

Role of $p14^{ARF}$ and $p15^{INK4B}$ promoter methylation in patients with lung cancer: a systematic meta-analysis

Xinmei Yang^{1,*}Lei Yang^{2,*}Wanrong Dai³Bo Ye⁴

¹Department of Oncology, the First Affiliated Hospital of Jiaying University, Jiaying, ²Cancer Center, The First Hospital of Jilin University, Changchun, ³Department of Pharmacy, The First Affiliated Hospital, Zhejiang University, Zhejiang, ⁴Department of Thoracic Surgery, Hangzhou Red Cross Hospital, Hangzhou, People's Republic of China

*These authors contributed equally to this work

Background: The cyclin-dependent kinase inhibitors $p14^{ARF}$ and $p15^{INK4B}$ are tumor suppressor genes that have been reported to be silenced through promoter methylation in many human cancers. However, the strength of association between $p14^{ARF}$ or $p15^{INK4B}$ promoter methylation and lung cancer remains unclear. Thus, we first determined whether $p14^{ARF}$ and $p15^{INK4B}$ promoter methylation played a key role in the carcinogenesis of lung cancer.

Methods: Eligible studies were selected from the online electronic databases. The pooled odds ratios or hazard ratios and 95% confidence intervals were calculated and summarized.

Results: Finally, 12 studies with 625 lung cancer samples and 488 nontumor samples were included under the fixed-effects model. The pooled odds ratio showed that $p14^{ARF}$ promoter methylation was observed to be significantly higher in non-small-cell lung cancer (NSCLC) than in nontumor samples ($P < 0.001$). No significant correlation was found between $p15^{INK4B}$ promoter methylation and lung cancer ($P = 0.27$). Subgroup analysis of ethnicity revealed that $p14^{ARF}$ promoter methylation was significantly related to the risk of NSCLC in Asian and Caucasian populations. Subgroup analysis of sample type demonstrated that $p14^{ARF}$ promoter methylation was correlated with the risk of NSCLC in tissue samples ($P < 0.001$), but not in bronchoalveolar lavage fluid and blood samples. $p14^{ARF}$ promoter methylation from one study was not significantly correlated with overall survival of patients with NSCLC. Promoter methylation of $p14^{ARF}$ and $p15^{INK4B}$ was not correlated with clinicopathological characteristics, such as gender status, smoking status, tumor differentiation, and tumor stage ($P > 0.05$).

Conclusion: Our findings suggested that $p14^{ARF}$ promoter methylation may play an important role in the carcinogenesis of lung cancer, but not $p15^{INK4B}$ promoter methylation. Promoter methylation of $p14^{ARF}$ and $p15^{INK4B}$ was not associated with clinicopathological parameters. However, more extensive large-scale studies are essential to further validate our study.

Keywords: $p14^{ARF}$, $p15^{INK4B}$, methylation, lung cancer, overall survival

Introduction

Lung cancer is one of the most common human malignant tumors and is the leading cause of cancer-related deaths in the world.¹ Based on global cancer statistics, ~1,824,700 new cases of lung cancer were clinically diagnosed, with an estimated 1,589,900 deaths in 2012.¹ Human lung cancer consists of two major types: small-cell lung cancer and non-small-cell lung cancer (NSCLC). NSCLC is the most common type and accounts for 85% of lung cancer cases, including adenocarcinoma, squamous cell carcinoma, large cell carcinoma, and adenosquamous carcinoma.^{2,3} Due to the lack of an early diagnostic method, >40% of NSCLC patients have developed metastases

Correspondence: Bo Ye
Department of Thoracic Surgery,
Hangzhou Red Cross Hospital,
No 28 East Huancheng Road,
Shangcheng District, Hangzhou
310003, People's Republic of China
Tel +86 571 56109841
Fax +86 571 56109598
Email kopmanncn@sohu.com

by the time of diagnosis,⁴ and the average 5-year survival rate is currently 18%.⁵ Therefore, early diagnosis is crucial for improving the survival rate.⁶

Epigenetic modifications are involved in tumor cancers, DNA methylation, as a crucial mechanism of epigenetic changes, leads to target gene silencing, and plays a key role in the carcinogenesis and progression of cancer.⁷⁻⁹ A large number of studies have reported that genes with aberrant promoter methylation are significantly correlated with lung cancer.¹⁰⁻¹³ Located on the human chromosome 9p21, the INK4/ARF locus encodes three cell-cycle inhibitory proteins involving *p15^{INK4B}*, *p14^{ARF}*, and *p16^{INK4A}*; *p16^{INK4A}* is encoded by cyclin-dependent kinase inhibitor 2A (*CDKN2A*), *p14^{ARF}* is encoded by an alternative reading frame of *CDKN2A*, and *p15^{INK4B}* is encoded by cyclin-dependent kinase inhibitor 2B (*CDKN2B*), which plays roles in the regulation of p53 and retinoblastoma pathways.¹⁴⁻¹⁶ Inactivation of *p15^{INK4B}*, *p14^{ARF}*, and *p16^{INK4A}* as tumor suppressor genes is one of the most common events in human cancers.¹⁷⁻¹⁹ The loss of *p15^{INK4B}* expression via promoter methylation has been reported in lung cancer cell lines.²⁰ Seike et al reported that aberrant methylation of *p15^{INK4B}* was not detected in lung cancer tissues.²¹ Wang et al reported that aberrant DNA methylation may be the most common mechanism of inactivating cancer-related genes in lung cancer, including *p15^{INK4B}*.²² Yanagawa et al reported that aberrant promoter methylation of *p14^{ARF}* was correlated with lung cancer.²³ Zhang et al reported that *p14^{ARF}* promoter methylation was not correlated with lung cancer.²⁴

Thus, there were also contradictory results concerning the methylation frequency of *p14^{ARF}* and *p15^{INK4B}* promoter in lung cancer samples. The current study was first analyzed to better identify the association between *p14^{ARF}* and *p15^{INK4B}* promoter methylation and lung cancer.

Materials and methods

Search for eligible studies

A systematic search was performed through online literature databases (PubMed, EMBASE, EBSCO, Wangfang, and Cochrane Library). The following search strategy was used: “methylation,” “hypermethylation,” or “promoter methylation”; and “lung cancer,” “lung carcinoma,” or “lung tumor”; and “*p14*,” “*p14^{ARF}*,” “*p15*,” “*p15^{INK4B}*,” “*CDKN2B*,” “cyclin-dependent kinase inhibitor 2.” The search was updated till June 24, 2016. Additionally, we manually searched the references of the selected studies to obtain additional studies. The full texts of all of the qualified studies were published.

Inclusion criteria

Studies were included if they met all the following criteria: 1) Study was an original case-control study on the association between *p14^{ARF}* or *p15^{INK4B}* gene promoter methylation and lung cancer; 2) Patients were diagnosed as lung cancer based on histopathological examination; 3) Study had sufficient data to calculate the pooled odds ratios (ORs) or hazard ratios (HRs) and 95% confidence intervals (CIs) for the meta-analysis; and 4) The most recent study with more information was selected when several publications contained duplicated data.

Data extraction

The data obtained from the publications included first author's name, year of publication, country, ethnicity, sample type, methylation detection method, histology, number of participants, overall survival (OS), and methylation frequency. The various sample types included tissue, bronchoalveolar lavage fluid (BALF), and blood samples, clinicopathological parameters, such as gender status, smoking status, tumor differentiation, and tumor stage. The selection of eligible studies and data extraction were independently performed by two reviewers (LY and WD).

Statistical analysis

The current meta-analysis was performed using the STATA software (version 12.0, Stata Corporation, College Station, TX, USA). The pooled ORs and 95% CIs were calculated to determine the correlation between *p14^{ARF}* or *p15^{INK4B}* gene promoter methylation and lung cancer. The pooled HR with 95% CI was used to evaluate the impact of *p14^{ARF}* promoter methylation on OS of NSCLC patients. Between-study heterogeneity was estimated based on the Cochran's *Q*-test and *I*² statistic.²⁵ A random-effects model was applied for the meta-analysis with significant heterogeneity (*I*²>50% and *P*<0.1); otherwise, the fixed-effects model was used.^{26,27} *P*-value <0.05 was considered to be significant.

Results

Study characteristics

As shown in Figure 1, 269 potentially relevant articles were obtained from the PubMed, EMBASE, EBSCO, Wangfang, and Cochrane Library databases. After a series of selection procedures, a total of 12 studies involving 625 lung cancer samples and 488 nontumor samples were included in the current study. Eight studies with 505 NSCLC samples and 419 nontumor samples analyzed the relationship between *p14^{ARF}* promoter methylation and NSCLC.^{23,24,28-33}

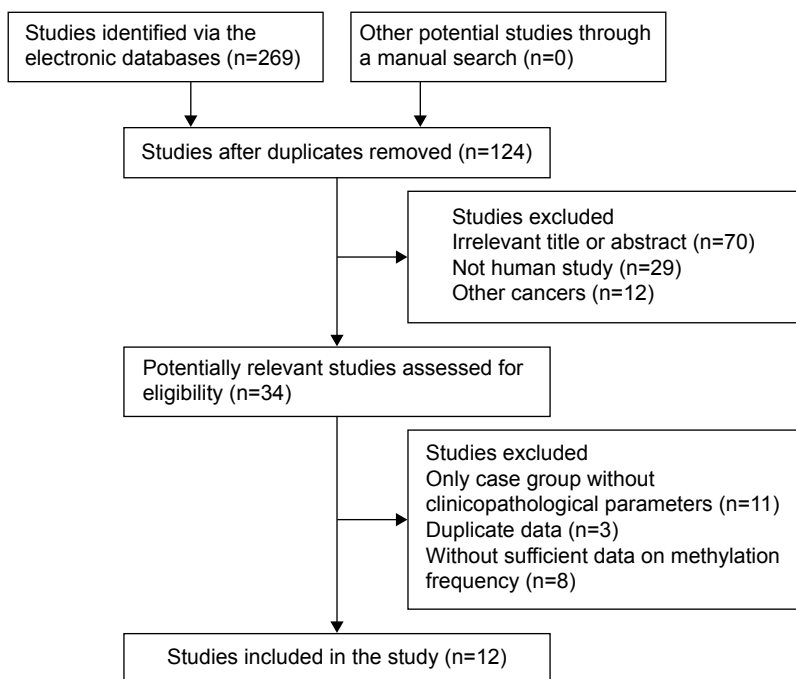


Figure 1 Flow diagram of the stepwise selection from relevant studies.

Of 8 studies, 7 studies used methylation-specific polymerase chain reaction and 1 study used quantitative fluorogenic real-time polymerase chain reaction. Four studies with 120 lung cancer samples and 69 nontumor samples evaluated the relationship between *p15^{INK4B}* promoter methylation and lung cancer.^{21,22,34,35} Of 4 studies, 3 studies used methylation-specific polymerase chain reaction detection and 1 study used 3-dimensional, polyacrylamide gel-based DNA microarray coupled with linker-polymerase chain reaction. Four studies evaluated the correlation of

p14^{ARF} promoter methylation with clinicopathological features.^{23,30,32,33} Two studies assessed the correlation of *p15^{INK4B}* promoter methylation with clinicopathological features.^{22,34} The general characteristics of the included studies are presented in Table 1.

Promoter methylation of *p14^{ARF}* and *p15^{INK4B}* in lung cancer

Substantial heterogeneity among studies was not detected (*p14^{ARF}*: $I^2=0.0\%$, $P=0.900$; *p15^{INK4B}*: $I^2=0.0\%$, $P=0.992$),

Table 1 The general characteristics of the included studies in the current meta-analysis

Gene	First author	Country	Ethnicity	Method	Histology	Sample	Cancer		Control		OS
							M+ %	Total	M+ %	Total [#]	
<i>p14^{ARF}</i>	Jarmalaite et al ³¹ (2003)	Finland	Caucasians	MSP	NSCLC	Tissue	8.7	46	2	51	0.57 (0.19–1.73)
	Topaloglu et al ³⁰ (2004)	USA	Caucasians	QRT-PCR	NSCLC	BALF	0	31	0	10	
	Fischer et al ²⁹ (2007)	Germany	Caucasians	MSP	NSCLC	Blood	30.4	92	0	14	
	Yanagawa et al ²³ (2007)	Japan	Asians	MSP	NSCLC	Tissue	7.9	101	0.99	101	
	De Jong et al ²⁸ (2009)	the Netherlands	Caucasians	MSP	NSCLC	BALF	0	10	0	18	
	Zhang et al ²⁴ (2011)	China	Asians	MSP	NSCLC	Tissue	6.4	78	1.3	78	
	Tian et al ³² (2006)	China	Asians	MSP	NSCLC	Tissue	17.5	40	2.5	40	
<i>p15^{INK4B}</i>	Hu et al ³³ (2014)	China	Asians	MSP	NSCLC	Tissue	33.6	107	12.1	107	
	Chaussade et al ³⁵ (2001)	France	Caucasians	MSP	NELC	Tissue	100	20	95	20	
	Seike et al ²¹ (2000)	Japan	Asians	MSP	LC	Tissue	0	27	0	27	
	Shimamoto et al ³⁴ (2004)	Japan	Asians	MSP	NSCLC	Tissue	11.1	45	0	10	
	Wang et al ²² (2008)	China	Asians	3DPCR	NSCLC	Tissue	7.1	28	0	12	

Note: [#]Total is the number of lung cancer or nontumor samples.

Abbreviations: 3DPCR, 3-dimensional (3-D), polyacrylamide gel-based DNA microarray coupled with linker-polymerase chain reaction; BALF, bronchoalveolar lavage fluid; LC, lung cancer; M+, methylation positive status; MSP, methylation-specific polymerase chain reaction; NELC, neuroendocrine lung cancer; NSCLC, non-small-cell lung cancer; OS, overall survival; QRT-PCR, quantitative fluorogenic real-time PCR.

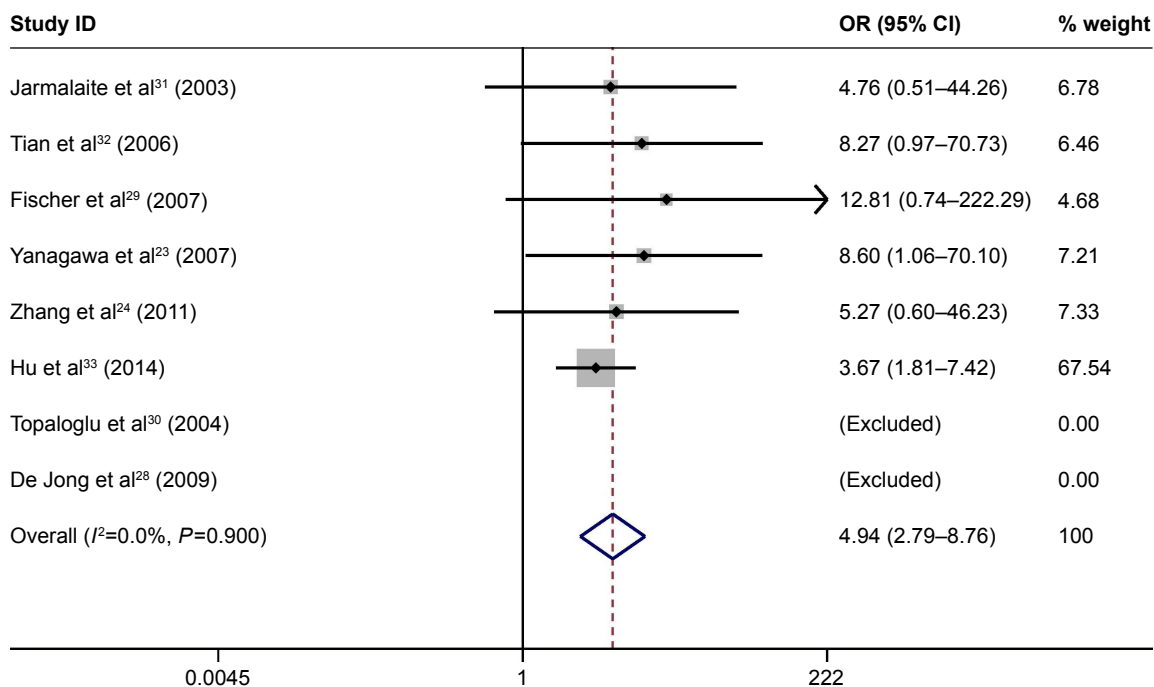


Figure 2 The pooled OR from eight studies with 505 NSCLC samples and 419 nontumor samples showing the association between *p14^{ARF}* promoter methylation and NSCLC in cancer versus nontumor samples, $I^2=0\%$, OR =4.54, 95% CI: 2.47–8.32, $P<0.001$.

Abbreviations: CI, confidence interval; NSCLC, non-small-cell lung cancer; OR, odds ratio.

as shown in Figures 2 and 3, indicating that our results were stable and reliable, with no evidence of heterogeneity. The pooled OR from 8 studies with 505 NSCLC samples and 419 nontumor samples showed that *p14^{ARF}* promoter methylation status was significantly higher in NSCLC than in nontumor samples (OR =4.94, 95% CI: 2.79–8.76, $P<0.001$) (Figure 2), which suggested that *p14^{ARF}* promoter methylation

may play a key role in the initiation of NSCLC. The pooled OR from 4 studies involving 120 lung cancer samples and 69 nontumor samples demonstrated that *p15^{INK4B}* promoter methylation status had a similar OR among lung cancer and nontumor samples (OR =2.76, 95% CI: 0.46–16.70, $P=0.27$) (Figure 3), indicating that *p15^{INK4B}* promoter methylation was not significantly associated with a risk of lung cancer.

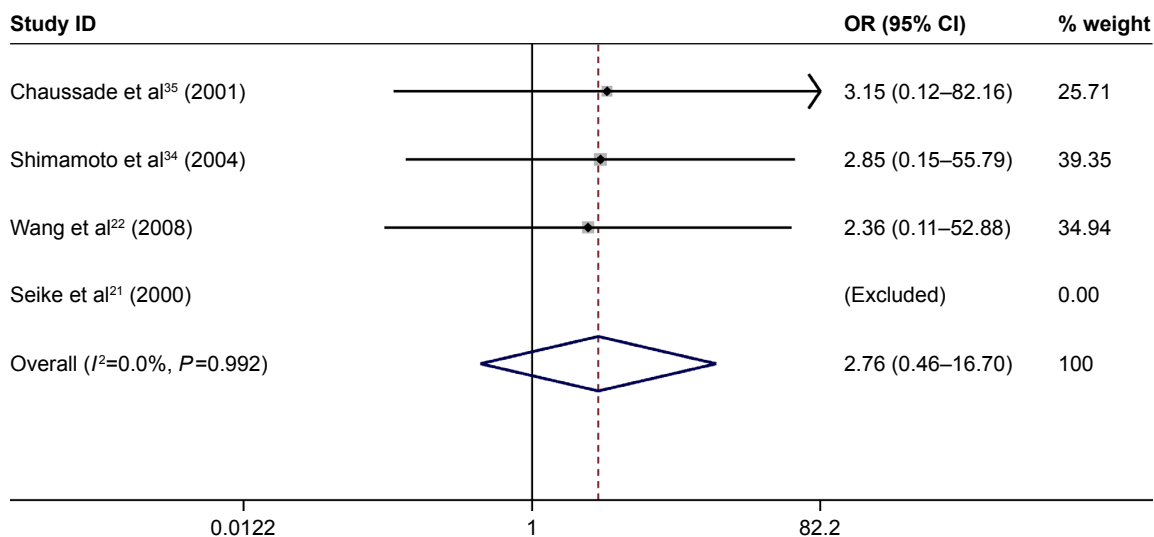


Figure 3 The pooled OR from four studies with 120 lung cancer samples and 69 nontumor samples showing the relationship between *p15^{INK4B}* promoter methylation and lung cancer in cancer versus nontumor samples, $I^2=0\%$, OR =2.76, 95% CI: 0.46–16.70, $P=0.27$.

Abbreviations: CI, confidence interval; OR, odds ratio.

Subgroup analyses of *p14^{ARF}* promoter methylation

Subgroup analyses based on the ethnic population (Asian population and Caucasian population) and sample type (tissue, BALF, and blood) were analyzed to find the different strength of association. Subgroup analysis of ethnicity revealed that *p14^{ARF}* promoter methylation was significantly correlated with an increased risk of NSCLC in Asians and Caucasians (OR =4.54, 95% CI: 2.47–8.32, $P<0.001$; OR =8.05, 95% CI: 1.39–46.50, $P=0.02$; respectively) (Figure 4). Subgroup analysis of sample type demonstrated that *p14^{ARF}* promoter methylation had a significantly increased risk of NSCLC in tissue samples (OR =4.55, 95% CI: 2.54–8.18, $P<0.001$), but not in BALF and blood samples (Figure 5). The results of BALF and blood samples should be carefully considered using smaller sample sizes.

Prognostic value of *p14^{ARF}* promoter methylation

The pooled HR from 1 study with 92 NSCLC patients demonstrated that *p14^{ARF}* promoter methylation was not significantly correlated with OS of patients with NSCLC (HR =0.57, 95% CI: 0.19–1.73).²⁹ Additional studies with larger

subjects are needed to assess the correlation between *p14^{ARF}* promoter methylation and OS in NSCLC in the future.

Association of *p14^{ARF}* and *p15^{INK4B}* promoter methylation with clinicopathological features

We determined whether *p14^{ARF}* and *p15^{INK4B}* promoter methylation was associated with clinicopathological characteristics, including four studies with 259 NSCLC patients and 2 studies with 73 patients with NSCLC, respectively. Our results showed that aberrant promoter methylation of *p14^{ARF}* was not correlated with clinicopathological characteristics ($P>0.05$), including tumor stage, tumor histology, gender status, tumor differentiation, smoking status, and lymph node status (Figure 6). Aberrant promoter methylation of *p15^{INK4B}* was also not associated with clinicopathological features ($P>0.05$), including gender status, tumor histology, tumor differentiation, and tumor stage (Figure 7).

Discussion

Tumor suppressor genes *p16^{INK4A}*, *p14^{ARF}*, and *p15^{INK4B}* encoding cell-cycle regulatory proteins play a crucial role in the negative regulation of the cell cycle and the inhibition of

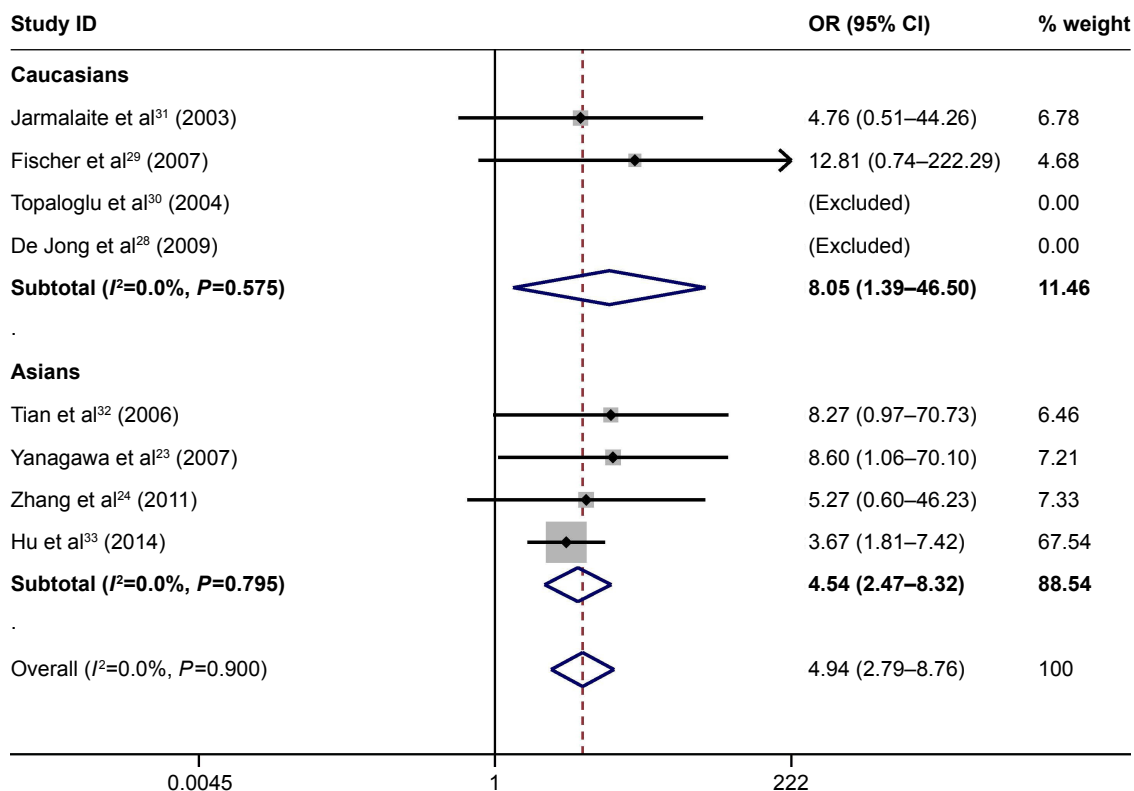


Figure 4 The pooled OR based on subgroup analysis of ethnicity showing the correlation between *p14^{ARF}* promoter methylation and different ethnicity in cancer versus nontumor samples, Asians: OR =4.54, 95% CI: 2.47–8.32, $P<0.001$; Caucasians: OR =8.05, 95% CI: 1.39–46.50, $P=0.02$.
Abbreviations: CI, confidence interval; OR, odds ratio.

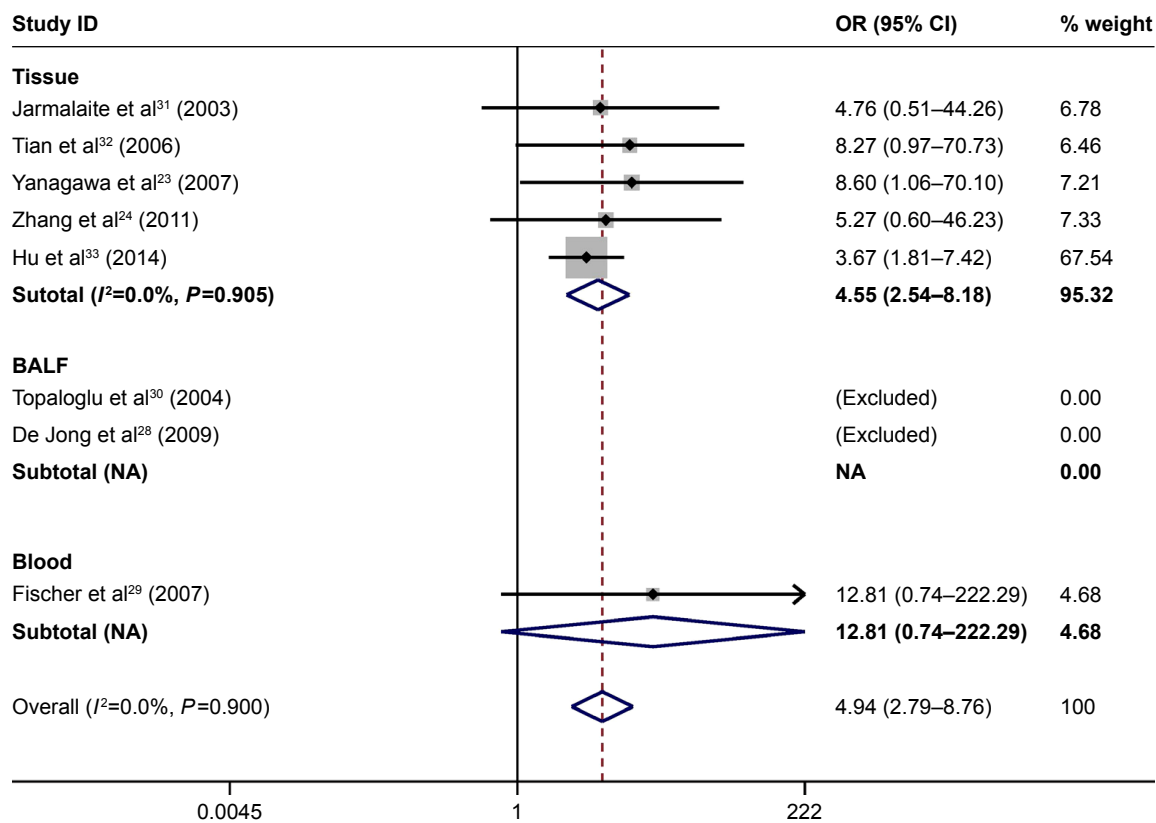


Figure 5 The pooled OR based on subgroup analysis of sample type showing the correlation between *p14^{ARF}* promoter methylation and different sample type in cancer versus nontumor samples, tissue: OR =4.55, 95% CI: 2.54–8.18, $P<0.001$; BALF: unmethylation; blood: OR =12.81, 95% CI: 0.74–222.29, $P=0.08$. **Abbreviations:** BALF, bronchoalveolar lavage fluid; CI, confidence interval; OR, odds ratio; NA, not applicable.

the cell proliferation.³⁶ The silencing of *p16^{INK4A}*, *p14^{ARF}*, and *p15^{INK4B}* genes by DNA methylation of the C-phosphate-G (CpG) islands of the promoter regions has been reported to be involved in the carcinogenesis and be an early biologic event in many cancers.^{37–40} Based on a meta-analysis, Gu et al reported that *p16^{INK4A}* promoter methylation may play a key role in the tumorigenesis of lung cancer.⁴¹ However, whether *p14^{ARF}* or *p15^{INK4B}* promoter methylation plays a crucial role in the carcinogenesis of lung cancer, which remains to be certified. The results were inconsistent with regard to *p14^{ARF}* and *p15^{INK4B}* promoter methylation rate in lung cancer. For example, Topaloglu et al reported that *p14^{ARF}* promoter was absent in methylation in NSCLC.³⁰ Fischer et al reported that *p14^{ARF}* promoter had a methylation frequency of 30.4% in NSCLC.²⁹ In addition, Seike et al showed that *p15^{INK4B}* promoter had no methylation,²¹ while Chaussade et al showed that *p15^{INK4B}* promoter had a methylation rate of 100% in lung cancer.³⁵ Therefore, we performed a meta-analysis to evaluate the strength of *p14^{ARF}* and *p15^{INK4B}* promoter methylation on lung cancer risk.

There was no evidence of the heterogeneity in the current meta-analysis, presenting no obvious publication bias. Our findings demonstrated that *p14^{ARF}* promoter methylation status

had a significantly higher OR in NSCLC than in nontumor samples, while significant correlation was not observed between *p15^{INK4B}* promoter methylation and lung cancer, suggesting that *p14^{ARF}* promoter methylation may play an important role in the initiation of NSCLC. However, the result of *p15^{INK4B}* promoter methylation should be prudent as only 120 lung cancer samples were included in our analysis.

Next, subgroup analyses of the ethnic population (Asians and Caucasians) and sample type (tissue, BALF, and blood) were conducted to find the different association between *p14^{ARF}* promoter methylation and different subgroups in NSCLC versus nontumor samples. Significant correlation between Asian population and Caucasian population in *p14^{ARF}* promoter methylation was found in subgroup analysis of ethnicity, which suggested that Asian and Caucasian populations were susceptible to *p14^{ARF}* promoter methylation. According to subgroup analysis of sample type, a significant association between *p14^{ARF}* promoter methylation and NSCLC was observed in tissue subgroup, but not in BALF and blood subgroups. The results should be carefully considered as only one or two studies with smaller subjects were analyzed in BALF and blood subgroups.

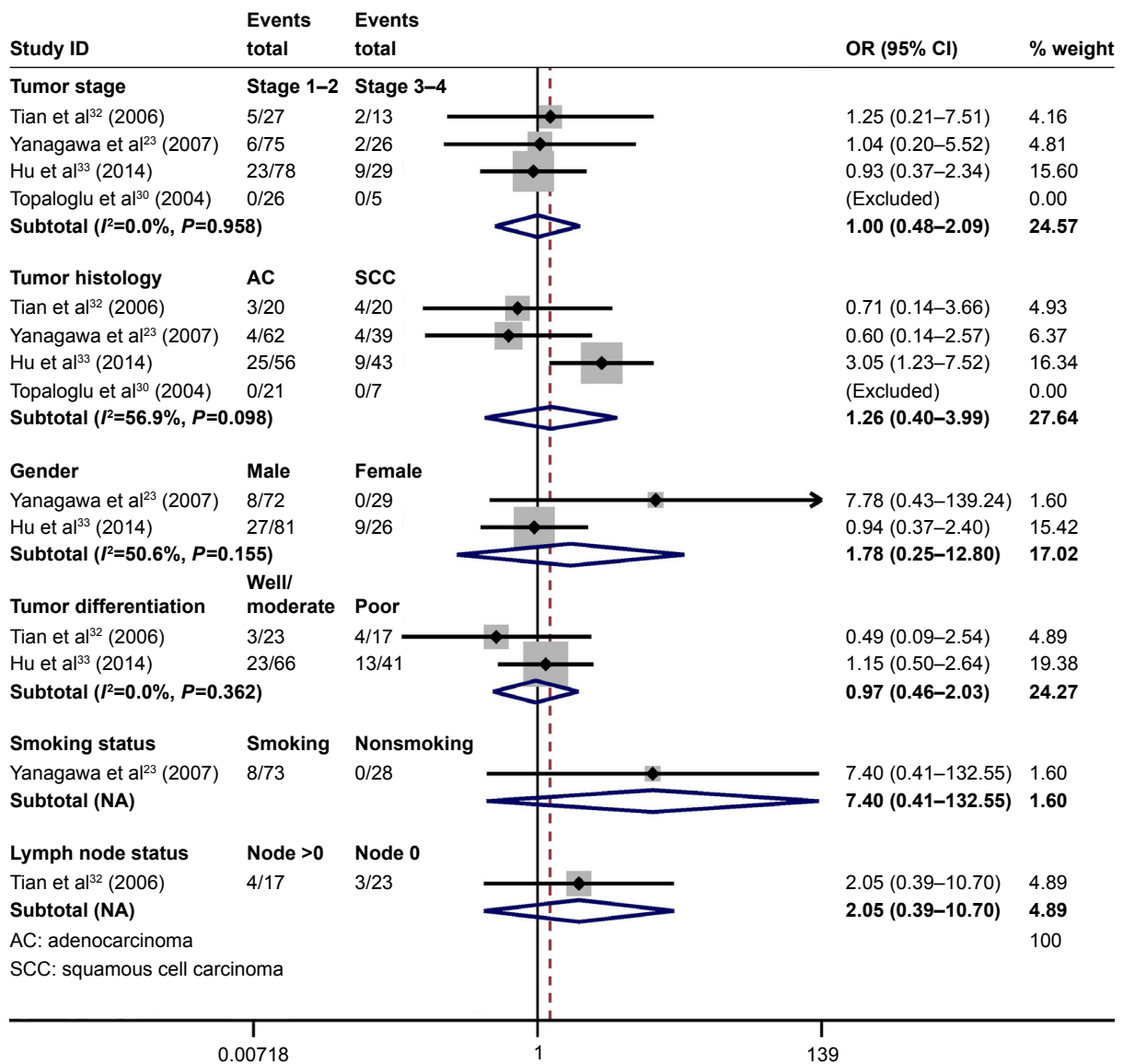


Figure 6 The pooled OR from four studies with 259 NSCLC samples showing the association of *p14^{ARF}* promoter methylation with clinicopathological characteristics, including tumor stage, tumor histology, gender status, tumor differentiation, smoking status, and lymph node status (all *P*>0.05).
Abbreviations: AC, adenocarcinoma; CI, confidence interval; NSCLC, non-small-cell lung cancer; OR, odds ratio; SCC, squamous cell carcinoma; NA, not applicable.

Previous several meta-analyses had evaluated the correlation of *p16^{INK4A}* methylation with clinicopathological parameters in lung cancer,^{11,42,43} which suggested that *p16^{INK4A}* methylation was associated with smoking status and tumor histology. However, the correlation of *p14^{ARF}* and *p15^{INK4B}* promoter methylation with clinicopathological features was not determined. Our findings suggested that *p14^{ARF}* and *p15^{INK4B}* promoter methylation was not correlated with clinicopathological characteristics, such as gender status, tumor histology, tumor differentiation, and tumor stage. Based on small sample sizes (*p14^{ARF}*: 259 NSCLC patients, *p15^{INK4B}*: 73 patients with NSCLC), more studies with larger sample size should be done in the future.

Limitations

There were several limitations in the present meta-analysis. First, there might be selection bias because eligible studies were restricted to articles published in English and Chinese, studies with other language and other styles, such as conference abstracts were missed. Second, the main ethnic population consisted of Asian and Caucasian populations, and other ethnicities, such as Africans, were insufficient. Third, only smaller subjects were included in BALF and blood subgroups; more studies with larger sample sizes are essential to further determine whether *p14^{ARF}* promoter methylation can become a promising biomarker based on BALF or blood detection. Fourth, only 1 study reported

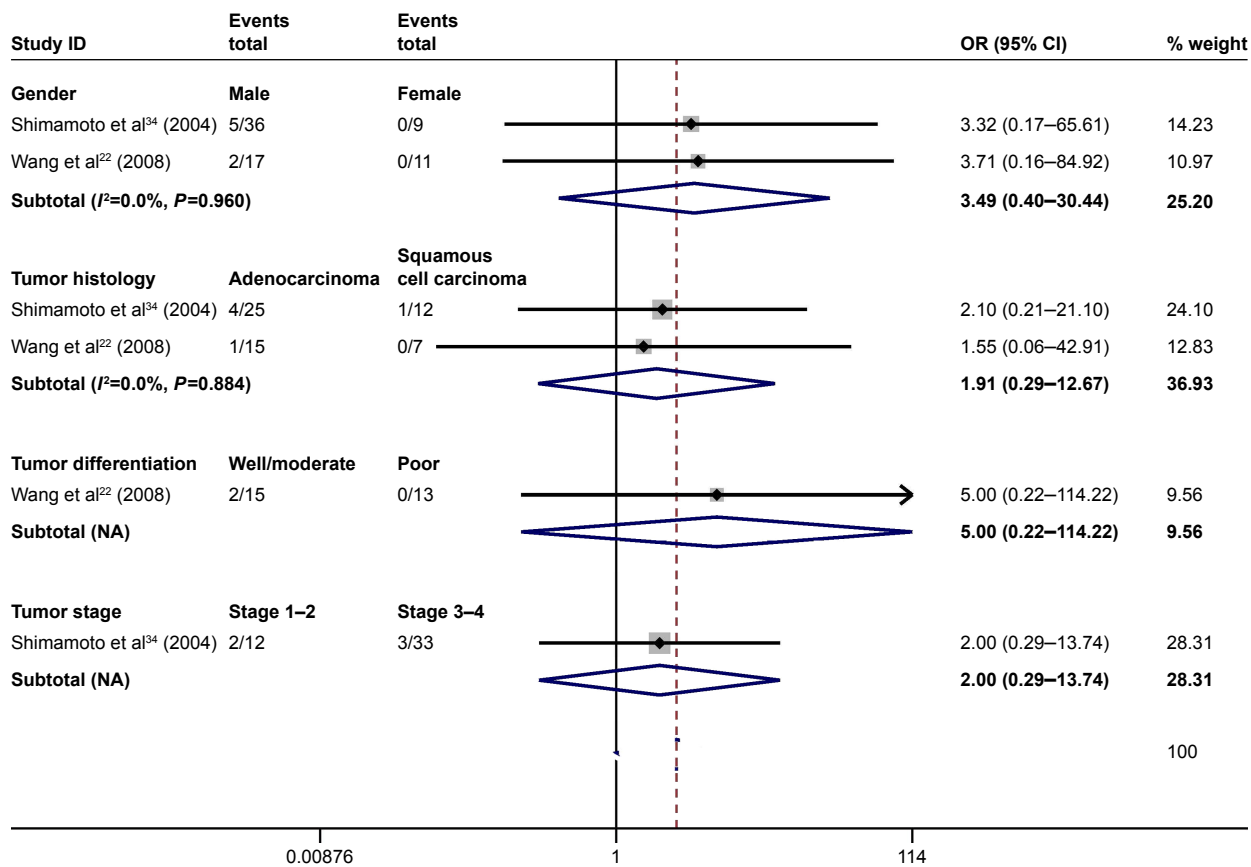


Figure 7 The pooled OR from two studies with 73 NSCLC samples showing the association of *p15^{INK4B}* promoter methylation with clinicopathological characteristics, including gender status, tumor histology, tumor differentiation, and tumor stage (all $P>0.05$).

Abbreviations: CI, confidence interval; NSCLC, non-small-cell lung cancer; OR, odds ratio; NA, not applicable.

that *p14^{ARF}* promoter methylation was not associated with the prognosis of NSCLC patients in OS; further large-scale studies with larger subjects are very necessary in the future. Finally, sample sizes on clinicopathological features were smaller in this study.

Conclusion

The results suggested that *p14^{ARF}* promoter methylation may play a pivotal role in the carcinogenesis of lung cancer, but not *p15^{INK4B}* promoter methylation. In addition, *p14^{ARF}* promoter methylation was a susceptible gene for Asians and Caucasians. Aberrant promoter methylation of *p14^{ARF}* and *p15^{INK4B}* was not associated with clinicopathological features. Additional studies with larger subjects are needed to further validate our results.

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Author contributions

All authors contributed toward data analysis, drafting and 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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