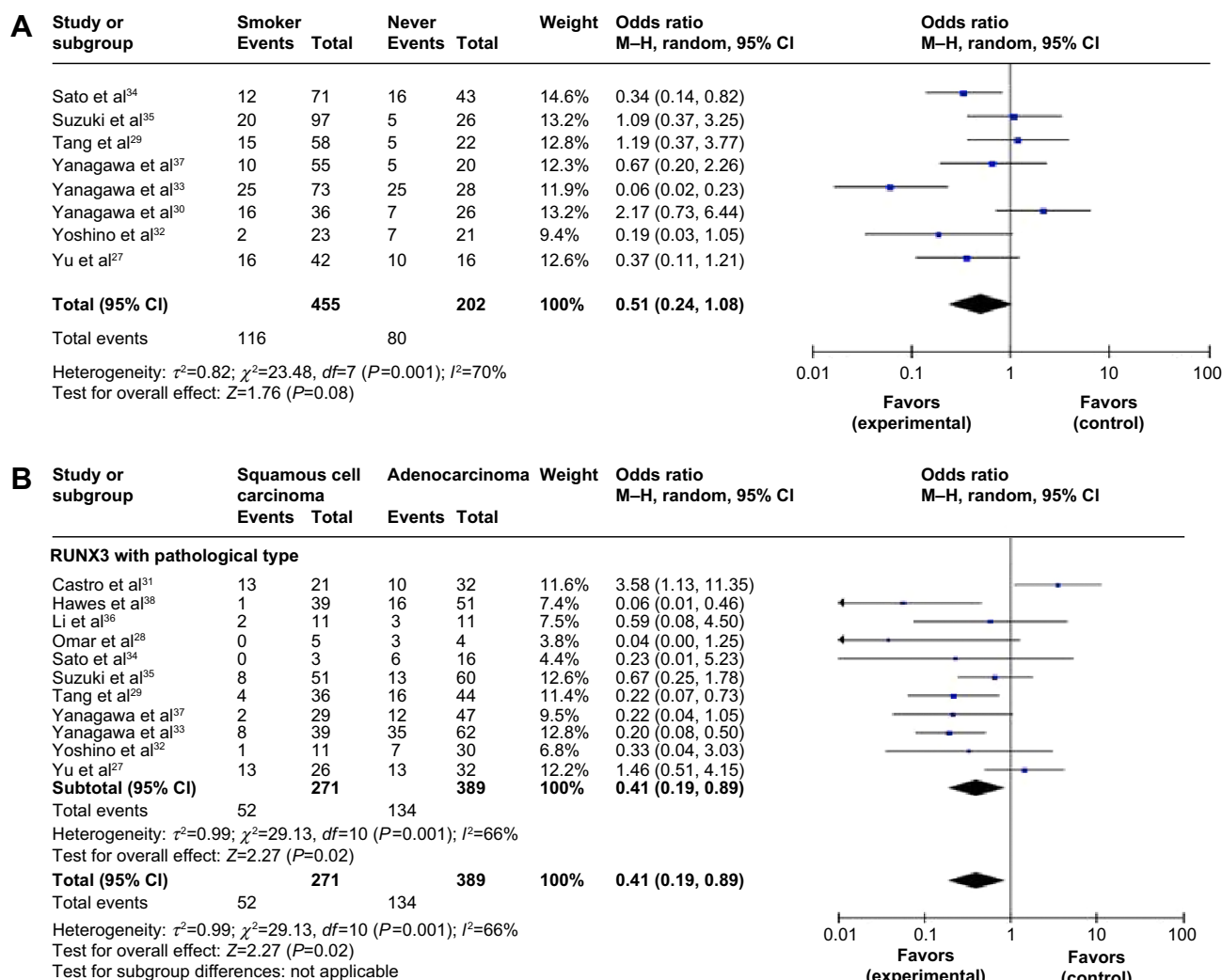


Figure 3 Relationship between the frequency of *RUNX3* hypermethylation and pathological types. **Notes:** (A) NSCLC patients (n=657) with smoking status pooled from eight studies. Aberrant *RUNX3* hypermethylation was not strongly associated with smoking status in NSCLC patients (OR 0.51, CI 0.24–1.08, $P=0.08$). (B) The pooled OR from eleven studies including 271 squamous cell carcinoma and 389 adenocarcinoma (OR 0.41, CI 0.19–0.89, $P=0.02$), indicating that *RUNX3* hypermethylation plays a more important role in the pathogenesis of adenocarcinoma. **Abbreviations:** CI, confidence interval; OR, odds ratio; M-H, Mantel-Haenszel; NSCLC, non-small cell lung cancer.

associated with smoking status in NSCLC patients. However, the pooled OR for four studies from Japan for patients with or without a smoking history is shown in Figure 5C ($I^2=50\%$, OR 0.93, CI 0.52–1.67, $P=0.82$), indicating that the heterogeneity was not strong, and that *RUNX3* hypermethylation is not significantly associated with smoking status in NSCLC patients.

Sensitivity analyses and publication bias

A sensitivity analysis, in which one study was removed at a time, was conducted to assess the stability of the results. The pooled ORs and HRs were not significantly changed, indicating that our analyses were stable. In the case of a relationship between *RUNX3* hypermethylation in NSCLC and in normal lung tissue, the overall OR ranged from 4.48

(95% CI 2.04–9.87) to 6.17 (95% CI 3.66–10.41). The funnel plots are largely symmetric (Figure 6), suggesting there was no publication bias in the meta-analysis of *RUNX3* methylation/expression and clinicopathological features.

Discussion

RUNX3 belongs to the runt-domain family of transcriptional factors, and its inactivation by promoter hypermethylation plays an important role during normal development and in tumorigenesis in several types of tumors, including NSCLC.^{11,16–18,46–56} To date, there have been some studies describing the precise expression, prognostic impact, and methylation status of *RUNX3* in NSCLC; however, the role of inactivation of *RUNX3* in NSCLC and its clinical significance have not been thoroughly investigated. We conducted

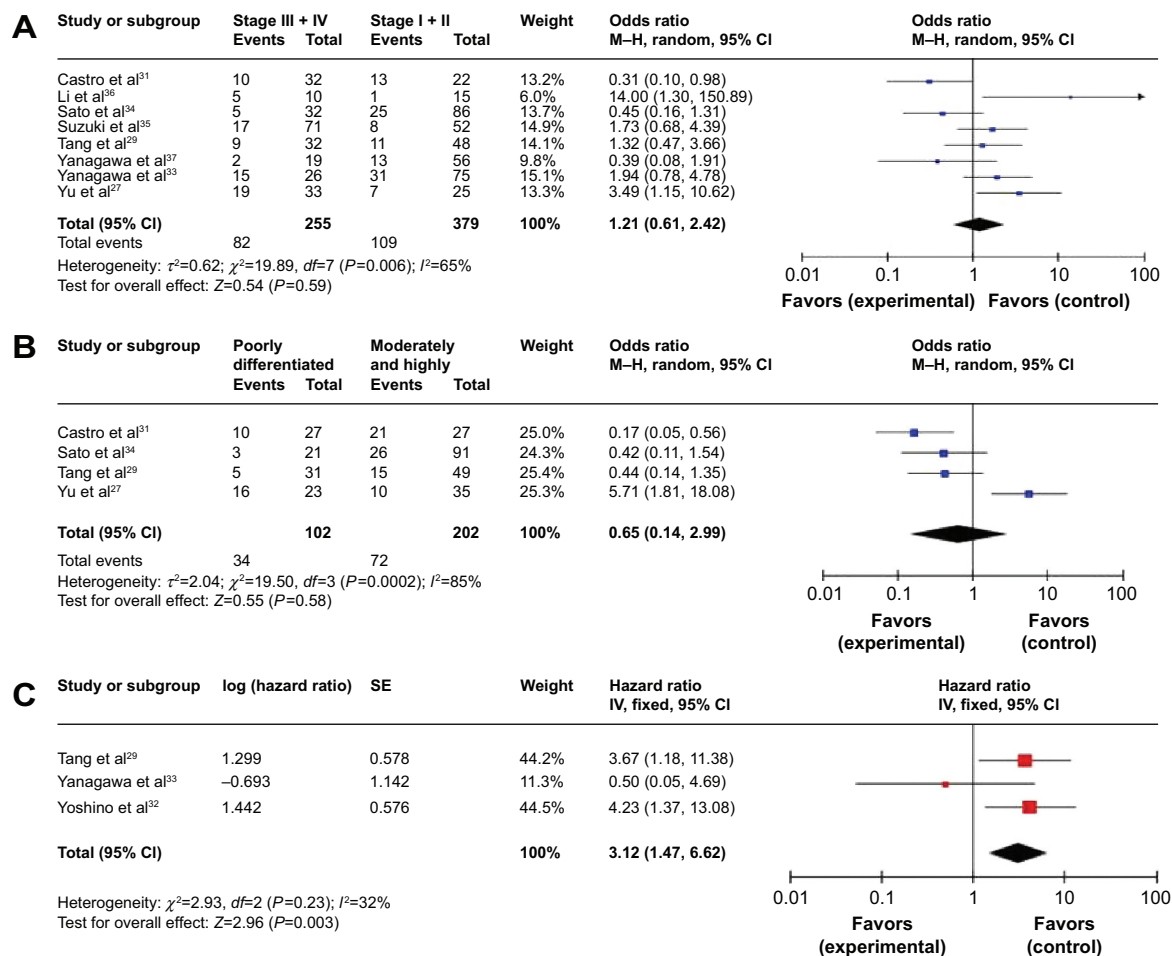


Figure 4 NSCLC patients (n=634) pooled from eight studies to assess whether or not aberrant *RUNX3* hypermethylation in NSCLC is associated with a more advanced stage of disease.

Notes: (A) Aberrant *RUNX3* hypermethylation was not significantly higher in advanced NSCLC (III and IV) than in early stage NSCLC (I and II; OR 1.21, CI 0.61–2.42, $P=0.59$). (B) Aberrant *RUNX3* hypermethylation was also not significantly higher in poorly differentiated NSCLC than that in moderately and highly differentiated NSCLC (OR 0.65, CI 0.14–2.99, $P=0.58$). (C) Three included studies investigated relationships between overall survival and *RUNX3* hypermethylation. The pooled HR for overall survival showed that *RUNX3* hypermethylation was associated with worse survival in NSCLC (HR 3.12, 95% CI 1.47–6.62, $P=0.003$).

Abbreviations: CI, confidence interval; HR, hazard ratio; OR, odds ratio; M-H, Mantel-Haenszel; NSCLC, non-small cell lung cancer; SE, standard error.

the present meta-analysis to determine the correlation between *RUNX3* hypermethylation and clinicopathological characteristics in NSCLC. Analysis of the pooled data showed that: NSCLC had higher hypermethylation than normal lung tissue (OR 6.17); *RUNX3* hypermethylation was not strongly associated with smoking status in NSCLC patients (OR 0.51); *RUNX3* hypermethylation played a more important role in the pathogenesis of adenocarcinoma (OR 0.41); aberrant *RUNX3* hypermethylation was not significantly higher in advanced NSCLC (III and IV) than that in early stage NSCLC (I and II; OR 1.21); aberrant *RUNX3* hypermethylation was not significantly higher in poorly differentiated NSCLC than in moderately and highly differentiated NSCLC (OR 0.65); and NSCLC patients with *RUNX3* hypermethylation had a lower survival rate than those without *RUNX3* hypermethylation (HR 3.12).

The results from the current study demonstrate that the hypermethylation rate of the *RUNX3* gene promoter in NSCLC was significantly higher than in normal lung tissues (OR 7.08), indicating that hypermethylation of the *RUNX3* promoter is common in NSCLC. Since changes in hypermethylation of the *RUNX3* promoter are reversible, drug treatment through demethylation may be useful to inhibit carcinogenesis, progression, and improve prognosis. This approach brings new direction and hope for cancer treatment through gene-targeted therapy. The results of this meta-analysis also suggest that *RUNX3* methylation status could be used as a stratified factor for prediction of NSCLC patient survival. Increasing numbers of studies suggest that determination of the DNA methylation status of tumor suppressor genes has the potential to provide additional information over and above existing cancer markers.

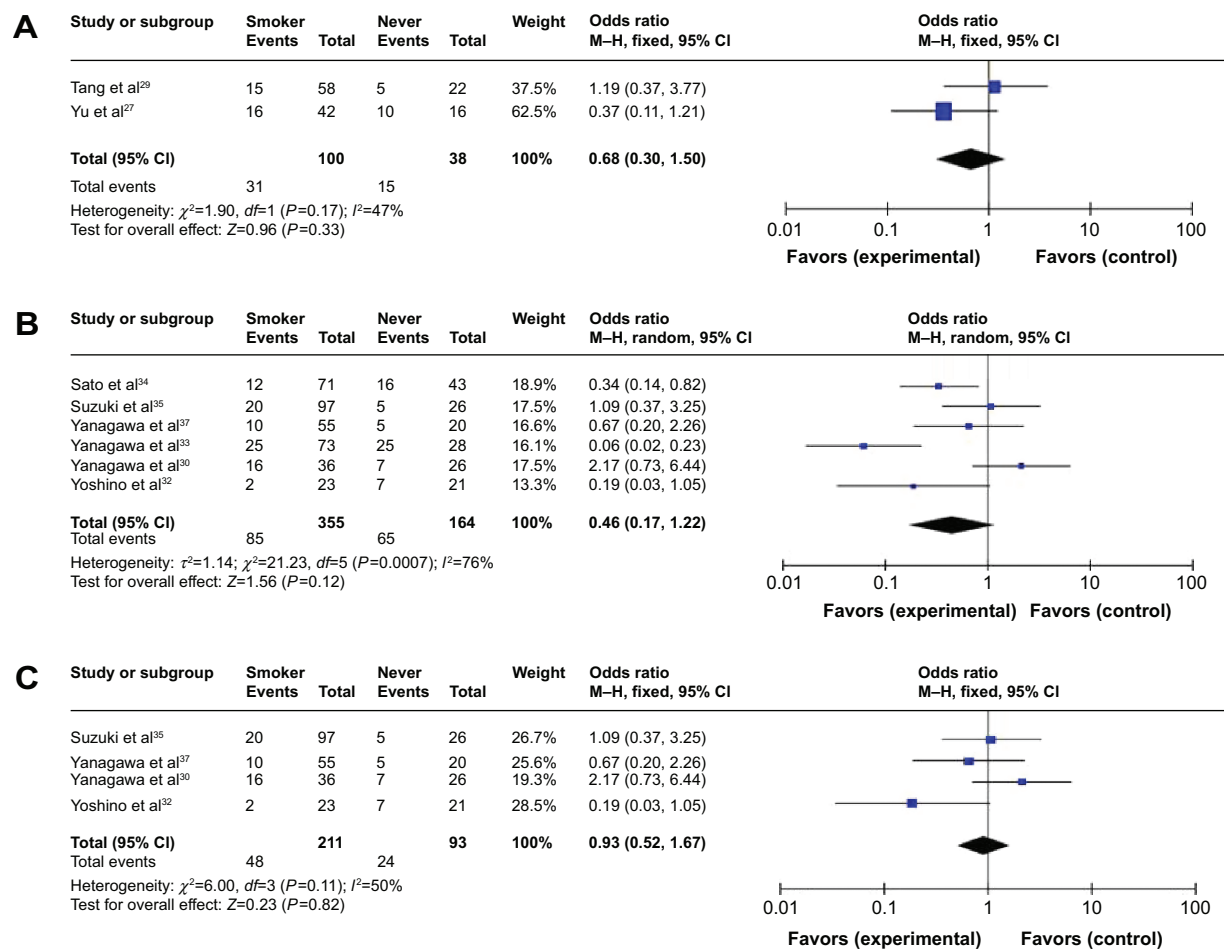


Figure 5 Subgroup analysis and meta-regression.

Notes: (A) Pooled OR for two studies from the People’s Republic of China for patients with or without a smoking history ($I^2=47%$, OR 0.68, CI 0.30–1.50, $P=0.33$). (B) Pooled OR from six studies from Japan for patients with or without smoking history ($I^2=76%$, OR 0.46, CI 0.17–1.22, $P=0.12$). (C) Pooled OR from four studies from Japan with or without smoking history ($I^2=50%$, OR 0.93, CI 0.52–1.67, $P=0.82$).

Abbreviations: CI, confidence interval; OR, odds ratio; M-H, Mantel-Haenszel.

For the group in which the heterogeneity was strong, we performed subgroup analysis and meta-regression to analyze the sources of the heterogeneity. In the case of the relationship between frequency of *RUNX3* hypermethylation and smoking status, the heterogeneity was determined in patients from the People’s Republic of China and Japan (Figure 5). The strong heterogeneity appeared to be coming from the studies in Japanese. However, the exact sources of the heterogeneity still need to be determined. Due to the small number of studies and limited information available, we were not able to determine the exact sources of the heterogeneity from the frequency of *RUNX3* hypermethylation, pathological types, and clinical stages.

Epigenetics is the study of heritable and age-related modifications of the genome that occur without a change in the primary DNA sequence. Epigenetic alterations, particularly aberrant DNA methylation, one of the best-characterized epigenetic modifications, contribute to tumor initiation and

progression.^{57,58} *RUNX3* exerts pleiotropic effects during tumor suppression, and also inhibits the oncogenic Wnt signaling pathway via formation of a complex with the TCF4- β -catenin complex and preventing it from binding to target genes such as *c-myc* and *cyclin D1*.^{46,59} *RUNX3* interacts with SMAD3/SMAD4 to activate inhibition of transforming growth factor-beta-dependent proliferation and apoptosis via activation of p21 and Bim. In addition, *RUNX3* destabilizes the hypoxia-inducible factor-1 alpha (*HIF-1 α*) protein by promoting hydroxylation of proline in *HIF-1 α* through binding to *HIF-1 α* /PHD2; thus, *RUNX3* could be a novel suppressor of *HIF-1 α* and of hypoxia-mediated angiogenesis.⁶⁰ Therefore, *RUNX3* can be considered as a tumor suppressor, and its inactivation could contribute to tumor progression and a poor prognosis. Although only three studies evaluated the relationship between overall survival and hypermethylation of *RUNX3* in NSCLC, they yielded very similar results.^{29,32,33} Based on this meta-analysis, we

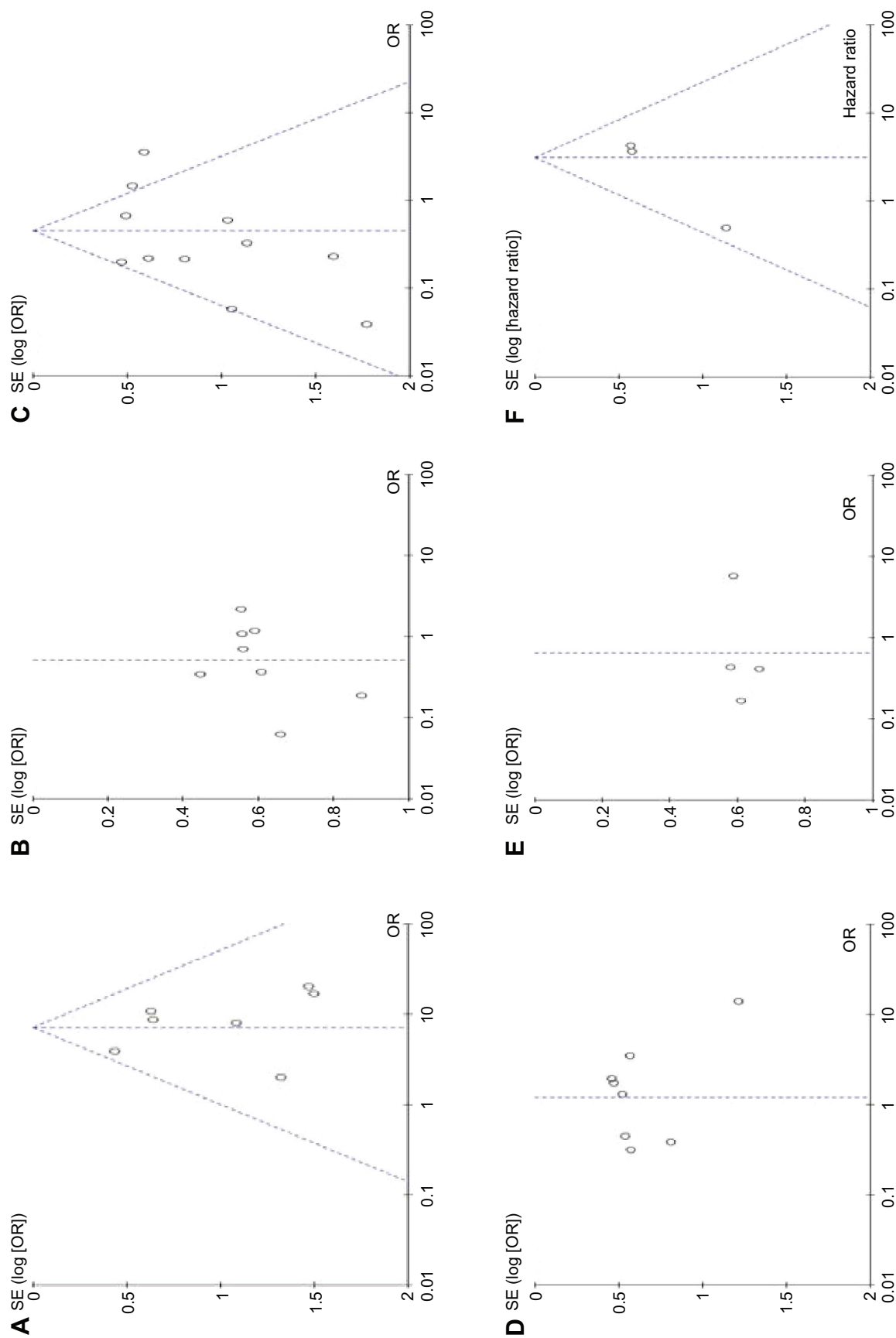


Figure 6 The funnel plots were largely symmetric, suggesting there was no publication bias in the meta-analysis of RUNX3 hypermethylation and clinicopathological features. **Notes:** (A) Funnel plot from seven studies comparing NSCLC and normal lung tissue. (B) Funnel plot from eight studies determining RUNX3 hypermethylation and smoking status in NSCLC patients. (C) Funnel plot from eleven studies comparing RUNX3 hypermethylation between squamous cell carcinoma and 389 adenocarcinoma. (D) Funnel plot from eight studies determining RUNX3 hypermethylation in early and advanced staged NSCLC. (E) Funnel plot from four studies in determining RUNX3 hypermethylation in moderately and highly differentiated NSCLC. (F) Funnel plot from three studies determining the relationship between RUNX3 hypermethylation and overall survival in NSCLC. **Abbreviations:** NSCLC, non-small cell lung cancer; OR, odds ratio; SE, standard error.

consider that hypermethylation of *RUNX3* in NSCLC tends to indicate a poor prognosis.

Consistent results were shown in the sensitivity analyses, and no evidence of publication bias was found. This study has several potential limitations. First, the possibility of information and selection bias along with unidentified confounders could not be completely excluded because all of the included studies were observational. Second, the searching strategy was restricted to articles published in English. Articles with potentially high-quality data that were published in other languages were not included because of the anticipated difficulties in obtaining accurate medical translations. Hence, caution should be taken when our findings are interpreted among the general populations.

Disclosure

The authors have no financial involvement with any organization or entity with a financial interest in the subject matter or materials discussed in this paper. The authors have no other conflicts of interest to report in regards to this paper.

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