

RESEARCH ARTICLE

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

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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

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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 β 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

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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 |

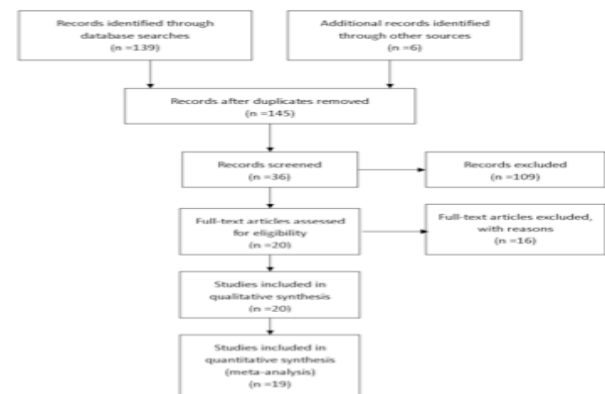
A, Autologous control, the control tissues from corresponding adjacent normal tissues; H, Heterogeneous control, the control tissues from non-cancerous respiratory diseases; MSP, methylation-specific PCR; n-MSP, nested methylation-specific PCR; M+, The number of tissues with methylation; M-, The number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

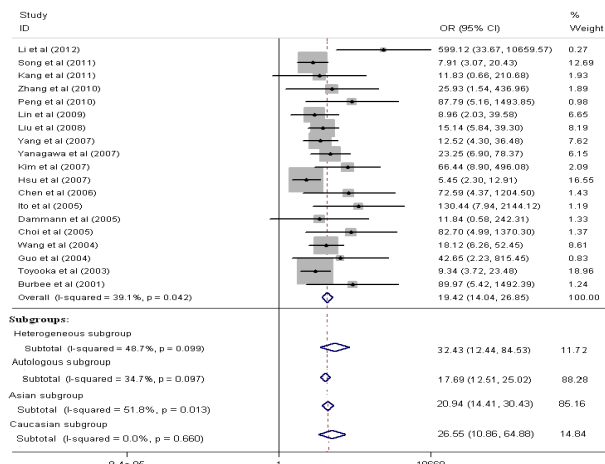


Figure 2. Forest Plot of RASSF1A Promoter Methylation in NSCLC Tissues vs. Normal Tissues and Subgroup Analyses

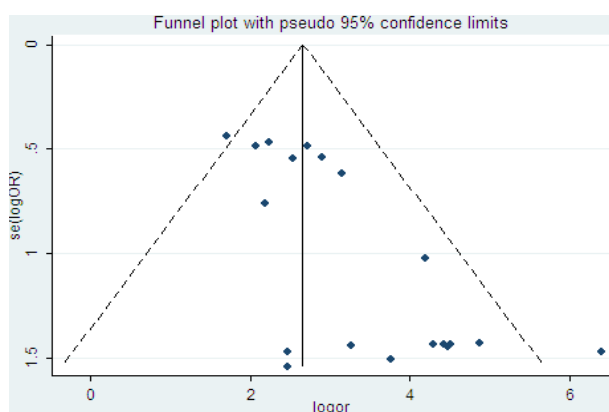


Figure 3. Funnel Plot, The Results Showed that the Standard Errors in Two of the Studies Exceeded the 95% Confidence Limit, and Egger’s Test Detected Evidence of Publication Bias (t=4.51, P = 0.000)

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 ² , 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 (I² = 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.

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