

Association between *Retinoic acid receptor-β* hypermethylation and NSCLC risk: a meta-analysis and literature review

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ABSTRACT

Emerging evidence indicates that *Retinoic acid receptor-β* (*RARβ*) is a tumor suppressor in many types of tumor. However, whether or not *RARβ* is a risk factor and is correlated to clinicopathological characteristics of non-small cell lung cancer (NSCLC) remains unclear. In this report, we performed a meta-analysis to determine the effects of *RARβ* hypermethylation on the incidence of NSCLC and clinicopathological characteristics in human NSCLC patients. Final valuation and analysis of 1780 cancer patients from 16 eligible studies was performed. *RARβ* hypermethylation was found to be significantly higher in NSCLC than in normal lung tissue, the pooled OR from 7 studies including 646 NSCLC and 580 normal lung tissues, OR = 6.05, 95% CI = 3.56-10.25, $p < 0.00001$. *RARβ* hypermethylation was significantly higher in adenocarcinoma (AC) compared to squamous cell carcinoma (SCC), pooled OR is 0.68 (95% CI = 0.52-0.89, $p = 0.005$). *RARβ* hypermethylation was also found to occur significantly higher in smoker ($n = 232$) than non-smoker ($n = 213$) (OR = 2.46, 95% CI = 1.54-3.93, $p = 0.0002$). Our results indicate that *RARβ* hypermethylation correlates well with an increased risk in NSCLC patients. *RARβ* gene inactivation caused by *RARβ* methylation contributes the NSCLC tumorigenesis and may serve as a potential risk factor, diagnostic marker and drug target of NSCLC.

INTRODUCTION

Non-small cell lung carcinoma (NSCLC) consists of squamous cell carcinoma (SCC), adenocarcinoma (AC), large cell carcinoma and others. There are approximately 80% of NSCLC cases in later stage where the treatment and prognosis is far from satisfactory [1]. Thus, identification of the risk factors and diagnostic markers is still needed for the prevention and the diagnosis of NSCLC patients. Epigenetic alterations, particularly aberrant DNA methylation, play a crucial role in cancer formation and progression [2-3]. Hypermethylation of CpG island in tumor suppressor gene promoter is a well-known and typical epigenetic changes in cancer [2-4]. Nowadays the assay of gene promoter hypermethylation has not only been recognized as a remarkable tool for diagnosis of cancer, but also a prognosis factor to predict the cancer risk in a variety of cancers including NSCLC [5].

Retinoic acid (RA) and its derivative, retinoid can bind its three retinoic acid receptors (RAR), *RARα*, *RARβ* and *RARγ*, are required for normal lung development [6-9]. Previous reports showed that *RARβ* was frequently epigenetically silenced in tumor progression, which demonstrated that *RARβ* belongs to a tumor suppressor protein [10-12]. *RARβ* gene mutation was not reported in NSCLC. The loss of coactivators, such as AF2 coactivators of the RAR-Thyroid Hormone Receptor complex are often lost in human lung cancer and the loss of AF-2 cofactors results in low levels of transcribed *RARβ* [13]. Silencing *RARβ* by promoter hypermethylation has been found as one mechanism that regulates alveolar and epithelial differentiation and lung tumorigenesis [14-15]. The hypermethylation of *RARβ* in NSCLC has been reported inconsistently [16-17], although some groups indeed found that the inactivation of the *RARβ* is caused by epigenetic progression, hypermethylation of *RARβ* gene in NSCLC [16-17]. In addition, it is not clear whether *RARβ*

Table 1: Clinical features of selected studies

Study	Country	Case No.	Methods	Goal
Li, et al 2014a[49]	China	167	Methylation specific PCR (MSP)	Determine whether tobacco exposure plays a role in gene methylation
Li, et al 2014b[37]	China	56	MSP	Determine the methylation status of three tumor suppressor genes in NSCLC
Zhao, et al 2012[50]	China	80	MSP	Detect methylation of the RAR β gene in tissues from NSCLC patients
Scesnaite, et al 2012[51]	Finland	212	MSP	Determine the methylation status of five tumor suppressor genes in NSCLC
Yanagawa, et al 2011[42]	Japan	62	MSP	Determine the methylation status of five tumor suppressor genes in NSCLC
Zhang, et al 2011[52]	China	200	MSP	Determine the methylation status of three tumor suppressor genes in NSCLC
Liu, et al 2010 [53]	China	80	MSP	Access the methylation status of six tumor suppressor genes in NSCLC
Hawes, et al 2010[54]	USA	117	Methy Light	Determine the DNA methylation status of 27 genes NSCLC
Kubo, et al 2009[55]	Japan	100	MSP	Examine the methylation status in five genes in NSCLC
Seng, et al 2008[56]	Australia	239	MSP	Investigate methylation status of three genes in NSCLC
Hsu, et al 2007[57]	China	82	MSP	Examine 19 genetic and epigenetic markers in NSCLC
Yanagawa, et al 2007[17]	Japan	101	MSP	Determine methylation in 10 genes in NSCLC
Kim, et al 2005a [36]	Korea	72	MSP	Examine the DNA methylation status of five NSCLC
Kim, et al 2005b[58]	Korea	61	MSP	Examine the methylation status of four tumor suppressor genes in NSCLC
Tomizawa, et al 2004[41]	Japan	120	MSP	Investigate aberrant methylation of three genes in NSCLC
Topaloglu, et al 2004[59]	USA	31	MSP	Examine the methylation status of eight tumor suppressor genes in NSCLC

is associated with any clinical characteristics of NSCLC and predicts the risk of diseases. The discrepancy of the published data underpin the necessity to study the role of *RAR β* inactivation by gene promoter hypermethylation in NSCLC. Therefore, we perform an evaluation on the role of *RAR β* hypermethylation in lung tumorigenesis using a quantitative meta-analysis as well as a systematic review.

RESULTS

Sixteen publications which fitted into the inclusion criteria were collected into in this study. One hundred and ninety one publications were not included for the reasons such as *in vivo* animal work or *in vitro* cell culture model, non-original articles (review and editorial), or irrelevant publications. Figure 1 shows sixteen studies from 2004 to 2014 were included in the current meta-analysis. NSCLC patients from different countries such as China, South

Korea, Japan, Finland, Australia, and USA was included and counted 1780 cases. Their demographic information and clinical parameters are formatted into Table 1.

By determine the *RAR β* promoter hypermethylation using lung tissues, the NSCLC patients showed significantly higher *RAR β* promoter hypermethylation frequency than those of normal individuals. That is the *RAR β* conveyed the positive association for promoter hypermethylation when comparing NSCLC tumors to normal controls, with the pooled OR of 6.05 (95% CI = 3.56-10.25, $p < 0.00001$) from 7 studies having both NSCLC tissues ($n = 646$) and normal lung tissues ($n = 580$) (Figure 2). To further determine the association of *RAR β* hypermethylation and two major pathological types, SCC and AC, total of 456 of SCC patients and 567 of AC patients in 8 studies were extracted. *RAR β* hypermethylation was remarkably higher in AC patients in comparison to SCC patients with pooled OR of 0.68

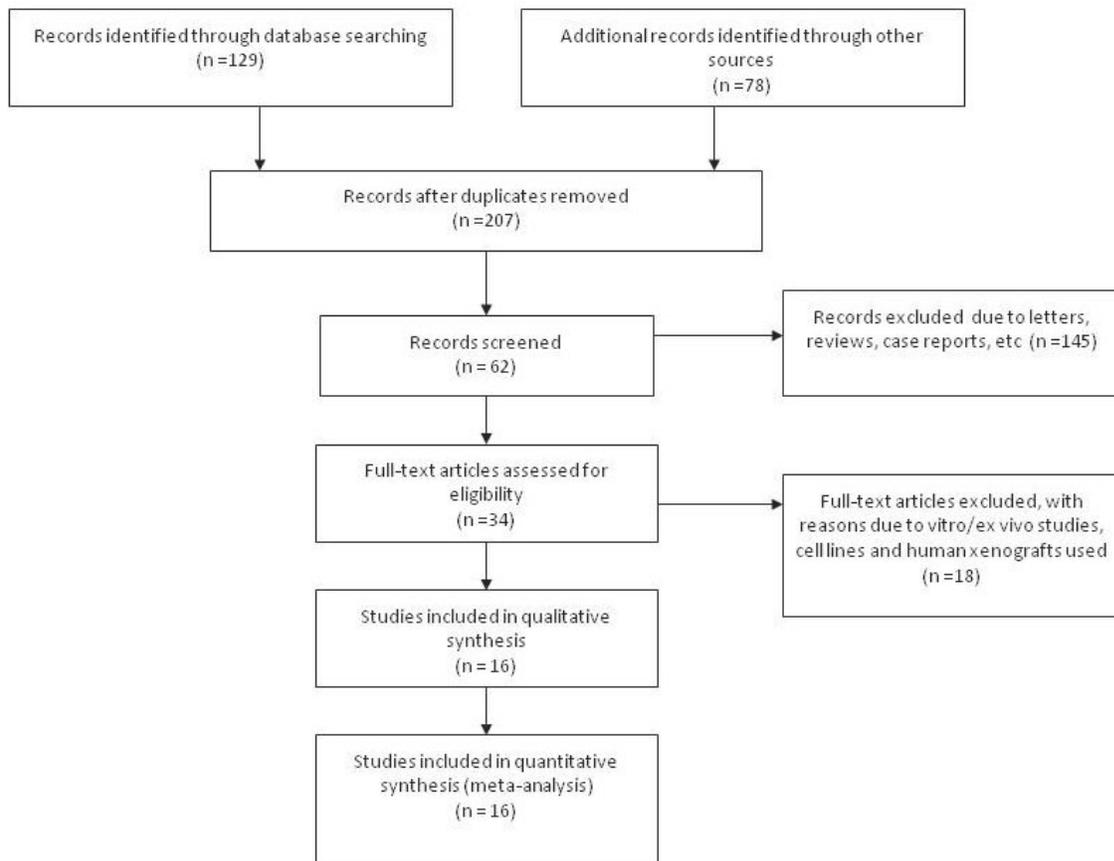


Figure 1: Flow chart of included study.

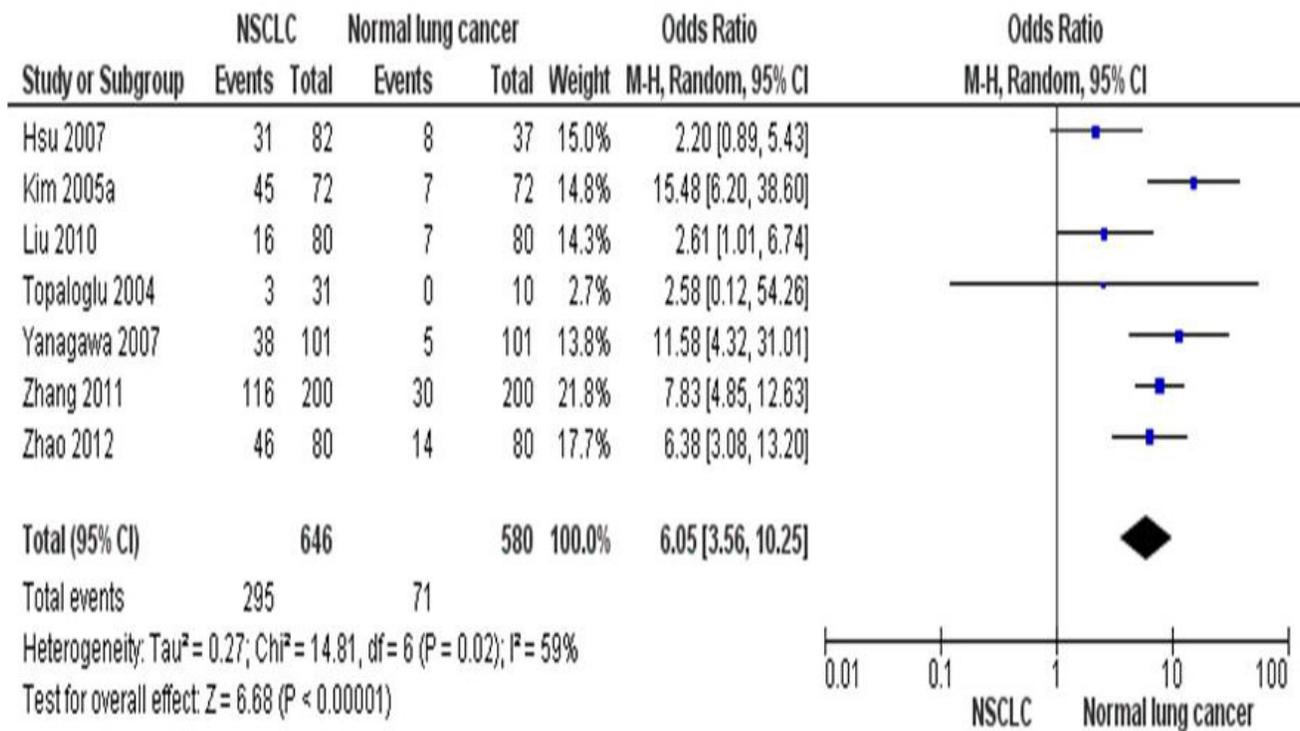


Figure 2: The combining estimates of the odds ratio from 7 selected studies containing lung tissues from 646 of NSCLC patients and 580 of normal individuals is 6.05(95% CI,3.56-10.25, $p < 0.00001$).

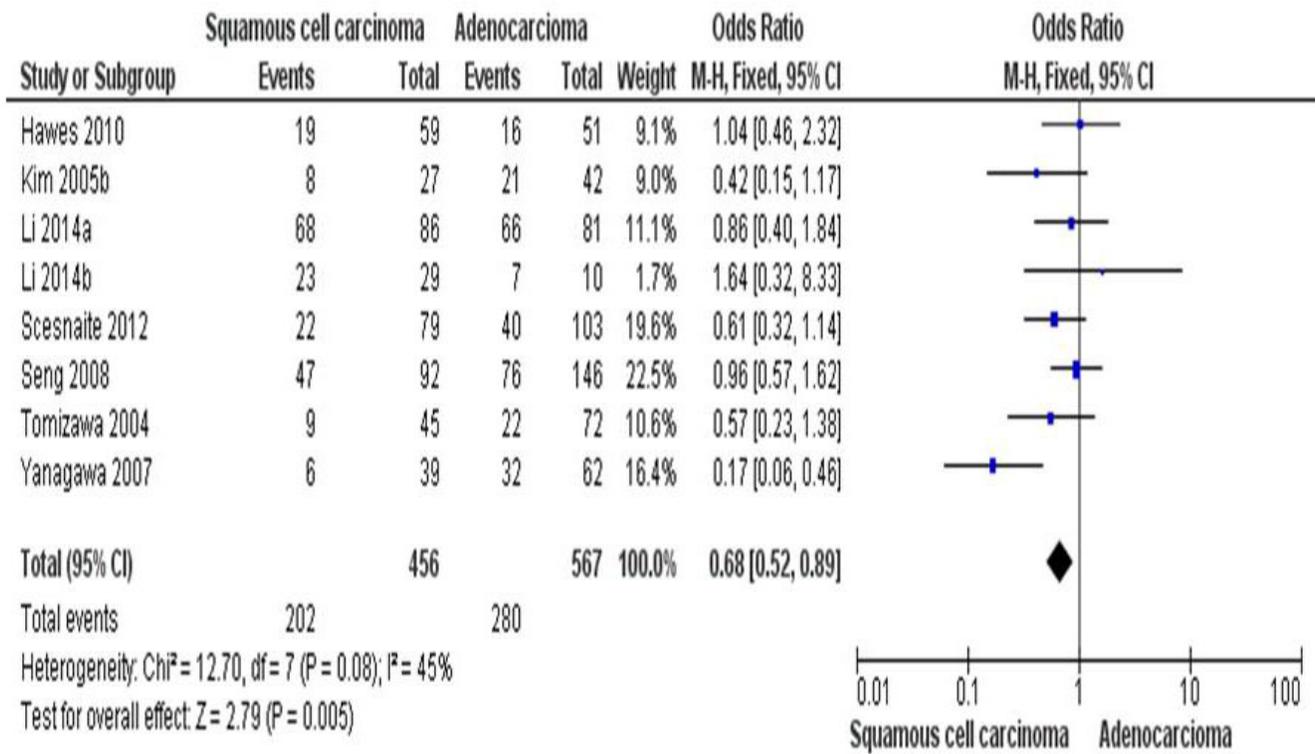


Figure 3: The combining estimates of the odds from 8 studies containing 456 of squamous cell carcinoma (SCC) and 567 of adenocarcinoma (AC) is 0.68 (95% CI, 0.52-0.89, $p = 0.005$).

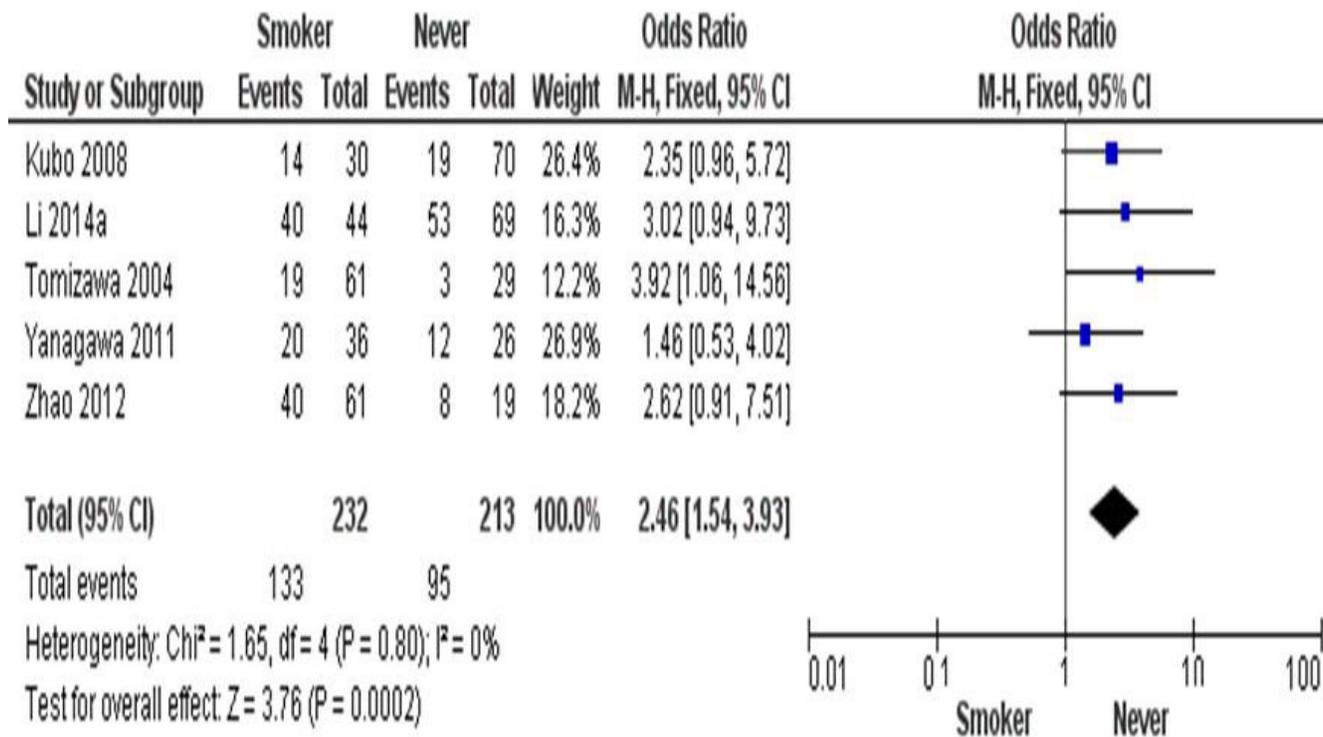


Figure 4: The combining estimates of the odds from 5 studies containing 232 and 213 NSCLC with and without smoking history is 2.46 (95% CI, 1.54-3.93, $p = 0.0002$).

(95% CI = 0.52-0.89, $p = 0.005$) (Figure 3). This result demonstrates that the hypermethylation *RARβ* may have a crucial function in the lung tumorigenesis especially in AC formation.

After analysis of combining estimates of the OR from 5 studies of 445 of NSCLC (Figure 4), the rate of hypermethylation of *RARβ* was detected significantly higher in smoker ($n = 232$) than non-smoker ($n = 213$) (OR = 2.46, 95% CI = 1.54-3.93, $p = 0.0002$). This indicates that *RARβ* conveyed the positive association for promoter hypermethylation when comparing NSCLC smoker and NSCLC non-smoker.

Finally we removed one study at a time to calculate the sensitivity which is a sign of the analysis result stability. Neither the pooled ORs nor HRs were extremely altered, demonstrating the acceptable stability of the results. The symmetrical funnel plots as shown in Figure 5A-C imply no publication biases exist in the current study.

DISCUSSION

DNA *methylation* is an epigenetic mechanism that carried out by specific *enzymes*, the DNA methyltransferase enzymes [18-19]. In Eucaryotes, only cytosine belonging to CG dinucleotides, also named CpG in the 5' position can be methylated, leading to the formation of 5-methylcytosine and are distributed

in promoter regions of genes [20-21]. Just similar to other tumor suppressor genes, the CpG islands of *RARβ* promoter are easily hypermethylated in malignant cells. Gene hypermethylation usually represses its transcription and plays an important role in the gene regulation. The *RARβ* gene expression can cause RA-dependent and RA-independent apoptosis and growth arrest, which is mediated through *RARα* [22-23]. *RARβ* protein results in the expression of a number of its target genes that mediate cell differentiation and death [10, 24-25]. *RARβ* is epigenetically altered in many different kinds of human cancers. Inactivation of *RARβ* by promoter hypermethylation contributes significantly to tumorigenesis of a variety of cancers including NSCLC [26-32]. Several groups have studied *RARβ* methylation status in NSCLC; but the lack of systemic and quantitative analysis of methylation of *RARβ* in NSCLC and its relationship with the clinical parameters really hampers the appreciation of mechanism of lung cancer as well as the development of novel tools for diagnosis and treatment of NSCLC patients. Analysis of the pooled data showed that there was a higher *RARβ* hypermethylation in NSCLC patients when compared to normal lung tissue, indicating that *RARβ* hypermethylation is risk factor in the carcinogenesis of NSCLC. *RARβ* hypermethylation was also found to correlated with pathological types, as well as smoking status. Our findings that NSCLC patients have high frequencies

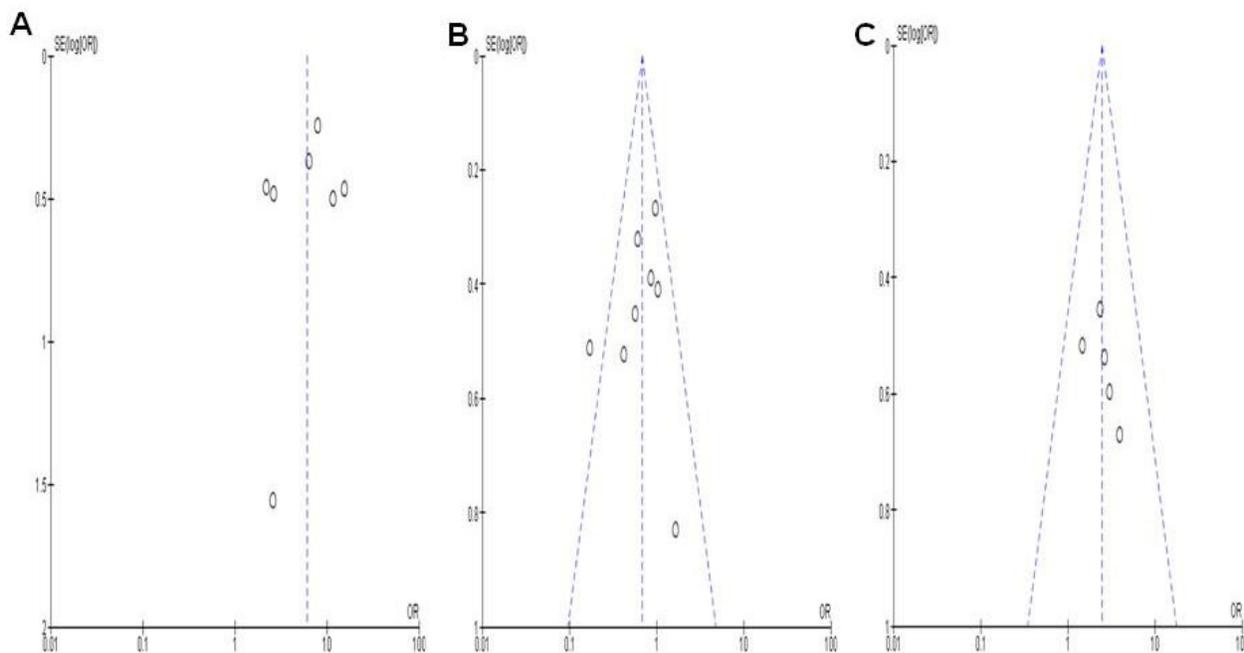


Figure 5: The symmetrical funnel plots demonstrates that no publication biases existed regarding *RARβ* hypermethylation and clinicopathological features. Panel A. showed the funnel plot from 7 studies comparing NSCLC and normal lung tissue. Panel B. showed the funnel plot from 8 studies comparing *RARβ* hypermethylation between squamous cell carcinoma (SCC) and adenocarcinoma (AC). Panel C showed the funnel plot from 5 studies examining the relationship between *RARβ* hypermethylation and the smokers/ non-smokers in NSCLC patients C. X axis: value of Odds ratio (OR); Y axis: Standard errors (SE) multiply log scale of OR.

of *RARβ* gene promoter methylation compared to the normal lung tissues, indicating that the detection of *RARβ* hypermethylation may provide a practical diagnostic marker for NSCLC patients. Unlike genetic changes, the reversible epigenetic modifications in *RARβ* gene by methylation can be demethylated and therefore would be practical to inhibit or delay carcinogenesis. Epigenetically silenced *RARβ* has been shown to be re-expressed in the presence of histone deacetylase (HDAC) inhibitors and DNA methyltransferase inhibitors (DNMT) inhibitors in *RARβ2* silent cancer cells [33]. Treatment with 9-cis RA and an HDAC inhibitor showed effective in a cancer xenografts [34]. Four lung tumor lines treated with 5-aza-2'-deoxycytidine, restored *RARβ* expression and resulted in decreased tumorigenicity [15]. In addition, curcumin can increase *RARβ* expression at the mRNA and protein levels in lung cancer A549 and H460 cells, indicating that curcumin is able to inhibit *RARβ* promoter methylation [35]. The clinical trials have shown the therapeutic potential by reactivation of tumor suppressor expression and may shed light on cancer treatment through gene-targeted therapy.

A number of studies detected and compared the different rate of *RARβ* hypermethylation; however, the results were contradictory due to limited number of patients from AC and SCC [36-37]. The combining estimates of the odds ratio from 8 publication having 456 SCC and 567 AC is OR = 0.68, 95% CI = 0.52-0.89, $p = 0.005$, which shows the rate of *RARβ* hypermethylation was higher in AC than in SCC. In addition, studies have also reported a correlation between DNA methylation and tobacco carcinogens [38-42]. The combining estimates of the odds ratio from 5 studies having 232 and 213 NSCLC with and without smoking history is OR = 2.46, 95% CI = 1.54-3.93, $p = 0.0002$, shows *RARβ* hypermethylation significantly increased in smoking NSCLC patients compared to non-smoking NSCLC.

Publication bias did not exist after sensitivity analyses. However the study has several potential restrictions for some reasons. The language was limited to articles published in English. Publications in other languages were excluded due to unavailability of accurate medical translation. In addition, the selection biases could be possibly existed due to observational studies. Cancer cells may silence or repress *RARβ* by mechanisms other than hypermethylation in NSCLC in order to initiate and promote their growth and resist treatment with RA. Therefore, cautions should be taken when our results are extrapolated to the general populations.

Taken together, our analysis showed a higher *RARβ* hypermethylation in NSCLC than normal lung tissues, higher in AC than in SCC, and higher in smokers than in non-smokers. *RARβ* promoter hypermethylation, which inactivated *RARβ* gene, may significantly contribute to the carcinogenesis and serve as a potential drug target and diagnostic marker for NSCLC patients.

MATERIALS AND METHODS

Inclusion and exclusion criteria

The database of Pubmed, Embase, and ISI web of knowledge were searched for publications from May 1, 1998 to December 2015 by the key words: “lung” and “cancer or tumor or neoplasm or carcinoma”, “methylation”, and “*RARβ* or retinoic acid receptor- β or NR1B2”. In addition, the author bibliographies were also searched manually from the retrieved publications for additional papers.

After withdraw of irrelevant and/or repeated publications from various resources, the remaining papers in the full text were evaluated for selection according to inclusion and exclusion criteria. All searched papers were retrieved. The cited references of selected studies were also determined for other relevant studies. Only one complete study was selected if same patient populations were repeatedly reported.

The inclusion criteria for eligibility include: (1) *RARβ* hypermethylation was determined in the lung tissues of primary NSCLC patients, (2) *RARβ* promoter hypermethylation was detected by polymerase chain reaction (PCR), (3) the data provided the information on both status of *RARβ* hypermethylation as well as clinicopathological indexes of the NSCLC patients (4) the statistic information was given such as individual hazard ratio (HR) of overall survival (OS) and 95 % confidence interval (CI). However, the Letters, reviews, case reports, conference abstracts, editorials, expert opinion, and all research which are involved *in vitro/ex vivo* studies were not included.

Extraction methods

We reviewed the following information in each eligible study, authorship, time of publication, sample collection, case numbers, clinicopathological information, the techniques used to calculate gene promoter methylation, methylation frequencies and/or gene product expression, and the process of follow-up. Disagreements between the analyzers were fully discussed until come to an agreement. Demographic data and clinical responses were formatted into a table. Heterogeneity of study was calculated to determine if the dataset were suitable for a meta-analysis.

The methodology of each study was evaluated and scored based on the REMARK and ELCWP guidelines [43-44].

Statistical analysis

Analysis was performed by the softwares of STATA 12.0 (Stata Corporation, TX, USA) and Review Manager 5.2 (Cochrane Collaboration, Oxford, UK). The pooled ratios of *RARβ* promoter hypermethylation and 95% confidence intervals (95% CI) were calculated. The frequency of *RARβ* hypermethylation was analyzed and correlated to various tumor phenotypes. Heterogeneity among studies was calculated by Cochran's Q test [45] which showed by the I^2 [46-47]. When heterogeneity did not exist (I^2 values < 50%), a fixed effect model was utilized. On the contrary, a random-effects model was utilized to pool individual data together. In addition, potential sources of heterogeneity were identified by further subgroup analyses. The existence of an association between *RARβ* promoter hypermethylation and clinicopathological indexes were determined by a pooled OR. P values less than 0.05 was considered significant level.

Publication bias was assessed by using a method reported by Egger et al [48]. We also explored reasons for statistical heterogeneity using meta-regression, subgroup analysis, and sensitivity analysis. The analysis of meta-regression and publication bias were performed by using STATA version 10.0.

CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

Authors' contribution

YL, DL, HL participated in the design of the study; YL, DL performed experiments; YL, YM, and HL analyzed data; YL, HL wrote the manuscript; All authors reviewed the manuscript.

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