

## RESEARCH ARTICLE

# Associations Between RASSF1A Promoter Methylation and NSCLC: A Meta-analysis of Published Data

Wen-Jian Liu, Xiao-Hong Tan, Bao-Ping Guo, Qing Ke, Jie Sun, Hong Cen\*

### Abstract

**Background:** RASSF1A has been reported to be a candidate tumor suppressor in non-small cell lung cancer (NSCLC). However, the association between RASSF1A promoter methylation and NSCLC remains unclear, particularly in regarding links to clinicopathologic features. **Methods:** Eligible studies were identified through searching PubMed, EMBASE, Cochrane Library and China National Knowledge Infrastructure (CNKI) databases. Studies were pooled and odds ratios (ORs) with corresponding confidence intervals (CIs) were calculated. Funnel plots were also performed to evaluate publication bias. **Results:** Nineteen studies involving 2,063 cases of NSCLC and 1,184 controls were included in this meta-analysis. A significant association was observed between RASSF1A methylation and NSCLC in the complete data set (OR = 19.42, 95% CI: 14.04-26.85,  $P < 0.001$ ). Pooling the control tissue subgroups (heterogeneous/autologous) gave pooled ORs of 32.4 (95% CI, 12.4-84.5) and 17.7 (95% CI, 12.5-25.0) respectively. Racial subgroup (Caucasian/Asian) analysis gave pooled ORs of 26.6 (95% CI, 10.9-64.9) and 20.9 (95% CI, 14.4-30.4) respectively. The OR for RASSF1A methylation in poorly-differentiated vs. moderately/well-differentiated NSCLC tissues was 1.88 (95% CI, 1.32-2.68,  $P < 0.001$ ), whereas there were no significant differences in RASSF1A methylation in relation to gender, pathology, TNM stage and smoking behavior among NSCLC cases. **Conclusion:** This meta-analysis suggests a significant association between RASSF1A methylation and NSCLC, confirming the role of RASSF1A as a tumor suppressor gene. Large-scale and well-designed case-control studies are needed to validate the associations identified in the present meta-analysis.

**Keywords:** RAS associations domain family protein 1A - non-small cell lung cancer - methylation - odds ratio

*Asian Pacific J Cancer Prev*, 14 (6), 3719-3724

### Introduction

As of 2008, lung cancer was the most commonly diagnosed cancer and the leading cause of cancer death in males, as well as the fourth most commonly diagnosed cancer and second leading cause of cancer death in females (Jemal et al., 2011). Primary lung cancer may be divided into two categories according to histopathology: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). 80% of primary lung cancers are non-small cell lung cancer (NSCLC), which has a long asymptomatic latency and poor prognosis. Despite advances in both detection and treatment of NSCLC, the overall 5-year survival rate remains less than 15% (Jemal et al., 2011). Once tumor cells have spread, the long-term prognosis is poor because no curative treatments are available. Thus, identification of biomarkers for early detection of NSCLC is of great importance. Previous studies have shown that a number of tumor-related and tumor suppressor genes (TSGs) were frequently methylated in NSCLC and they might be involved in the pathogenesis and progression of NSCLC (Agathangelou et al., 2005; Anglim et al., 2008; Choi et al., 2008; Zhang et al., 2011; Kontic et al., 2012). Several potential TSGs have been described as frequently

silenced by methylation in NSCLC. p16, MGMT, hMLH1, H-cadherin, CDH-1, DAPK, TMS1, RAR $\beta$ 2 and RASSF1A are all TSGs that are frequently methylated in NSCLC patients. In particular, RAS association domain containing protein 1A (RASSF1A) is widely investigated. RASSF1A, is located within a 120-kb region of chromosome 3p21, a site of frequent epigenetic modifications in NSCLC (Brauch et al., 1987). RASSF1A is functionally involved in cell cycle control, microtubule stabilization, cellular adhesion, motility and apoptosis (Agathangelou et al., 2005). It shares high sequence homology with a known mouse protein (Nore1) and may serve as the effector that regulates Ras induced apoptosis by binding Ras in a guanosine triphosphate dependent manner (Hesson et al., 2007). RASSF1A could also lead to cell cycle arrest by participating in the retinoblastoma family cell cycle checkpoint. Inhibition of cyclinD1 by RASSF1A occurs post-transcriptionally and may be at the level of translational control (Shivakumar et al., 2002). Previous studies have reported that epigenetic inactivation of RASSF1A by methylation of CpG islands in the promoter region [NC\_000075.5 (107,453,580–107,454,373)] was observed in several cancers. RASSF1A was also associated with clinicopathological factors in various

Department of Chemotherapy, The Affiliated Tumor Hospital of Guangxi Medical University, Nanning, China \*For correspondence: cen\_hong@163.com

cancers, including cervical (Yu et al., 2003), gastric (To et al., 2002), bladder (Chan et al., 2003), nasopharyngeal (Kwong et al., 2002) and NSCLC (Dammann et al., 2000). Furthermore, RASSF1A promoter methylation has been identified as an independent indicator of poor prognosis for NSCLC in a meta-analysis of 19 published studies (Wang et al., 2011). All of these findings suggest that RASSF1A promoter methylation might play a pivotal role in the development of NSCLC.

To date, the prevalence of RASSF1A methylation is exhibited in 30–40% of NSCLC, and is mainly detected by methylation-specific PCR (MSP) analysis (Burbee et al., 2001; Toyooka et al., 2003; Guo et al., 2004; Wang et al., 2004; Choi et al., 2005; Dammann et al., 2005; Ito et al., 2005; Chen et al., 2006; Hsu et al., 2007; Kim et al., 2007; Yanagawa et al., 2007; Yang et al., 2007; Liu et al., 2008; Lin et al., 2009; Peng et al., 2010; Zhang et al., 2010; Kang et al., 2011; Song et al., 2011; Li et al., 2012). However, association of RASSF1A promoter methylation with NSCLC have mostly been investigated in studies with small sample sizes or other limitations in study design, leading to conflicting results, especially in the relationship between RASSF1A promoter methylation and clinicopathologic features among NSCLC. Some studies have reported that RASSF1A methylation was found more frequently in smoking patients (Dammann et al., 2005; Yang et al., 2007) and as their tumors progressed (Yang et al., 2007). And Kim et al showed that the methylation rate of RASSF1A was significantly higher in cases of adenocarcinoma than in squamous cell carcinoma (47% vs. 36%,  $P < 0.05$ ) (Kim et al., 2007). Other studies demonstrated no association between RASSF1A methylation and the individual clinical features such as gender, pathology, TNM stage, and smoking behavior (Ito et al., 2005; Chen et al., 2006; Peng et al., 2010; Kang et al., 2011; Song et al., 2011). Some researchers found no statistically significant associations between RASSF1A methylation and NSCLC differentiation (Ito et al., 2005; Kang et al., 2011; Song et al., 2011). However, other studies have demonstrated statistically relevant associations (Guo et al., 2004; Wang et al., 2004; Peng et al., 2010; Zhang et al., 2010). Hence, we conducted a meta-analysis of the published studies to derive a more precise estimation of the associations.

## Materials and Methods

### Study selection

This study involved searching a series of computerized databases, including PubMed, EMBASE, Cochrane Library and China National Knowledge Infrastructure (CNKI). Database searches were performed up to February 2013 with no language limitation. Studies were identified via an electronic search with the keywords: 'non-small cell lung carcinoma', 'non-small cell lung cancer', 'NSCLC', 'RAS association domain family protein 1A', 'RASSF1A', 'methylation' and 'hypermethylation'. Additional studies were found via the reference lists of the identified articles. Two independent reviewers (Liu and Tan) screened the titles and abstracts identified by the electronic search to identify relevant studies. Our inclusion

criteria were as follows: (1) Studies primarily evaluating the incidence of RASSF1A methylation in NSCLCs and corresponding control tissues; (2) Measurement of DNA methylation must be performed on surgically resected primary tumor samples; (3) A case-control study. Our exclusion criteria were: (1) RASSF1A methylation conducted in the cell lines, body fluids such as sputum, peritoneal fluid and serum; (2) Unavailable raw data on the amount of methylation among cases and controls; (3) Review papers. We also used a manual search for relevant articles, including original articles and reviews, to identify additional studies. To avoid duplication of data, we carefully checked the authors' names and the different research institutions involved. If there was any disagreement between the two authors, it was settled by discussion with a third author (Guo) until a consensus was reached.

### Data extraction

The following data for each eligible study were extracted: first author's name, publication year, patient ethnicity, sample size, and RASSF1A promoter methylation status in extracted NSCLC tissues and controls. We also collected the clinical characteristics of NSCLC patients (gender, pathological type, differentiation, TNM stage and smoking behavior) for further research. We only discussed the two most common pathological types, adenocarcinoma (AC) and squamous cell carcinoma (SCC) in our meta-analysis. Although the precise definition of non-smoker status varied slightly among the studies, we classified patients as ever-smokers (former smokers and/or current smokers regardless of the extent of smoking) or never-smokers in our meta-analysis. Rates of RASSF1A promoter methylation were collected using standard data extraction forms. We did not contact the author of the primary study to request the information and did not require a minimum number of patients for a study included in our meta-analysis. Point estimates for selected variables were extracted and checked by the other two reviewers (Ke and Sun).

### Statistical analysis

The odds ratio (OR) with corresponding 95% confidence intervals (CIs) was used to assess the effect of the associations between the RASSF1A promoter methylation and NSCLC risk in cases and controls. Subgroup analyses of the ORs of RASSF1A promoter methylation in NSCLC tissues vs. control tissues were performed according to control types (autogenous and heterogeneous) and ethnicity (Caucasian and Asian). Differences in the RASSF1A promoter methylation status of NSCLC were also analyzed in relation to gender (male vs. female), pathological type (AC vs. SCC), differentiation grade (poorly vs. moderately/well), TNM stage (TNMIII, IV vs. TNMI, II), and smoking behavior (ever-smokers vs. never-smokers). According to the heterogeneity statistic  $I^2$ , a fixed effect or a random-effects model was selected. An effects model was selected according to the heterogeneity statistic  $I^2$ : a fixed effect model was used when  $I^2 < 50\%$ , following the Mantel-Haenszel method, otherwise a random-effects model was applied.

**Table 1. Main Characteristics of the Included Studies**

Study/country	Year	Race	Histology /control	Methods	Male/female	Patients (M+/M-)	Control (M+/M-)	Median age (year)
Li et al/China	2012	Asian	NSCLC/H	MSP	not clear	48/8	0/52	52.6±16.2
Song et al/China	2011	Asian	NSCLC/A	MSP	58/20	31/47	6/72	59
Kang et al/China	2011	Asian	NSCLC/H	MSP	46/7	10/43	0/24	54.3
Zhang et al/China	2010	Asian	NSCLC/H	MSP	121/29	58/92	0/20	59.0±9.0
Peng et al/China	2010	Asian	NSCLC/H	MSP	55/27	52/30	0/25	56.6±9.4
Lin et al/China	2009	Asian	NSCLC/H	MSP	80/44	53/71	2/24	not clear
Liu et al/China	2008	Asian	NSCLC/A	MSP	44/16	40/20	7/53	60.83±10.16
Yang et al/China	2007	Asian	NSCLC/A	MSP	36/17	30/23	5/48	57.0±10.0
Yanagawa et al/Japan	2007	Asian	NSCLC/A	MSP	72/29	42/59	3/98	68.1
Kim et al/Korea	2007	Asian	NSCLC/A	MSP	80/19	40/59	1/98	63±8.4
Hsu et al/Taiwan	2007	Asian	NSCLC/A	n-MSP	45/18	30/33	9/54	69
Chen et al/Japan	2006	Asian	NSCLC/A	MSP	65/49	44/70	0/57	not clear
Ito et al/Japan	2005	Asian	NSCLC/A	MSP	85/53	44/94	0/138	not clear
Dammann et al /Germany	2005	Caucasian	NSCLC/A	MSP	not clear	7/9	0/7	not clear
Choi et al/Korea	2005	Asian	NSCLC/A	MSP	99/17	47/69	0/60	63
Wang et al/USA	2004	Caucasian	NSCLC/A	MSP	72/47	46/73	4/115	not clear
Guo et al/USA	2004	Caucasian	NSCLC/A	MSP	not clear	12/8	0/14	66.1±10.9
Toyooka et al/USA	2003	Mix	NSCLC/A	MSP	355/159	191/323	5/79	not clear
Burbee et al/USA	2001	Caucasian	NSCLC/A	MSP	76/31	32/75	0/104	61

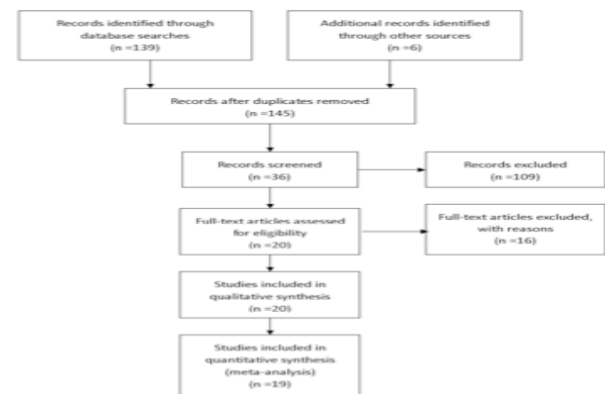
A, Autologous control, the control tissues from corresponding adjacent normal tissues; H, Heterogeneous control, the control tissues from non-cancerous respiratory diseases; MSP, methylation-specific PCR; n-MSP, nested methylation-specific PCR; M+, The number of tissues with methylation; M-, The number of tissues with unmethylation

If the heterogeneity was significant, a Galbraith plot and meta-regression analyses were employed to analyze the sources of the heterogeneity. We also evaluated possible publication bias with funnel plots and Egger's test. The significance of the intercept was determined by the t-test ( $P < 0.05$  was considered representative of statistically significant publication bias). All statistical analyses were conducted with Stata statistical software (version 11.0, Stata Corporation, College Station, Texas, USA). Results were shown in forest plots, where the sizes of the boxes for individual studies were inversely proportional to the variances of the log relative risks, and the horizontal lines represent a 95% confidence interval (CI).

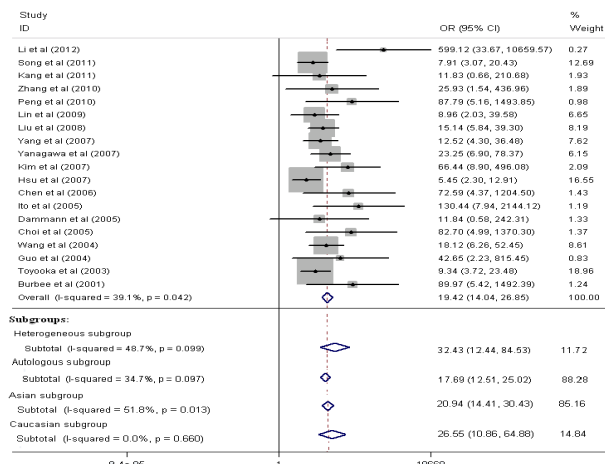
## Results

### Study characteristics

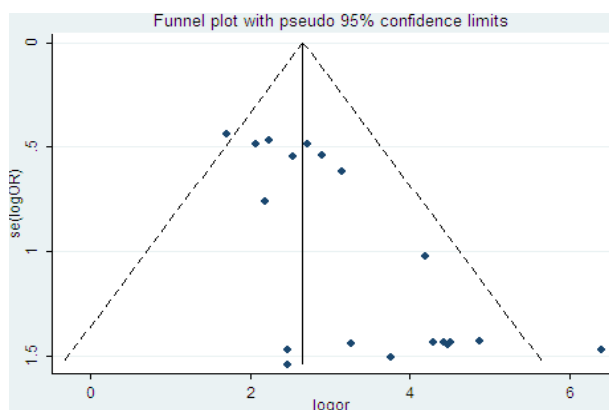
There were 145 articles relevant to the search terms described previously. Of these, 109 were initially excluded after further examining titles and abstracts (35 not about NSCLC; 28 with other gene methylation; 12 on serum; 11 on cell lines, 7 on sputum; 9 review articles; 7 on tumor biological behavior). Investigators retrieved the remaining 36 citations for full text evaluation. Upon further review, 8 articles were not case-control studies, 5 articles were not target methylation, and 3 were of inadequate data for meta-analysis, excluding them from the study. Moreover, one was excluded as a duplicate publication (Yanagawa et al., 2003). Ultimately, 19 publications met the inclusion criteria (Figure 1) (Burbee et al., 2001; Toyooka et al., 2003; Guo et al., 2004; Wang et al., 2004; Choi et al., 2005; Dammann et al., 2005; Ito et al., 2005; Chen et al., 2006; Hsu et al., 2007; Kim et al., 2007; Yanagawa et al., 2007; Yang et al., 2007; Liu et al., 2008; Lin et al., 2009; Peng et al., 2010; Zhang et al., 2010; Kang et al., 2011; Song et al., 2011; Li et al., 2012) and were included in the meta-analysis. Detailed characteristics of the 19 studies are listed in Table 1. Overall, 5 of the included articles

**Figure 1. Flow Diagram of Study Selection**

were published in Chinese (Yang et al., 2007; Peng et al., 2010; Zhang et al., 2010; Kang et al., 2011; Song et al., 2011), and the others were published in English (Burbee et al., 2001; Toyooka et al., 2003; Guo et al., 2004; Wang et al., 2004; Choi et al., 2005; Dammann et al., 2005; Ito et al., 2005; Chen et al., 2006; Hsu et al., 2007; Kim et al., 2007; Yanagawa et al., 2007; Liu et al., 2008; Lin et al., 2009; Li et al., 2012). The aforementioned 19 studies provided data regarding RASSF1A promoter methylation in 2063 cases of NSCLC tissues and 1184 controls of non-tumor tissues, with a total sample size of 3247 cases. The median age of the study participants ranged from 52.6 to 69 years. All of the specimens in the 19 studies were surgically obtained from NSCLC patients and histologically confirmed. Controls were mainly matched for sex and age. Among the controls, 14 studies were autogenous (tissues from corresponding adjacent normal tissues) (Burbee et al., 2001; Toyooka et al., 2003; Guo et al., 2004; Wang et al., 2004; Choi et al., 2005; Dammann et al., 2005; Ito et al., 2005; Chen et al., 2006; Hsu et al., 2007; Kim et al., 2007; Yanagawa et al., 2007; Yang et al., 2007; Liu et al., 2008; Song et al., 2011), and 5 were heterogeneous (tissues from non-cancerous respiratory diseases) (Lin et al., 2009; Peng et al., 2010; Zhang et al.,



**Figure 2. Forest Plot of RASSF1A Promoter Methylation in NSCLC Tissues vs. Normal Tissues and Subgroup Analyses**



**Figure 3. Funnel Plot, The Results Showed that the Standard Errors in Two of the Studies Exceeded the 95% Confidence Limit, and Egger’s Test Detected Evidence of Publication Bias (t=4.51, P = 0.000)**

2010; Kang et al., 2011; Li et al., 2012). Among the 19 retrieved articles, 18 studies (Burbee et al., 2001; Toyooka et al., 2003; Guo et al., 2004; Wang et al., 2004; Choi et al., 2005; Dammann et al., 2005; Ito et al., 2005; Chen et al., 2006; Kim et al., 2007; Yanagawa et al., 2007; Yang et al., 2007; Liu et al., 2008; Lin et al., 2009; Peng et al., 2010; Zhang et al., 2010; Kang et al., 2011; Song et al., 2011; Li et al., 2012) used methylation-specific PCR (MSP), while the remaining study (Hsu et al., 2007) used nested methylation-specific PCR (n-MSP) to explore RASSF1A promoter methylation. Furthermore, 14 were Asian (Choi et al., 2005; Ito et al., 2005; Chen et al., 2006; Hsu et al., 2007; Kim et al., 2007; Yanagawa et al., 2007; Yang et al., 2007; Liu et al., 2008; Lin et al., 2009; Peng et al., 2010; Zhang et al., 2010; Kang et al., 2011; Song et al., 2011; Li et al., 2012), 4 were Caucasian (Burbee et al., 2001; Guo et al., 2004; Wang et al., 2004; Dammann et al., 2005), and one involved mixed ethnicities from United States, Australia, Japan and Taiwan (Toyooka et al., 2003). The pooled OR for RASSF1A promoter methylation in NSCLC tissues compared with non-tumor tissues was 19.42 (95% CI, 14.04-26.85,  $P < 0.001$ ), suggesting an increased likelihood of RASSF1A promoter methylation in NSCLC tissues compared with normal tissue (Figure 2). There was a low degree of heterogeneity among the

**Table 2. RASSF1A promoter methylation in relation to gender, pathology, differentiation, TNM stages and smoke behavior among NSCLC cases**

Patient characteristics	Methylation (%)	OR (95% CIs)	Heterogeneity test (I <sup>2</sup> , P-value)
Gender			
Male	39.6	1.12(0.87-1.65)	0.0%, 0.269
Female	34.96		
Pathological type			
AC	39.85	1.12(0.90-1.40)	1.5%, 0.306
SCC	38.76		
Differentiation			
Poorly	45.41	1.88(1.32-2.68)	0.0%, 0.001
Moderately/well	31.76		
TNM stage			
TNM III, IV	43.64	1.15(0.84-1.57)	24.3%, 0.392
TNM I, II	38.27		
Smoking behavior			
Ever-smokers	42.34	1.25(0.90-1.73)	61.6%, 0.181
Never-smokers	37.16		

AC, adenocarcinoma; SCC, squamous cell carcinoma; CI, confidence interval; OR, odds ratio

19 studies (I<sup>2</sup> = 39.1%, P = 0.042).

*Subgroup analysis*

14 studies calculated the OR of RASSF1A promoter methylation in NSCLC cases and autogenous controls (Burbee et al., 2001; Toyooka et al., 2003; Guo et al., 2004; Wang et al., 2004; Choi et al., 2005; Dammann et al., 2005; Ito et al., 2005; Chen et al., 2006; Hsu et al., 2007; Kim et al., 2007; Yanagawa et al., 2007; Yang et al., 2007; Liu et al., 2008; Song et al., 2011), and the other 5 studies involved heterogeneous tissue as controls (Lin et al., 2009; Peng et al., 2010; Zhang et al., 2010; Kang et al., 2011; Li et al., 2012). The OR in the autologous control subgroup was 17.69 (95%CI, 12.51-25.02;  $P < 0.001$ ) and the OR in the heterogeneous control subgroup was 32.43 (95%CI, 12.44-84.53;  $P < 0.001$ ) (Figure 2). In the subgroup analysis of ethnicity, the OR for the Caucasian subgroup was 26.55 (95% CI, 10.86-64.88;  $P < 0.001$ ) from 4 studies (Burbee et al., 2001; Guo et al., 2004; Wang et al., 2004; Dammann et al., 2005), for the Asian subgroup was 20.94 (95% CI, 14.41-30.43;  $P < 0.001$ ) from 14 studies (Choi et al., 2005; Ito et al., 2005; Chen et al., 2006; Hsu et al., 2007; Kim et al., 2007; Yanagawa et al., 2007; Yang et al., 2007; Liu et al., 2008; Lin et al., 2009; Peng et al., 2010; Zhang et al., 2010; Kang et al., 2011; Song et al., 2011; Li et al., 2012) (Figure 2).

*Associations between RASSF1A promoter methylation and clinicopathologic features in NSCLC cases*

We also conducted an analysis of the relationship between clinicopathologic features and RASSF1A promoter methylation among NSCLC cases (Table2). We found no significant associations between RASSF1A methylation and the following clinicopathologic features: gender, pathological type, TNM stage and smoking behavior. However, there was a relationship between RASSF1A and differentiation status; the OR for poorly-differentiated NSCLC vs. moderately/well-differentiated NSCLC was 1.88 (95% CI, 1.32-2.68,  $P < 0.001$ ). Detailed are listed in Table 2.

### Publication bias

To ensure the quality of this study, we performed a Begger's funnel plot and Egger's tests to eliminate the publication bias of included studies. Egger's test was used to provide statistical evidence of funnel plot symmetry (Figure 3) and detected evidence of publication bias ( $t = 4.51, P = 0.000$ ).

## Discussion

NSCLC involves complex, multistep and heterogeneous malignant tumorigenesis. Gene methylation is a major source of epigenetic modification in mammals, and changes in methylation patterns play a key role in tumorigenesis in humans. Particularly, promoter CpG island methylation is related to inactivation and silencing, resulting in decreased expression of tumor suppressors, chromosome inactivation and affects the development of tumors (Baylin et al., 2005; Hesson et al., 2007; Akhavan-Niaki et al., 2013). Aberrant methylation within the promoter region of RASSF1A has been reported in various tumors, including NSCLC (Dammann et al., 2000). The role of RASSF1A promoter methylation in NSCLC is controversial, especially in the relationship between RASSF1A promoter methylation and clinical features among NSCLC patients (Burbee et al., 2001; Toyooka et al., 2003; Guo et al., 2004; Wang et al., 2004; Choi et al., 2005; Dammann et al., 2005; Ito et al., 2005; Chen et al., 2006; Kim et al., 2007; Yang et al., 2007; Zhang et al., 2010; Kang et al., 2011; Song et al., 2011). We therefore performed a meta-analysis to derive a more precise estimation of the associations between RASSF1A gene methylation and NSCLC.

This meta-analysis is based on 19 studies including a total of 2063 cases of NSCLC tissues and 1184 cases of non-tumor tissues. The overall OR for RASSF1A promoter methylation status in NSCLC tissues vs. non-tumor tissues was 19.42 (95%CI, 14.04-26.85,  $P < 0.001$ ) based on a fixed-effects model. And the result suggests a significantly increased likelihood of RASSF1A promoter methylation in NSCLC tissues compared with non-tumor tissues (Figure 2). Subgroup analysis showed an OR in the autologous control subgroup of 17.69 (95% CI, 12.51-25.02;  $P < 0.001$ ), and an OR of 32.44 (95% CI, 12.44-84.53;  $P < 0.001$ ) in the heterogenous control subgroup. This indicates an increased likelihood of RASSF1A methylation in NSCLC cases compared with heterogeneous controls than autologous controls. The OR also differed in subgroups with different races: the OR in the Caucasian subgroup was 26.55 (95% CI, 10.86-64.88;  $P < 0.001$ ), while the Asian subgroup OR was 20.94 (95% CI, 14.41-30.43;  $P < 0.001$ ). Analysis of racial subgroups showed that RASSF1A methylation was more strongly associated with increased risk of NSCLC in the Caucasian population.

Additionally, we also conducted an analysis of the relationship between the clinicopathologic features and RASSF1A methylation among NSCLC cases. The pooled OR in poorly-differentiated vs. moderately/well-differentiated NSCLC was 1.88 (95% CI, 1.32-2.68,  $P < 0.001$ ), indicating an increasing likelihood of RASSF1A

promoter methylation in poorly-differentiated rather than moderately/well-differentiated tissues. However, there was no statistically significant association between RASSF1A promoter methylation and the individual clinical features among NSCLC cases: gender ( $P > 0.1$ ), pathology ( $P > 0.1$ ), TNM stages ( $P > 0.1$ ) and smoking behavior ( $P > 0.1$ ).

No significant relationship with RASSF1A methylation was observed in NSCLC patients with different TNM stage or smoking behavior. Although some studies have reported RASSF1A methylation was more frequently in smokers (Dammann et al., 2005; Yang et al., 2007) and in patients with more advanced TNM stages (Yang et al., 2007), the overall results of the current meta-analysis failed to support the existence of such a relationship. Other factors such as gender and disease pathology also demonstrated no relationship with RASSF1A promoter methylation. This is contrary to the conclusion drawn by Kim et al that the methylation rate of RASSF1A was significantly higher in adenocarcinoma cases than squamous cell carcinoma cases (47% vs. 36%,  $P < 0.05$ ) (Kim et al., 2007). Reasons for inconsistencies among these studies is that they are single case-control studies with small sample sizes, low statistical power, specific ethnic backgrounds, and have other limitations in study design. Our aggregated results found that RASSF1A promoter methylation was increased in poorly-differentiated NSCLC compared with moderately/well-differentiated NSCLC, suggesting that poorly-differentiated NSCLC had a higher RASSF1A methylation OR than moderately/well-differentiated cancer tissues. This suggests that RASSF1A promoter methylation may be related to poor prognosis, as suggested in a meta-analysis mentioned previously (Wang et al., 2011).

This meta-analysis quantitatively assessed the relationship of RASSF1A promoter methylation between NSCLC tissues and non-tumor tissues, as well as the association between RASSF1A promoter methylation and clinicopathologic features among NSCLC patients using well-designed case-control studies. To our knowledge, this has not been presented in other meta-analyses or reviews. However, there are several potential limitations in this study. The possibility of information and selection biases and unidentified confounders cannot be excluded completely because all of the included studies were observational. Secondly, most studies included in this meta-analysis were conducted in an Asian population, which may produce selection bias. Additionally, publication bias existed in some comparisons, which may potentially influence the results of our meta-analysis. Since the number of studies included in the subgroup analyses was small, the results lack sufficient evidence to confirm an associations in a definitive manner. Finally, for some included case-control studies, our results were based on unadjusted estimates, whereas more precise analysis could be performed if individual data were available. Hence, caution should be taken before applying our results to the general population.

In conclusion, we found significant associations between RASSF1A promoter methylation and NSCLC. Moreover, the incidence of methylation was higher in

NSCLC vs. heterogeneous tissues than autologous tissues. RASSF1A methylation was more strongly associated with increased risk of NSCLC in Caucasian than Asian populations. RASSF1A methylation was also associated with the differentiation state of NSCLC, suggesting that RASSF1A might play a more significant role in NSCLC tissue differentiation. However, gender, pathology, TNM stage and smoking status showed no significant associations with RASSF1A methylation among NSCLC tissues.

## References

- Agathangelou A, Cooper WN, Latif F (2005). Role of the Ras-association domain family 1 tumor suppressor gene in human cancers. *Cancer Res*, **65**, 3497-508.
- Akhavan-Niaki H, Samadani AA (2013). DNA Methylation and Cancer Development: Molecular Mechanism. *Cell Biochem Biophys*.
- Anglim PP, Alonzo TA, Laird-Offringa IA (2008). DNA methylation-based biomarkers for early detection of non-small cell lung cancer: an update. *Mol Cancer*, **7**, 81.
- Baylin SB (2005). DNA methylation and gene silencing in cancer. *Nat Clin Pract Oncol*, **2**, S4-11.
- Brauch H, Johnson B, Hovis J, et al (1987). Molecular analysis of the short arm of chromosome 3 in small-cell and non-small-cell carcinoma of the lung. *N Engl J Med*, **317**, 1109-13.
- Burbee DG, Forgacs E, Zochbauer-Muller S, et al (2001). Epigenetic inactivation of RASSF1A in lung and breast cancers and malignant phenotype suppression. *J Natl Cancer Inst*, **93**, 691-9.
- Chan MW, Chan LW, Tang NL, et al (2003). Frequent hypermethylation of promoter region of RASSF1A in tumor tissues and voided urine of urinary bladder cancer patients. *Int J Cancer*, **104**, 611-6.
- Chen H, Suzuki M, Nakamura Y, et al (2006). Aberrant methylation of RASGRF2 and RASSF1A in human non-small cell lung cancer. *Oncol Rep*, **15**, 1281-5.
- Choi JE, Kim DS, Kim EJ, et al (2008). Aberrant methylation of ADAMTS1 in non-small cell lung cancer. *Cancer Genet Cytogenet*, **187**, 80-4.
- Choi N, Son DS, Song I, et al (2005). RASSF1A is not appropriate as an early detection marker or a prognostic marker for non-small cell lung cancer. *Int J Cancer*, **115**, 575-81.
- Dammann R, Li C, Yoon JH, et al (2000). Epigenetic inactivation of a RAS association domain family protein from the lung tumour suppressor locus 3p21.3. *Nat Genet*, **25**, 315-9.
- Dammann R, Strunnikova M, Schagdarsurengin U, et al (2005). CpG island methylation and expression of tumour-associated genes in lung carcinoma. *Eur J Cancer*, **41**, 1223-36.
- Guo M, House MG, Hooker C, et al (2004). Promoter hypermethylation of resected bronchial margins: a field defect of changes? *Clin Cancer Res*, **10**, 5131-6.
- Hesson LB, Cooper WN, Latif F (2007). The role of RASSF1A methylation in cancer. *Dis Markers*, **23**, 73-87.
- Hsu HS, Chen TP, Hung CH, et al (2007). Characterization of a multiple epigenetic marker panel for lung cancer detection and risk assessment in plasma. *Cancer*, **110**, 2019-26.
- Ito M, Ito G, Kondo M, et al (2005). Frequent inactivation of RASSF1A, BLU, and SEMA3B on 3p21.3 by promoter hypermethylation and allele loss in non-small cell lung cancer. *Cancer Lett*, **225**, 131-9.
- Jemal A, Bray F, Center MM, et al (2011). Global cancer statistics. *CA Cancer J Clin*, **61**, 69-90.
- Kang CY, Zhou HC, Tang SP, Xiao H (2011). Values of promoter hypermethylation of FHIT, p16, MGMT and RASSF1A genes in plasma in the diagnosis of lung cancer. *Tumor*, **31**, 729-34.
- Kim DS, Cha SI, Lee JH, et al (2007). Aberrant DNA methylation profiles of non-small cell lung cancers in a Korean population. *Lung Cancer*, **58**, 1-6.
- Kontic M, Stojic J, Jovanovic D, et al (2012). Aberrant promoter methylation of CDH13 and MGMT genes is associated with clinicopathologic characteristics of primary non-small-cell lung carcinoma. *Clin Lung Cancer*, **13**, 297-303.
- Kwong J, Lo KW, To KF, et al (2002). Promoter hypermethylation of multiple genes in nasopharyngeal carcinoma. *Clin Cancer Res*, **8**, 131-7.
- Li W, Deng J, Jiang P, et al (2012). Methylation of the RASSF1A and RAR(beta) genes as a candidate biomarker for lung cancer. *Exp Ther Med*, **3**, 1067-71.
- Lin Q, Geng J, Ma K, et al (2009). RASSF1A, APC, ESR1, ABCB1 and HOXC9, but not p16INK4A, DAPK1, PTEN and MT1G genes were frequently methylated in the stage I non-small cell lung cancer in China. *J Cancer Res Clin Oncol*, **135**, 1675-84.
- Liu Z, Zhao J, Chen XF, et al (2008). CpG island methylator phenotype involving tumor suppressor genes located on chromosome 3p in non-small cell lung cancer. *Lung Cancer*, **62**, 15-22.
- Peng Z, Shan C, Wang H (2010). [Value of promoter methylation of RASSF1A, p16, and DAPK genes in induced sputum in diagnosing lung cancers]. *Zhong Nan Da Xue Xue Bao Yi Xue Ban*, **35**, 247-53.
- Shivakumar L, Minna J, Sakamaki T, Pestell R, White MA (2002). The RASSF1A tumor suppressor blocks cell cycle progression and inhibits cyclin D1 accumulation. *Mol Cell Biol*, **22**, 4309-18.
- Song H, Yi J, Zhang Y, Wang R, Chen L (2011). DNA methylation of tumor suppressor genes located on chromosome 3p in non-small cell lung cancer. *Chin J Lung Cancer*, **14**, 233-8.
- To KF, Leung WK, Lee TL, et al (2002). Promoter hypermethylation of tumor-related genes in gastric intestinal metaplasia of patients with and without gastric cancer. *Int J Cancer*, **102**, 623-8.
- Toyooka S, Maruyama R, Toyooka KO, et al (2003). Smoke exposure, histologic type and geography-related differences in the methylation profiles of non-small cell lung cancer. *Int J Cancer*, **103**, 153-60.
- Wang J, Lee JJ, Wang L, et al (2004). Value of p16INK4a and RASSF1A promoter hypermethylation in prognosis of patients with resectable non-small cell lung cancer. *Clin Cancer Res*, **10**, 6119-25.
- Wang J, Wang B, Chen X, Bi J (2011). The prognostic value of RASSF1A promoter hypermethylation in non-small cell lung carcinoma: a systematic review and meta-analysis. *Carcinogenesis*, **32**, 411-6.
- Yanagawa N, Tamura G, Oizumi H, et al (2003). Promoter hypermethylation of tumor suppressor and tumor-related genes in non-small cell lung cancers. *Cancer Sci*, **94**, 589-92.
- Yanagawa N, Tamura G, Oizumi H, et al (2007). Promoter hypermethylation of RASSF1A and RUNX3 genes as an independent prognostic prediction marker in surgically resected non-small cell lung cancers. *Lung Cancer*, **58**, 131-8.
- Yang ZH, Cai YY, Sun LH (2007). Methylation of antioncogen at 3p in non-small cell lung cancer. *Chin J Cancer Prev Treat*, **14**, 363-5.
- Yu MY, Tong JH, Chan PK, et al (2003). Hypermethylation of the tumor suppressor gene RASSF1A and frequent concomitant loss of heterozygosity at 3p21 in cervical cancers. *Int J Cancer*, **105**, 204-9.
- Zhang H, Zhang S, Zhang Z, et al (2010). Prognostic value of methylation status of RASSF1A gene as an independent factor of non-small cell lung cancer. *Chin J Lung Cancer*, **13**, 311-6.
- Zhang Y, Wang R, Song H, et al (2011). Methylation of multiple genes as a candidate biomarker in non-small cell lung cancer. *Cancer Lett*, **303**, 21-8.