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## Meta-analyses of gene methylation and smoking behavior in non-small cell lung cancer patients

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Aberrant DNA methylation can be a potential genetic mechanism in non-small cell lung cancer (NSCLC). However, inconsistent findings existed among the recent association studies between cigarette smoking and gene methylation in lung cancer. The purpose of our meta-analysis was to evaluate the role of gene methylation in the smoking behavior of NSCLC patients. A total of 116 genes were obtained from 97 eligible publications in the current meta-analyses. Our results showed that 7 hypermethylated genes (including *CDKN2A*, *RASSF1*, *MGMT*, *RARB*, *DAPK*, *WIF1* and *FHIT*) were significantly associated with the smoking behavior in NSCLC patients. The further population-based subgroup meta-analyses showed that the *CDKN2A* hypermethylation was significantly associated with cigarette smoking in Japanese, Chinese and Americans. In contrast, a significant association of *RARB* hypermethylation and smoking behavior was only detected in Chinese but not in Japanese. The genes with altered DNA methylation were likely to be potentially useful biomarkers in the early diagnosis of NSCLC.

on-small-cell lung carcinoma (NSCLC) is a major type of epithelial lung cancer causing a serious hazard to human health<sup>1,2</sup>. The mortality of lung cancer was higher than that of any other malignant tumors among men and women in China<sup>3</sup>, and the incidence of lung cancer has been rising year by year<sup>4</sup>. Early detection is the key method to improve the survival of lung cancer patients<sup>5</sup>.

Cigarette smoking is the top risk factor which is attributed to more than four out of five cases of lung cancers<sup>6</sup>. Up to date, a handful of candidate genes have been investigated the association with cigarette smoking<sup>7,8</sup>. Epigenetic inhibition of gene expression is a potential cause for initiation and progression of lung cancer<sup>8</sup>. DNA methylation, a reversible epigenetic modification, plays an important role in gene expression at the very early stage of NSCLC<sup>9</sup>. Identification of aberrantly-methylated smoke-related genes may have potential in the early diagnosis of NSCLC.

Aberrant promoter methylation of smoking related genes has been reported in lung cancer patients<sup>7</sup>, but an unanimous conclusion could not be reached even in the same ethnic population<sup>10,11</sup>. Although several metaanalyses have reported the results of individual genes in patients with NSCLC, most of the current methylation studies were involved with a small number of samples that might produce spurious results. The purpose of our study was to perform an overview of all the candidate genes rather than single gene associated with smoking in NSCLC patients. Our meta-analysis would help identify the genes with robust findings across the studies in different ethnic populations.

#### **Methods**

Selection criteria of eligible studies. We searched the available studies through October 27, 2014 in the PubMed (English), CNKI and Wanfang (Chinese) literature databases using the corresponding combination: "(smok\* OR nonsmok\* OR clinic\*) AND lung cancer AND (methylation OR epigenetic silencing)". The inclusion criteria for the studies involved in this meta-analysis met the following criteria: 1) they were conducted in non-small cell lung cancer patients; 2) the subjects in every study comprised nonsmokers and smokers (former smokers and/or current smokers); 3) odds ratio (OR) with 95% confidential interval (CI) was included.

**Data Extraction.** All the datasets were extracted independently by two reviewers using a standard protocol. For each eligible study, we collected information regarding the names of first authors, publication year, ethnicity, country of origin, histology of lung cancer, types of biological specimen, number of participants, methylation status, smoking status, the method for methylation detection, and OR with 95% CI.

**Meta-analysis.** The meta-analysis was performed using the Review Manager software (version 5.2, Cochrane Collaboration, Oxford, United Kingdom)<sup>12</sup>. The combined ORs and the corresponding 95% CIs were calculated and demonstrated in the forest plots using the fixed or the random effects model. A fixed-effect model was applied for the meta-analysis with moderate heterogeneity (I<sup>2</sup> < 50%), otherwise a random-effect model was used<sup>13</sup>. Funnel plots were used to check whether there was obvious publication bias among the involved studies. P values less than 0.05 were considered to be significant.

#### Results

As shown in the Fig. 1, our initial search for the genetic studies of NSCLC retrieved 1724 articles from PubMed, CNKI and Wanfang literature databases. Among the retrieved studies, there were 1061 irrelevant studies, 412 studies without methylation data, 77 studies without the histological types of lung cancer, 15 studies with duplicate data, 26 only involved in risk factors, and 36 studies without smoking status of participants. At last, a total of 97 eligible association studies between smoking and nonsmoking NSCLC patients were included in the current meta-analyses. There were 14 genes involved in at least three studies, 13 genes involved in two studies and 89 genes involved in one study.

Smoking behavior was evaluated for its contribution to the methylation status of 116 genes in NSCLC patients. Among these genes, 14 hypermethylated and 12 hypomethylated genes were shown to be associated with the smoking behavior. Six hypermethylated and eight hypomethylated genes were reported to be associated with smoking in one study (p < 0.05, Dataset 1). In addition, four hypermethylated and one hypomethylated genes were reported to be associated with smoking behavior in two independent studies (p < 0.05, Dataset 1).

For 14 genes reported in at least three studies (Dataset 1), no evidence of statistical heterogeneity was observed for nine genes, including *CDKN2A* (I<sup>2</sup> = 38%), *MGMT* (I<sup>2</sup> = 19%), *CDH13* (I<sup>2</sup> = 38%), *RARB* (I<sup>2</sup> = 0%), *DAPK* (I<sup>2</sup> = 27%), *WIF1* (I<sup>2</sup> = 0%), *MLH1* (I<sup>2</sup> = 0%), *PTEN* (I<sup>2</sup> = 0%) and *CDH1* (I<sup>2</sup> = 0%). No visual bias was shown in the meta-analyses of the above nine genes (Fig. 2). Our data also demonstrated a significant heterogeneity of the rest five genes

that comprised *RASSF1* ( $I^2 = 57\%$ ), *RUNX3* ( $I^2 = 56\%$ ), *SFRP1* ( $I^2 = 55\%$ ), *FHIT* ( $I^2 = 52\%$ ) and *APC* ( $I^2 = 69\%$ ). Therefore, randomeffect tests were applied for meta-analysis of the above five genes. Their funnel plots were shown in Fig. 2.

As shown in Table 1, meta-analysis of CDKN2A gene was involved with 36 studies between 2957 smoking and 1192 nonsmoking NSCLC patients. Our results indicated that CDKN2A hypermethylation was significantly associated with smoking risk in NSCLC patients (the overall OR = 2.33, 95% CI = 1.96-2.77, p < 0.00001). Meta-analysis of the association between RASSF1 hypermethylation and smoking behavior among 1046 smoking NSCLC patients and 441 nonsmoking NSCLC patients indicated a statistical difference (the overall OR=1.75, 95% CI=1.15-2.65, p=0.008). The same consequences were also found in the other five genes including *MGMT* (the overall OR = 2.51, 95% CI = 1.81-3.46, p < 0.00001), RARB (the overall OR = 1.77, 95% CI = 1.29-2.42, p = 0.0004), DAPK (the overall OR = 2.04, 95% CI = 1.40-2.99, p = 0.0002), WIF1 (the overall OR = 1.62, 95% CI = 1.04-2.53, p = 0.03) and *FHIT* (the overall OR = 2.18, 95% CI = 1.33–5.95, p = 0.007). Our meta-analyses were unable to find statistical association with smoking for the methylation of the rest 7 genes, including CDH13, RUNX3, SFRP1, MLH1, APC, PTEN and CDH1.

Subgroup meta-analyses by ethnic populations were also performed for *CDKN2A*, *RASSF1*, *CDH13* and *RARB*. As shown in Fig. 3, our results showed that significant association between cigarette smoking and *CDKN2A* hypermethylation existed in Japanese from 10 studies (OR = 3.61, 95% CI = 2.52–5.18), Americans from 6 studies (OR = 1.60, 95% CI = 1.15–2.22) and Chinese from 12 studies (OR = 2.47, 95% CI = 1.39–4.41). In addition, there was a significant ethnic difference in the meta-analysis of *RARB* gene. We found a statistical difference between *RARB* hypermethylation and smoking status in Chinese (OR = 2.05, 95% CI = 1.39–3.03,  $I^2 = 0$ ) but not in Japanese (p = 0.50,  $I^2 = 94\%$ , Fig. 4). Subgroup meta-analyses of *RASSF1* and *CDH13* were unable to observe any significant results in each ethnic population (Fig. 5 and Fig. 6). The funnel plots of four genes showed there was no publication bias among the above meta-analyses (Fig. 7).

#### Discussion

In the present study, a comprehensive overview of genetic association studies was performed for the susceptibility of smoking



Figure 1 | Flow diagram of selecting studies for meta-analysis.



Figure 2 | Funnel plots for the relationship between 14 genes and NSCLC in the meta-analyses.

patients of NSCLC. Our meta-analyses mainly focused on 14 tumor suppressor genes ( $\geq$ 3 studies per gene). Seven NSCLC-associated genes (*CDKN2A*, *RASSF1*, *MGMT*, *RARB*, *DAPK*, *WIF1* and *FHIT*) hypermethylation showed significant evidences in the smoking NSCLC patients compared with nonsmoking NSCLC patients. Subgroup meta-analyses by ethnic populations found that *CDKN2A* hypermethylation was a common risk factor of cigarette smoking in NSCLC patients, however, *RARB* hypermethylation was only found in Chinese.

Smoking is the main cause of lung cancer<sup>14,15</sup>, which can be associated with the calcium signaling and the other signaling pathways<sup>16-19</sup>. Aberrant methylation of gene promoters induced by cigarette carcinogens was shown to be associated with cancer occurrence and tumor suppressor gene inactivation<sup>20</sup>. Cigarette smoking was shown to induce DNMT1 accumulation and subsequent hypermethylation of the tumor suppressor gene that might cause tumorigenesis and poor prognosis in NSCLC patients<sup>21</sup>.

Cyclin-dependent kinase inhibitor 2A (*CDKN2A*) has two alternative transcripts that playing an important role in the p53 and pRB tumor suppressor pathways<sup>22</sup>. Tobacco carcinogens were shown to raise promoter methylation and then down-regulate the expression of *CDKN2A* 

gene<sup>23</sup>, which might lead to tumorigenesis. Ras-association domain family (RASSF1) and O-6-methylguanine DNA methyltransferase (MGMT) gene are both involved in lung cancer development in different human lung cell lines<sup>24,25</sup>. Cigarette smoking led to the changes in expression and promoter methylation of MGMT and RASSF1<sup>26</sup>. Retinoic acid receptor beta (RARB) encoded a receptor mediating cellular signaling in embryonic morphogenesis, cell growth and differentiation<sup>27,28</sup>. Death-associated protein kinase (DAPK), a tumor suppressor gene, encodes a novel serine/threonine kinase linked with loss of expression in cancers<sup>29,30</sup>. Fragile histidine triad (FHIT) gene is located in the most common fragile site that is easy to be rearranged with the exposure in tobacco and other carcinogens, finally causing malignant transformation<sup>31</sup>. WNT inhibitory factor 1 (WIF1), as another tumor suppressor gene, has been found to be epigenetically silenced in various cancers including NSCLC<sup>32</sup>. Our results indicated that the hypermethylation of these genes were associated with cigarette smoking in the development of NSCLC.

Our meta-analyses aimed to provide a comprehensive list of aberrantly methylated genes in the smoking NSCLC patients. Compared with the meta-analysis of *CDKN2A* by Zhang et al<sup>33</sup>, our study was involved with a total of 2957 smoking patients and 1192 nonsmoking

Table 1   Characteristics of 14 genes# among smoking behavior								
Gene	Studies (n)	Overall OR (95% CI)°	<b> </b> <sup>2</sup>	Pvalue	smoking/non-smoking NSCLC patients (n)			
CDKN2A	36	2.33 [1.96, 2.77]	38%	< 0.00001	2957/1192			
RASSF1	14	1.75 [1.15, 2.65]	57%	0.008	1046/441			
MGMT	8	2.51 [1.81, 3.46]	19%	< 0.00001	478/339			
RARB	7	1.77 [1.29, 2.42]	0%	0.0004	507/279			
DAPK	7	2.04 [1.40, 2.99]	27%	0.0002	427/192			
FHIT	5	2.81 [1.33, 5.95]	52%	0.007	406/112			
CDH13	7	1.42 [0.93, 2.18]	38%	0.11	300/184			
RUNX3	6	0.67 [0.34, 1.34]	56%	0.26	303/156			
SFRP1	6	1.40 [0.75, 2.61]	55%	0.29	358/190			
MLH1	4	0.82 [0.46, 1.44]	0%	0.48	225/86			
PTEN	3	1.47 [0.74, 2.91]	0%	0.27	229/98			
CDH1	3	0.61 [0.31, 1.21]	0%	0.16	132/61			
WIF1	5	1.62 [1.04, 2.53]	0%	0.03	346/161			
APC	3	1.36 [0.41, 4.55]	69%	0.62	89/66			

l<sup>2</sup> stands for heterogeneity;

Γ...

P value stands for significant or insignificant results;

a: Overall OR describes the likelihood of gene methylation and smoking status observed in NSCLC patients;

# means only the genes over 3 studies or equal to 3 studies were displayed, the other genes were listed in Dataset 1.

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Study or Subaroup	Even	ts Total	nonsmokers		Weight M-H Eized 95% CI Ve		I Year	Mult Eixed 95% Cl	
CDKN2A-Japanese	13 1010	Lyonis	Total	Treight	111-11, 1 1xcd, 30 / 0	Treat	11-11, 11/20, 55/5 G		
Yanagawa 2002	· .	13 37	4	21	9.1%	2 30 [0 64 8 29]	2002		
Yanagawa 2003		21 55	2	20	5.0%	5.56 [1.17, 26.42]	2003		
Tovooka 2004		75 250	11	105	29.7%	3.66 [1.85, 7.23]	2004		
Nakata 2006		24 73	3	28	8.0%	4.08 [1.12, 14.88]	2006		
Toyooka 2006		24 86	7	78	14.5%	3.93 [1.58, 9.74]	2006		
Naoki Yanagawa 2007(P	14)	8 73	0	28	1.7%	7.40 [0.41, 132.55]	2007		
Naoki Yanagawa 2007(P	16)	24 73	3	28	8.0%	4.08 [1.12, 14.88]	2007		
Takafumi Kubo 2009		12 30	11	70	10.9%	3.58 [1.35, 9.47]	2009		
Mitsuru Yoshino 2009		7 23	4	21	8.0%	1.86 [0.46, 7.58]	2009		
Naoki Yanagawa 2011		7 36	2	26	5.1%	2.90 [0.55, 15.26]	2011		
Subtotal (95% CI)		736		425	100.0%	3.61 [2.52, 5.18]		•	
Total events	2	15	47						
Heterogeneity: Chi <sup>2</sup> = 2.0	3, df = 9 (P =	= 0.99); l	= 0%						
Test for overall effect: Z =	6.97 (P < 0	.00001)							
CDKN2A-Americar	ns								
Sanchez-Cespedes 2001		7 33	5	14	9.8%	0.48 [0.12, 1.92]	2001		
Kim 2001		19 172	2	13	4.7%	2.19 [0.47, 10.25]	2001		
Jie Wang 2004		12 81	16	38	18.5%	1.48 [0.68, 3.22]	2004		
Divine 2005		31 157	18	46	23.8%	1.66 [0.85, 3.24]	2005	+	
Yang Liu 2006		51 81	13	41	11.3%	3.66 [1.65, 8.13]	2006		
Tessema 2009		67 100	48	75	32.0%	1.14 [0.61, 2.14]	2009		
Subtotal (95% CI)		624		227	100.0%	1.60 [1.15, 2.22]		•	
Total events	2	97	102						
Heterogeneity: Chi <sup>2</sup> = 8.3	5, df = 5 (P	= 0.14); l	= 40%						
Test for overall effect: Z = 2.78 (P = 0.005)									
								0.01 0.1 1 10 100	
								Decreased Risk Increased Risk	
								Odda Patia	
Study or Subaroup	smokers	nor	smokers			Odds Ratio		U00S Ratio	
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CDKN2A-Chinese Jun Wu 2002 Liang Zhang 2003 Ming Liu 2004 Zhenhua Yang 2005 Zhang Zhixue 2006 Yasong Wang 2007 Lei Xian 2009 Wang 2010 Chen-ye Zhang 2011 L. Li 2013 Zhiliang Hu 2014 Ping Xiao 2014 Subtotal (95% Cl) Total events Heterogeneity: Tau <sup>2</sup> = 0. Test for overall effect: Z	smokers <u>Events</u> Te 9 15 59 20 18 38 13 9 82 31 31 33 60; Chi <sup>2</sup> = 3 = 3.07 (P =	nor tal Eve 42 30 83 30 43 73 20 20 44 77 77 60 99 0.39, df = 0.002)	association Tota   7 18   4 10   9 22   1 19   0 4   1 22   13 25   10 36   5 30   20 301   93 11 (P = 0.1)	I Wei   3 8.   0 7.   3 10.   5 3.   5 8.   5 8.   5 8.   5 9.   4 12.   9 9.   100. 100.   1001); 1 <sup>2</sup>	ght M-   9% 6%   3% 3   0% 1   1% 2   9% 2%   2% 7%   6% 0%   = 64% 3	Odds Ratio H. Random, 95% CI 0.43 [0.13, 1.42] 1.50 [0.35, 6.42] 3.82 [1.46, 10.01] 36.00 [4.19, 309.67] 7.98 [0.42, 153.44] 21.71 [2.77, 170.40] 1.71 [0.51, 5.74] 2.13 [0.68, 6.68] 2.65 [1.37, 5.09] 3.37 [1.16, 9.75] 3.37 [1.16, 9.75] 0.61 [0.25, 1.52] 2.47 [1.39, 4.41]	Year 2002 2003 2004 2005 2006 2007 2009 2010 2011 2013 2014 2014	M-H. Random, 95% CI	

#### Figure 3 | Forest plots of the association studies between *CDKN2A* and NSCLC.

patients, which provided a much larger sample size than 2037 smoking patients and 765 nonsmoking patients in the study by Zhang et al<sup>33</sup>. In addition, our study was involved with the meta-analysis of 14 genes, among which 10 genes (including *MGMT*, *DAPK*, *FHIT*, *CDH13*, *RARB*, *SFRP1*, *PTEN*, *CDH1*, *WIF1* and *APC*) hadn't been included by previous meta-analysis studies. Moreover, the meta-analysis by Liu et al.<sup>34</sup> showed that *RASSF1* gene promoter methylation was not associated with smoking behaviour in NSCLC patients. Our meta-analysis of *RASSF1* methylation involved with 14 studies, which was 6 more than that of Liu et al.<sup>34</sup>. The smaller sample size might be the reason for the negative results in the meta-analysis by Liu et al.<sup>34</sup>.

Previous studies found mixed results regarding to racial disparities in NSCLC<sup>35</sup>. Our population-based subgroup meta-analyses showed





#### Figure 4 | Forest plots of the association studies between *RARB* and NSCLC.

smokers		nonsmokers			Odds Ratio		Odds Ratio	
Study or Subgroup	Events	Total	Events	Total	Weight	M-H. Random, 95% CI	Year	M-H, Random, 95% Cl
RASSF1-Chinese								
Zhenhua Yang 2007	26	34	4	19	20.6%	12.19 [3.13, 47.39]	2007	
Hui Zhang 2010	39	108	19	42	27.6%	0.68 [0.33, 1.41]	2010	
Guizhi Liu 2010	28	48	18	48	26.6%	2.33 [1.03, 5.29]	2010	
Haizhu Song 2011	20	49	11	29	25.2%	1.13 [0.44, 2.89]	2011	
Subtotal (95% CI)		239		138	100.0%	1.95 [0.69, 5.49]		-
Total events	113		52					
Heterogeneity: Tau <sup>2</sup> = 0.88	8; Chi <sup>2</sup> = '	15.16, d	f = 3 (P =	0.002);	l <sup>2</sup> = 80%			
Test for overall effect: Z =	1.26 (P =	0.21)						
RASSF1-Japanese								
Yoshio Tomizawa 2002	22	62	13	48	21.1%	1.48 [0.65, 3.37]	2002	
Hong Chen 2006	26	72	18	42	21.8%	0.75 [0.35, 1.64]	2006	
Naoki Yanagawa 2007	32	73	10	28	19.9%	1.40 [0.57, 3.46]	2007	
Takafumi Kubo 2009	18	30	13	70	19.3%	6.58 [2.55, 16.95]	2009	
Naoki Yanagawa 2011	18	36	9	26	18.0%	1.89 [0.67, 5.34]	2011	
Subtotal (95% CI)		273		214	100.0%	1.76 [0.87, 3.55]		◆
Total events	116		63					
Heterogeneity: Tau <sup>2</sup> = 0.43	3; Chi <sup>2</sup> = '	12.35, d	f = 4 (P =	0.01); 1	<sup>2</sup> = 68%			
Test for overall effect: Z = 1.59 (P = 0.11)								
								0.02 0.1 1 10 50
								Decreased Risk Increased Risk
smokers		nonsmo	okers		Odds Ratio		Odds Ratio	
Study or Subgroup	Events	Total	Events	Tota	Weight	M-H, Fixed, 95% Cl	Year	M-H. Fixed, 95% CI
RASSF1-American	ns							
Jie Wang 2004	33	81	13	38	64.9%	1.32 [0.59, 2.95]	2004	
Carmen J. Marsit 2005	80	165	3	1:	3 17.7%	3.14 [0.83, 11.81]	2005	
Lela Buckingham 2010	15	90	2	1	7 17 4%	1 50 [0 31 7 25]	2010	
Subtotal (95% CI)		336	-	68	3 100.0%	1.67 [0.90, 3.12]		•
Total events	128		18					
120 = 10								
Tot for overall of fact $7 = 1.2$ , $y_1 = 2.(F = 0.00)$ , $1 = 0.70$								
rescior overall effect: Z =	- 1.02 (P	- 0.10)						
								0.01 0.1 1 10 100

0.01 0.1 1 10 100 Decreased Risk Increased Risk

Figure 5 | Forest plots of the association studies between *RASSF1* and NSCLC.



Decreased Risk Increased Risk

Figure 6 | Forest plots of the association studies between *CDH13* and NSCLC.



Figure 7 Funnel plots for the relationship between four gene methylation and smoking exposure among NSCLC by region subgroup.

that significant association of *CDKN2A* hypermethylation existed in Japanese, Chinese and American populations. However, *RARB* hypermethylation was only found in Chinese but not in Japanese.

There were several potential limitations in our study. Firstly, selection bias might exist since publications only in English and Chinese were included in the current meta-analyses, which could affect the results in a certain extent. Secondly, the main ethnicities of the current meta-analyses were Chinese, Japanese and Americans. Studies in other ethnic populations were needed in the near future to explore the contribution of gene methylation to the smoking in NSCLC. Thirdly, the effects of genetic factors on NSCLC risk might be confounded by gender<sup>36</sup> and age<sup>37</sup>, therefore better design were warranted to avoid these confounding factors in the future. Lastly, our meta-analysis focused on gene with at least three independent studies, and this might prevent those genes reported in two large scale smokers-nonsmokers studies from being included in the current meta-analysis.

In conclusion, we identified significant associations between seven genes (*CDKN2A*, *RASSF1*, *MGMT*, *RARB*, *DAPK*, *WIF1* and *FHIT*) and smoking behavior in non-small cell lung cancer patients. *CDKN2A* hypermethylation was a common risk factor of smoking behavior in NSCLC patients, however, *RARB* hypermethylation was only found as a risk factor of smoking in Chinese but not in other populations.

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#### **Author contributions**

T.H., X.C., S.D. and M.Y. contributed to the conception, design and final approval of the submitted version. Q.H., Z.D., H.M., Y.X., Y.F., H.Y., R.W. and C.Z. contributed to the

meta-analysis, interpretation of data and completion of figures and tables. All the authors read and approved the final manuscript.

#### Additional information

Competing financial interests: The authors declare no competing financial interests.

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