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Clinicopathological significance and a potential drug target of *RARβ* in non-small-cell lung carcinoma: a meta-analysis and a systematic review

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Abstract: Lung cancer is the leading cause of cancer-related mortality in men worldwide. Aberrant *RARβ* promoter methylation has been frequently investigated in non-small-cell lung carcinoma (NSCLC), the most common form of lung cancer. The aim of present study was to carry out a meta-analysis and a systematic review to evaluate clinicopathological significance of *RARβ* promoter hypermethylation in NSCLC. A systematic literature search was carried out. The data were extracted and assessed by two reviewers independently. The Cochrane software Review Manager 5.2 was used to conduct the review. Odds ratios (ORs) with 95% corresponding confidence intervals (CIs) were calculated. A total of 18 relevant articles were available for meta-analysis which included 1,871 participants. The frequency of *RARβ* hypermethylation was significantly increased in NSCLC than in nonmalignant lung tissue, and the pooled OR was 5.69 ($P<0.00001$). *RARβ* hypermethylation was significantly more frequently observed in adenocarcinoma (AC) than in squamous cell carcinoma (SCC), and the pooled OR was 1.47 ($P=0.005$). Hypermethylation of *RARβ* gene in NSCLC was 2.46 times higher in smoking than in nonsmoking individuals, and the pooled OR was 2.46 ($P=0.0002$). *RARβ* hypermethylation rate was not significantly correlated with stage of the disease and sex. *RARβ* gene methylation status was not associated with prognosis of patients with NSCLC. In conclusion, *RARβ* promoter hypermethylation significantly increased in NSCLC than in non-neoplastic lung tissue and is predominant in AC, suggesting that *RARβ* methylation contributes to the development of NSCLC, especially AC. *RARβ* gene is a potential novel target for development of personalized therapy in patients with NSCLC, and is promising in restoration of retinoic acid-target gene induction via demethylation of *RARβ1′* promoter.

Keywords: NSCLC, *RARβ*, methylation, tumor suppressor gene, drug target, RA-resistance, meta-analysis, odds ratio

Introduction

Lung cancer is the leading cause of cancer-related mortality in men worldwide.[1] Lung cancers can be classified into two major histological groups: small-cell-lung carcinoma and non-small-cell lung carcinoma (NSCLC). NSCLC consists of adenocarcinoma (AC), squamous cell carcinoma (SCC), large cell carcinoma, and others.[2] NSCLC is the most common form, and although the advent of targeted therapies has improved outcomes in a subset of patients, the overall 5-year survival rate remains less than 15%.[3] It is critical to identify a molecular predictive marker for monitoring its progression and prognosis. Aberrant DNA methylation is a commonly observed epigenetic modification in human malignancies including NSCLC. Hypermethylation in promoter regions of many tumor suppressor genes inactivates gene transcription and contributes

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to the development and progression of various cancers by abolishing tumor suppressor gene function.[4–6] Currently, many genes were identified as tumor-specifically methylated by using a genome-wide approach to investigate CpG island methylation in a large number of NSCLC patients. Approximately, half of the methylated genes are involved in the regulation of gene expression and cell adhesion, and some of them could be prognostic markers.[7,8] Retinoic acid (RA) and its derivatives (retinoids) are required for normal lung development, cell growth, and differentiation, and a deficiency in RA is a risk factor for the development of lung cancer.[9–12] RA activity is mediated primarily by retinoic acid receptors (RAR), including RARα, RARβ, and RARγ, which belong to the nuclear receptor superfamily of transcription factors. *RARβ* gene is located at chromosome 3p24, a region frequently deleted in lung cancer. Loss of expression of *RARβ* during cancer development is associated with tumorigenesis and retinoid resistance; induction of its expression, on the other hand, can suppress carcinogenesis.[13] Previous studies have reported that *RARβ* hypermethylation was frequently observed in NSCLC tissue, but the reported rates of *RARβ* hypermethylation in NSCLC were remarkably diverse due to smaller number of patients. In addition, several studies have revealed an association between DNA methylation and tobacco carcinogens in animal models as well as in NSCLC patients, but the results were inconsistent.[14–18] In the present study, we systematically reviewed the studies of *RARβ* promoter hypermethylation in the process of NSCLC onset and progression, and quantified the association between *RARβ* promoter hypermethylation and the NSCLC by using meta-analysis methods. In addition, we analyzed the relationship of *RARβ* promoter hypermethylation with clinical features in NSCLC patients and discussed the tumor suppressor function, RA resistance, as well as the clinical significance of *RARβ* in NSCLC.

Materials and methods

Search strategy

We conducted a literature search for articles from the earliest data to August 2015 in PubMed, EMBASE, and Web of Science using the search terms: "lung" and "cancer or tumor or neoplasm or carcinoma", "methylation", and "*RARβ* or *retinoic acid receptor-β*". We also screened manually the reference lists of retrieved articles for additional articles. There were 150 articles identified from PubMed, 67 articles from EMBASE, and 340 articles from Web of Science. A total of 557 articles were screened by article titles and abstracts.

Selection criteria

Studies were selected based on the following inclusion criteria: 1) studies that reported the relationship between *RARβ* hypermethylation and NSCLC clinicopathological parameters and prognosis; 2) *RARβ* hypermethylation evaluated in the primary NSCLC tissues; 3) *RARβ* hypermethylation examined by methylation-specific polymerase chain reaction (MSP); and 4) studies provided sufficient information to estimate hazard ratio (HR) about overall survival (OS) and 95% confidence interval (CI). The exclusion criteria included the following: 1) reviews, letters, case reports, expert opinion, conference abstracts, editorials; 2) all publications regarding in vitro/ex vivo studies, cell lines, and human xenografts; 3) studies in which RARβ protein expression was investigated, and *RARβ* hypermethylation was not reported; and 4) studies in which same population or overlapping data were used.

Although our search did not have language limits initially, for the full-text reading and final evaluation we only performed the review of the studies published in English language. After exclusion of nonrelevant and/or redundant publications from different databases, the 44 remaining papers were evaluated in the full-text version for inclusion and exclusion criteria.

Data extraction and methodological assessment

Two authors (XS, KS) independently reviewed and extracted data from eligible studies. Disagreements were resolved by discussion and consensus. If they could not reach a consensus, a third author (SZ) was consulted. The following information was recorded for each study: the first author name, year of publication, sample source, number of cases, clinicopathological parameters, cancer tumor node metastasis stage, methylation detection method, and methylation rate and site. Data for study characteristics and clinical responses were summarized and organized into a table format. Heterogeneity of investigation was evaluated to determine whether or not the data of the various studies could be analyzed for meta-analysis.

For the methodological evaluation of the studies, three investigators (DPY, ZL, and YH) read through each publication independently, and they assessed and scored them according to the Newcastle Ottawa Quality Assessment Scale (NOQAS). The three readers provided the quality scores and compared them, and then they reached a consensus value for each item. Those scales allocate a maximum of nine points

for the quality of selection, comparability, exposure, and outcomes for study participants. The Newcastle–Ottawa scale scores ranged from 0 to 9, and a study with a score of 7 or more indicates a good quality.

Statistical analysis

The meta-analysis was conducted using Review Manager 5.2 (Cochrane Collaboration, Software Update, Oxford, UK). Odds ratios (ORs) with its 95% CIs were calculated. The assessment of statistical heterogeneity was done by using the Cochran's Q statistic and I^2 tests. When the I^2 value was below 50%, a fixed effect model was used, and when the I^2 value was 50% or greater, a random effect model was used. We also explored reasons for statistical heterogeneity using sensitivity analysis. The pooled frequency of *RARβ* hypermethylation and 95% CIs were estimated. The frequency of *RARβ* hypermethylation was compared in different tumor characteristics. The pooled OR was estimated for the association between *RARβ* hypermethylation and clinicopathological features. *P*-values tailed less than 0.05 were considered statistically significant. Publication bias was assessed by using a method reported by Egger et al.[19]

Results

Identification of relevant studies and study quality

Eighteen articles published from 2004 to 2014 were eligible for this meta-analysis, as shown in Figure 1. The quality of included articles was assessed by using NOQAS. Those scales allocate a maximum of 9 points for the quality of selection, comparability, exposure, and outcomes for study participants. The NOS scores ranged from 0 to 9, and a study with a score of 7 or more indicates a good quality. Among those studies, six scored 9 points, nine scored 8 points, and three scored 7 points. Hence, the studies were of a relatively high quality (data not shown).

Study characteristics

A total of 1,871 NSCLC patients from the People's Republic of China, Japan, Korea, Finland, Australia, and the USA

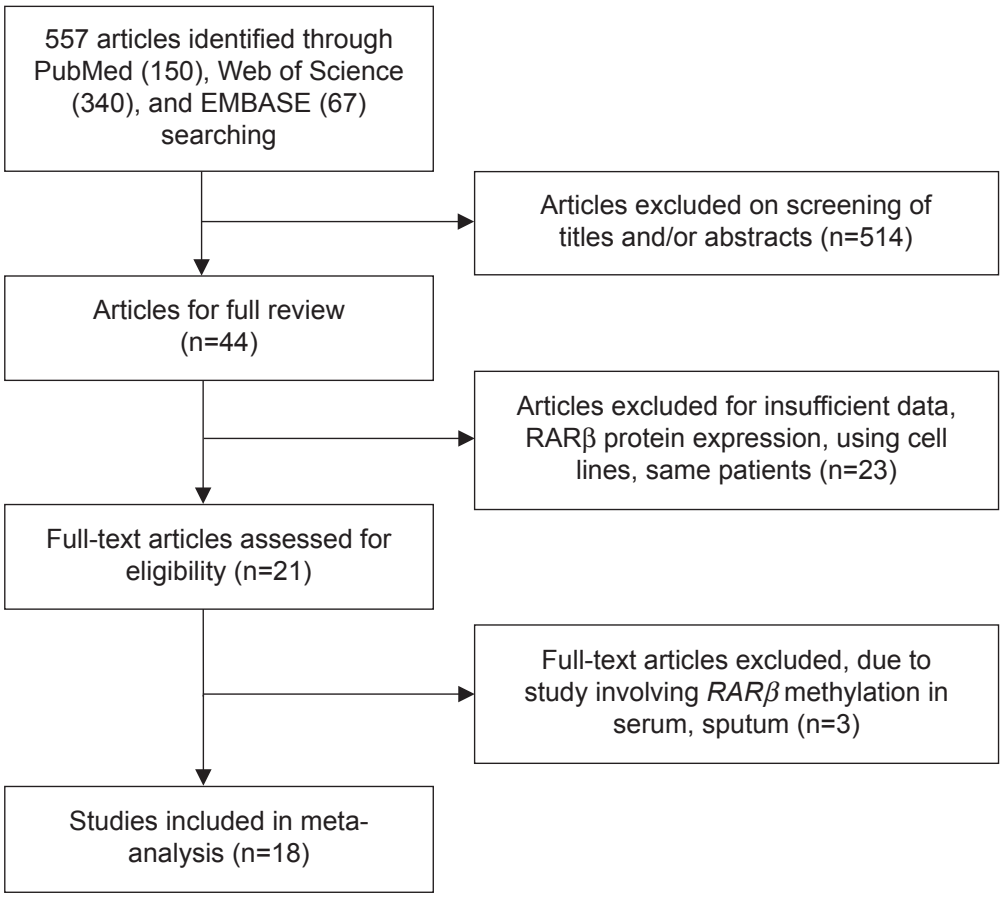

Figure 1 Schematic flow diagram for selection of included studies.

Table 1 Main characteristics of included studies

Author	Year	Country	Sex (F/M)	Histology (AC/SCC)	Stage (low/advanced)	Smoking (–/+)	Methods	Methylation site
Li et al[44]	2014	People's Republic of China	63/104	81/86	102/65	69/44	MSP	Promoter
Zhao et al[45]	2012	People's Republic of China	12/68	35/45	50/30	19/61	MSP	Promoter
Zhang et al[46]	2011	People's Republic of China	NA	NA	NA	NA	MSP	Promoter
Li et al[40]	2014	People's Republic of China	NA	10/29	34/22	NA	MSP	Promoter
Scesnaite et al[47]	2012	Finland	83/129	103/79	35/71	2/104	MSP	Promoter
Yanagawa et al[18]	2011	Japan	41/69	NA	44/18	26/36	MSP	Promoter
Liu et al[48]	2010	People's Republic of China	21/59	22/37	60/20	33/47	MSP	Promoter
Hawes et al[49]	2010	USA	45/72	51/59	93/24	5/112	Methy light	Promoter
Kubo et al[50]	2009	Japan	62/38	NA	52/48	70/30	MSP	Promoter
Umemura et al[33]	2008	Japan	1/10	6/4	5/6	2/9	MSP	promoter
Seng et al[51]	2008	Australia	84/155	146/92	86/153	NA	MSP	promoter
Hsu et al[52]	2007	People's Republic of China	NA	33/37	48/31	18/56	MSP	promoter
Yanagawa et al[34]	2007	Japan	29/72	62/39	33/68	26/36	MSP	promoter
Katayama et al[53]	2007	Japan	10/24	32/1	0/34	NA	MSP	promoter
Kim et al[39]	2005	Korea	NA	NA	NA	NA	MSP	promoter
Kim et al[43]	2005	Korea	NA	42/27	31/30	NA	MSP	promoter
Tomizawa et al[17]	2004	Japan	43/77	45/72	32/86	29/61	MSP	Promoter
Topaloglu et al[54]	2004	USA	NA	21/7	26/5	NA	Real-time PCR	Promoter

Abbreviations: F, female; M, male; AC, adenocarcinoma; SCC, squamous cell carcinoma; MSP, methylation-specific PCR; PCR, polymerase chain reaction; NA, not available.

were enrolled. The variables from 18 relevant studies are listed in Table 1.

The correlation of *RARβ* hypermethylation with clinicopathological features

The frequency of *RARβ* hypermethylation was significantly increased in NSCLC than in nonmalignant lung tissue, and the pooled OR was 5.69 with 95% CI: 3.32–9.76, z=6.32, $P<0.00001$ (Figure 2). *RARβ* hypermethylation was significantly more frequently observed in AC than in SCC, and the pooled OR was 1.47 with 95% CI: 1.12–1.93, z=2.79, P=0.005 (Figure 3). *RARβ* hypermethylation rate was not significantly correlated with stage of the disease, and the pooled OR was 0.82 with 95% CI: 0.63–1.08, z=1.4, P=0.16 (Figure 4). *RARβ* methylation was 2.46 times higher in smoking than in nonsmoking individuals, and the pooled OR was 2.46 with 95% CI: 1.54–3.93, z=3.76, P=0.0002 (Figure 5). *RARβ* gene hypermethylation had no correlation with patient sex, and the pooled OR was 1.20 with 95% CI: 0.79–1.84, z=0.85, P=0.40 (Figure 6). *RARβ* gene methylation status was not associated with prognosis of patients with NSCLC, the pooled HR was 1.05 with 95% CI: 0.68–1.64, z=0.23, P=0.82 (Figure 7).

Sensitivity analyses and publication bias

A sensitivity analysis was conducted by removing one study from the meta-analysis at a time, and the overall results were not significantly affected. The pooled ORs or HRs were not significantly changed, indicating the stability of our analyses. The funnel plots were largely symmetric (Figure 8A–F), suggesting there were no significant publication biases in the meta-analysis of *RARβ* hypermethylation and clinicopathological features.

Discussion

The human *RARβ* gene is located on chromosome 3, locus 3p24, and consists of five distinct isoforms: hRARβ1′, hRARβ1, hRARβ2, hRARβ4, and hRARβ5.[20,21] Among those five isoforms, hRARβ1 and hRARβ2 are the functional isoforms that activate distinct cassettes of target genes with different biological effects.[22] hRARβ1 is a fetal isoform, which may play an important role during development in human.[23] The hRARβ1′ was identified in normal lung tissue and bronchial epithelial cells. The absence of hRARβ1′ is associated with retinoid resistance in human lung carcinogenesis.[24,25] The reexpression of hRARβ1′ in lung cancer cells restored RA-target gene induction and growth suppression following retinoid treatment.[25] The hRARβ2 is the retinoid-inducible splicing isoform, and multiple hRARβ2 transcript variants are expressed in both normal and neoplastic cells.[26,27] Both the hRARβ1′ and hRARβ2 transcripts are obtained from the transcription start sites of P2 promote which contains a CpG-rich region that is susceptible to methylation-induced silencing.[28–31]

Study or subgroup	NSCLC Events	Total	Non-neoplastic lung Events	Total	Weight (%)	Odds ratio M–H, random, 95% CI
Hsu et al[52]	31	82	8	37	14.7	2.20 (0.89–5.43)
Kim et al[39]	45	72	7	72	14.5	15.48 (6.20–38.60)
Liu et al[48]	16	80	7	80	14.0	2.61 (1.01–6.74)
Topaloglu et al[54]	3	31	0	10	2.8	2.58 (0.12–54.26)
Umemura et al[33]	0	11	1	20	2.4	0.57 (0.02–15.06)
Yanagawa et al[34]	38	101	5	101	13.6	11.58 (4.32–31.01)
Zhang et al[46]	116	200	30	200	20.8	7.83 (4.85–12.63)
Zhao et al[45]	46	80	14	80	17.1	6.38 (3.08–13.20)
Total (95% CI)		**657**		**600**	**100**	**5.69 (3.32–9.76)**
Total events	295		72			

Heterogeneity: τ^2=0.30; χ^2=16.91, *df*=7 (*P*=0.02); I^2=59%
Test for overall effect: *Z*=6.32 (*P*<0.00001)

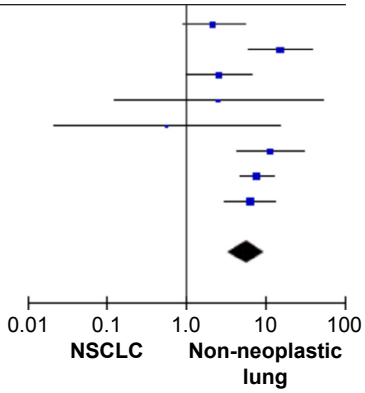

Figure 2 Forest plot for *RARβ* hypermethylation in NSCLC and non-neoplastic lung tissue.
Abbreviations: NSCLC, non-small-cell lung carcinoma; CI, confidence interval; *df*, degree of freedom; M–H, Mantel–Haenszel.

Study or subgroup	AC Events	Total	SCC Events	Total	Weight (%)	Odds ratio M–H, fixed, 95% CI
Hawes et al[49]	16	51	19	59	13.9	0.96 (0.43–2.15)
Kim et al[43]	21	42	8	27	5.6	2.38 (0.85–6.61)
Li et al[40]	66	81	68	86	14.1	1.16 (0.54–2.50)
Li et al[44]	7	10	23	29	4.1	0.61 (0.12–3.09)
Scesnaite et al[47]	40	103	22	79	17.5	1.65 (0.87–3.09)
Seng et al[51]	76	146	47	92	31.8	1.04 (0.62–1.75)
Tomizawa et al[17]	22	72	9	45	8.9	1.76 (0.73–4.27)
Yanagawa et al[34]	32	62	6	39	4.1	5.87 (2.15–15.99)
Total (95% CI)		**567**		**456**	**100**	**1.47 (1.12–1.93)**
Total events	280		202			

Heterogeneity: χ^2=12.70, *df*=7 (*P*=0.08); I^2=45%
Test for overall effect: *Z*=2.79 (*P*=0.005)

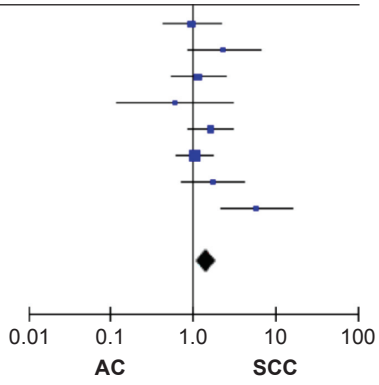

Figure 3 Forest plot for *RARβ* hypermethylation in AC and SCC.
Abbreviations: AC, adenocarcinoma; SCC, squamous cell carcinoma; CI, confidence interval; *df*, degree of freedom; M–H, Mantel–Haenszel.

Study or subgroup	Advanced stage Events	Total	Low stage Events	Total	Weight (%)	Odds ratio M–H, fixed, 95% CI
Kim et al[43]	15	30	15	31	6.5	1.07 (0.39–2.91)
Kubo et al[50]	12	48	21	52	13.4	0.49 (0.21–1.16)
Li et al[40]	48	65	86	102	15.5	0.53 (0.24–1.13)
Li et al[44]	16	22	29	34	5.5	0.46 (0.12–1.75)
Scesnaite et al[47]	21	71	5	35	4.2	2.52 (0.86–7.39)
Seng et al[51]	80	153	43	86	23.2	1.10 (0.65–1.86)
Tomizawa et al[17]	17	86	14	32	14.5	0.32 (0.13–0.76)
Yanagawa et al[34]	27	68	11	33	7.9	1.32 (0.55–3.15)
Zhao et al[45]	16	30	30	50	9.3	0.76 (0.31–1.90)
Total (95% CI)		**573**		**455**	**100**	**0.82 (0.63–1.08)**
Total events	252		254			

Heterogeneity: χ^2=14.67, *df*=8 (*P*=0.07); I^2=45%
Test for overall effect: *Z*=1.40 (*P*=0.16)

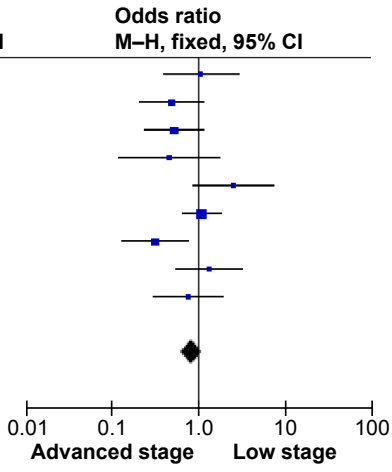

Figure 4 Forest plot for *RARβ* hypermethylation in advanced and low stage of NSCLC.
Abbreviations: NSCLC, non-small-cell lung carcinoma; CI, confidence interval; *df*, degree of freedom; M–H, Mantel–Haenszel.

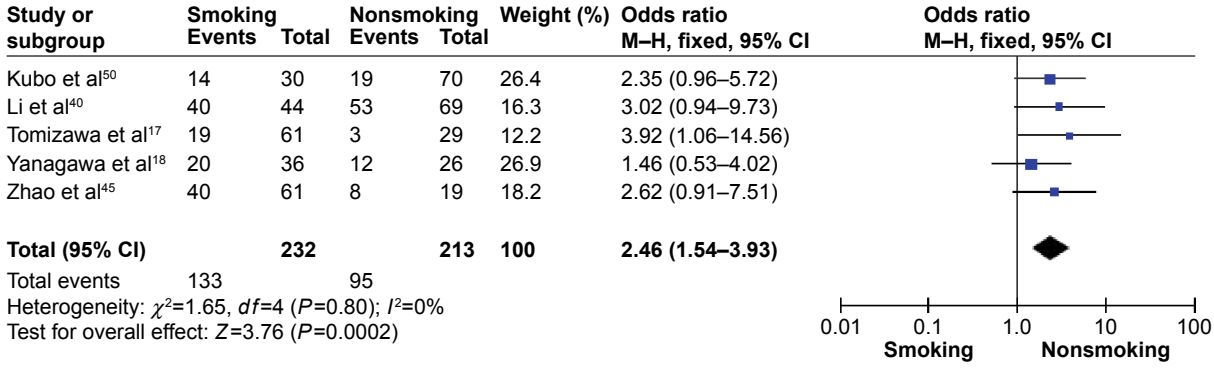

Figure 5 Forest plot for *RARβ* hypermethylation of NSCLC in smoking and nonsmoking individual.
Abbreviations: NSCLC, non-small-cell lung carcinoma; CI, confidence interval; *df*, degree of freedom; M–H, Mantel–Haenszel.

Aberrant methylation of the promoter regions of genes is a major mechanism of gene silencing in tumor.[32] A number of studies reported that *RARβ* promoter hypermethylation was observed in NSCLC, but the rate was remarkably diverse due to small power.[33,34] In the present study, we conducted a meta-analysis and pooled 18 studies which included 1,871 NSCLC patients. Our data showed *RARβ* promoter hypermethylation significantly increased by 5.69 times in NSCLC than in non-neoplastic lung tissue, indicating that *RARβ* promoter hypermethylation contributed to the initiation and development of NSCLC. On the other hand, the translation of hRARβ1′ initiates at the P2 promoter, and *RARβ* promoter hypermethylation suppresses hRARβ1′ expression that is required for RA-target gene induction. Therefore, *RARβ* promoter hypermethylation could lead to the incident of retinoid resistance following retinoid treatment. Confirmation studies need to be carried out in future. *RARβ* promoter hypermethylation is a reversible event; drug treatment through demethylation may be used not only to delay carcinogenesis and the progression of NSCLC, but also to restore RA-target gene induction. Virmani et al demonstrated that the treatment of lung cancer cell lines with demethylation agent 5-aza-2′-deoxycytidine (5-AZA-CdR) can restore *RARβ* expression.[32] A Phase I/II trial in patients with stage IV NSCLC suggests that 5-AZA-CdR may have some clinical activity against metastatic NSCLC.[35] Recently, curcumin, a potent cancer preventive agent,[36] was also reported to significantly decrease *RARβ* promoter methylation and elevate *RARβ* expression at the messenger RNA and protein levels in lung cancer A549 and H460 cells.[37] Taken together, demethylation of *RARβ* promoter could suppress the development of NSCLC, and potentially restores RA-target gene induction. *RARβ* is a promising gene target for the development of personalized therapy in patients with NSCLC.

Hua et al conducted a meta-analysis and reported that the frequency of *RARβ* promoter hypermethylation increased in

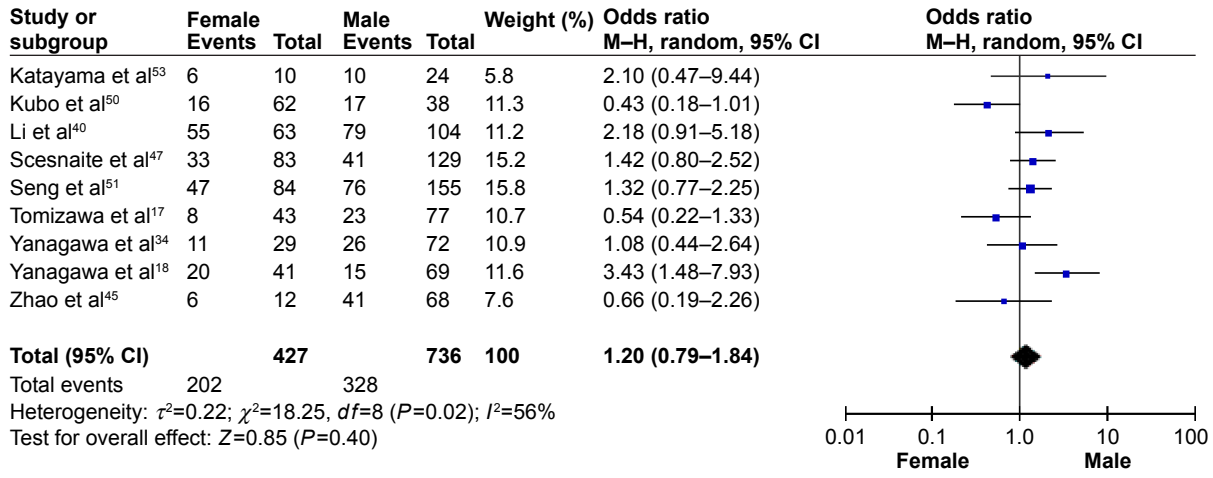

Figure 6 Forest plot for *RARβ* hypermethylation of NSCLC in different sexes.
Abbreviations: NSCLC, non-small-cell lung carcinoma; CI, confidence interval; *df*, degree of freedom; M–H, Mantel–Haenszel.

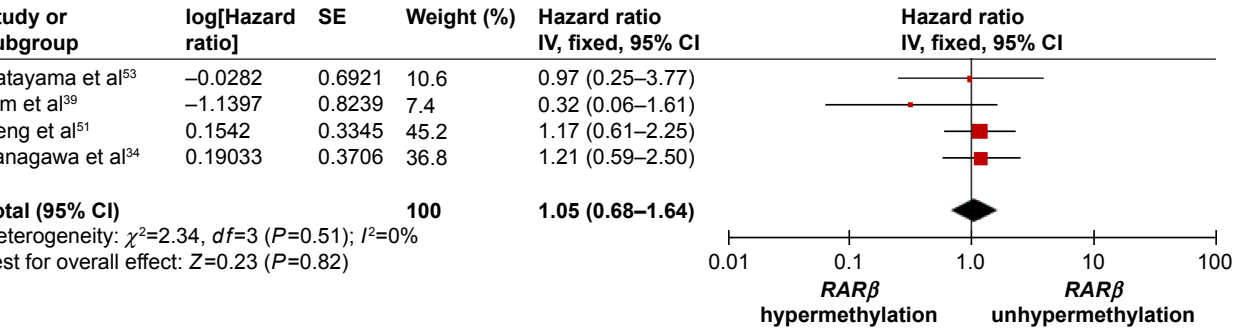

Figure 7 Forest plot for the association between *RARβ* hypermethylation and risk of NSCLC.
Abbreviations: NSCLC, non-small-cell lung carcinoma; CI, confidence interval; *df*, degree of freedom; SE, standard error of the mean.

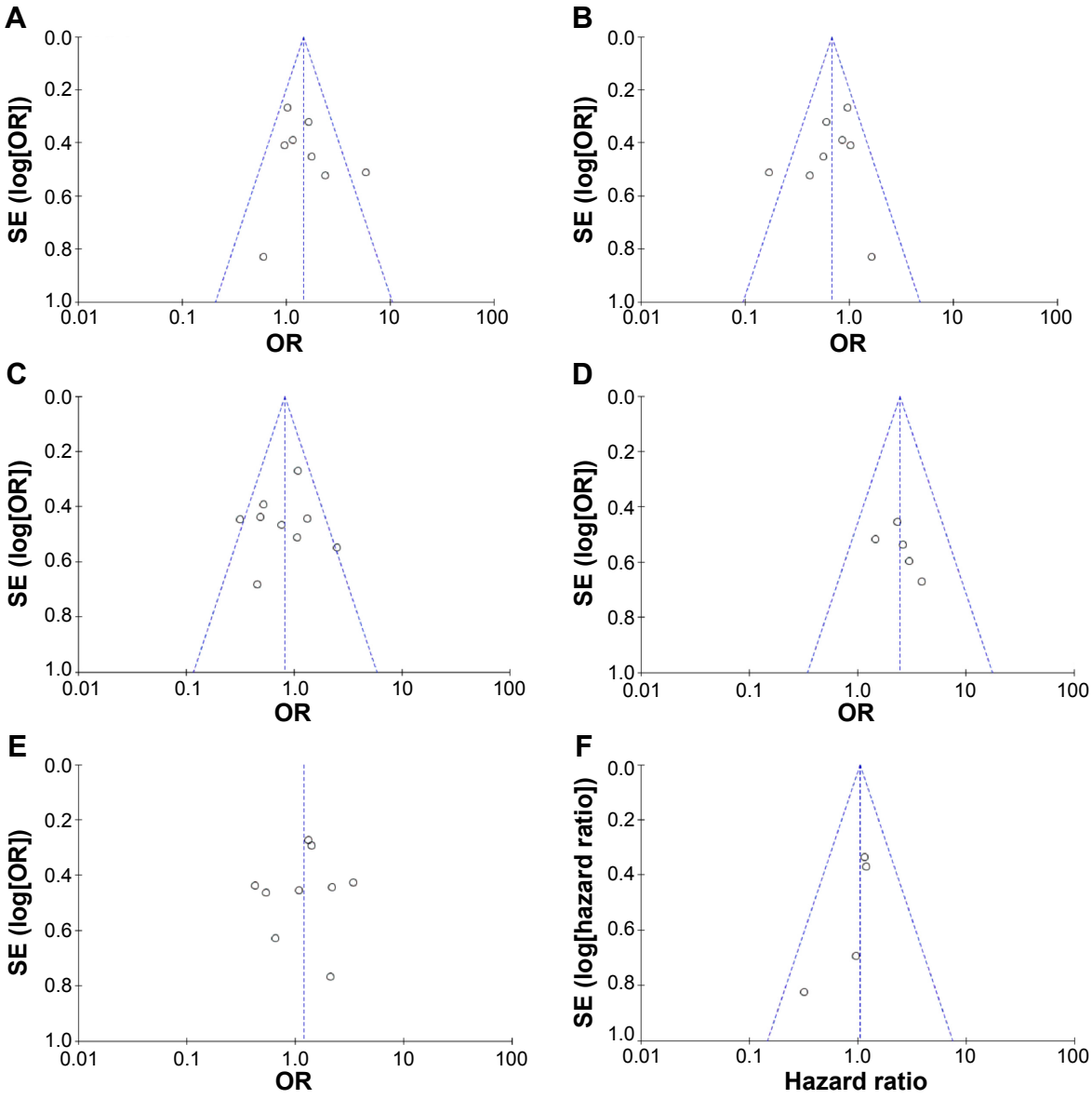

Figure 8 Funnel plot for publication bias.
Notes: (**A**) *RARβ* hypermethylation in NSCLC and non-neoplastic lung tissue; (**B**) *RARβ* hypermethylation in AC and SCC; (**C**) *RARβ* hypermethylation in advanced and low stage of NSCLC; (**D**) *RARβ* hypermethylation of NSCLC in smoking and nonsmoking individual; (**E**) *RARβ* hypermethylation of NSCLC in different sexes; (**F**) the association between *RARβ* hypermethylation and the risk of NSCLC.
Abbreviations: NSCLC, non-small-cell lung carcinoma; AC, adenocarcinoma; SCC, squamous cell carcinoma; SE, standard error of the mean; OR, odds ratio; *df*, degree of freedom.

NSCLC than autologous controls, suggesting the methylation status could be a valuable diagnostic tool for NSCLC.[38] However, they only accessed eleven studies. Our analysis included additional seven studies and showed more convincing results. In addition, we also found that the rate of *RARβ* hypermethylation was increased in AC than SCC and that there was a strong correlation between methylation status of *RARβ* and smoking in patients with NSCLC.

The rate of *RARβ* hypermethylation between AC and SCC has been compared in previous studies and the results were contradictory as the studies included limited number of patients.[39,40] We pooled eight studies and analyzed the frequency of *RARβ* promoter hypermethylation in AC and SCC. The rate of *RARβ* promoter hypermethylation was higher in AC than SCC, suggesting that the molecular mechanisms could be different between AC and SCC. Additionally, *RARβ* promoter hypermethylation significantly increased in smoking NSCLC patients compared to nonsmoking NSCLC patients, indicating that smoking induced gene methylation which may lead to malignant growth and cancer development.[41,42]

Several studies reported the correlation between methylation status of *RARβ* and survival in NSCLC patients, and the results were inconsistent.[34,43] In the present meta-analysis, no significant association between *RARβ* promoter methylation status and survival was found. Additionally, the rate of *RARβ* promoter hypermethylation was similar between lower and advanced stages of disease. Our study only included the articles published in English or Chinese language, and did not select some relevant papers published in other languages, which may result in certain publication bias. Hence, cautions should be taken when our findings are interpreted among the general populations.

Conclusion

In summary, *RARβ* promoter hypermethylation significantly increased in NSCLC tumor than in non-neoplastic lung tissue, suggesting that *RARβ* methylation contributes to the development of NSCLC. *RARβ* gene is a potential novel target for demethylation treatment in patients with NSCLC, and is promising in restoration of RA-target gene induction via demethylation of *RARβ1′* promoter. *RARβ* promoter hypermethylation is associated with smoking behavior as well as histologic subtypes of NSCLC in patients with NSCLC. The rate of *RARβ* promoter hypermethylation is higher in AC than SCC. *RARβ* methylation status is not correlated to survival of patients with NSCLC.

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Author contributions

All authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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