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Original Article

MLH1 promoter methylation in non-small cell lung cancer: a meta-analysis

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Abstract: Human mutL homolog 1 *(hMLH1)* is one of the DNA mismatch repair genes and plays a vital role in maintaining genomic stability. However, the association of *hMLH1* promoter methylation in non-small cell lung cancer (NSCLC) remains undetermined. A systematic literature search was conducted in the PubMed, Embase, Cochrane library, Web of Science, CNKI, and Wanfang databases to assess the associations of *hMLH1* methylation and NSCLC. The overall odds ratios (ORs) and 95% confidence intervals (95% CI) were calculated for the 11 studies included in our study involved in 1355 samples, comprising 707 NSCLC tissue samples and 648 controls. A significant association was observed between *hMLH1* methylation and NSCLC risk (OR = 7.24, P < 0.001). Furthermore, we stratified the association between *hMLH1* methylation and NSCLC risk. Subgroup analysis based on ethnicity showed a higher significance in the Asian group (OR = 10.67, P < 0.001) than in the mixed population group (P = 0.964). Subgroup analysis based on the detection methods showed a significant difference between the methylation specific polymerase chain reaction (MSP) and PCR group (all P < 0.05) and the differential DNA denaturation PCR (3DPCR) and MethyLight group (all P > 0.05). Meta-regression showed that the detection method was the major source of heterogeneity in our meta-analysis (P = 0.014). Sensitivity analysis showed the results were stable. However, the results should be interpreted with caution as only a few studies with small sample size were included in the mixed population, PCR, 3DPCR and MethyLight subgroups. A significant relationship between *hMLH1* methylation and gender, tumor histology or tumor stages was not found in our study. These results suggest that *hMLH1* promoter methylation is significantly associated with NSCLC risk, especially among Asians.

Keywords: *hMLH1*, methylation, NSCLC

Introduction

Lung cancer causes the highest number of cancer-related deaths in the world, accounting for approximately 15% of all cancer diagnoses [1]. Lung cancer types include non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). The most common histological category of lung cancer is NSCLC, accounting for approximately 80%-85% of cases [2]. The common subtypes of NSCLC are adenocarcinoma (AC), squamous cell carcinoma (SCC), large cell carcinoma and adenosquamous carcinoma [3, 4].

Epigenetic alterations play important roles in the initiation and progression of cancer. DNA methylation is an epigenetic modification causing target gene silencing, which can affect gene expression in cellular pathways [5, 6]. DNA mismatch repair genes have important roles in maintaining genomic stability. Genomic instability is associated with many human cancers, especially colorectal cancer and gastric cancer [7, 8]. The mutL homolog 1 (MLH1) gene, located on chromosome 3p21, encodes for one of the DNA mismatch repair (MMR) proteins [9, 10]. The tumor suppressor gene MLH1 has been reported to be silenced by promoter methylation in colorectal cancer and gastric cancer [11, 12].

The frequency of *MLH1* promoter methylation reported in NSCLC has been highly inconsistent, varying from 2% [13] to 72.4% [14]. Therefore, we performed a meta-analysis to further evaluate the association between the *MLH1* promoter methylation status and NSCLC risk and clinicopathological features.

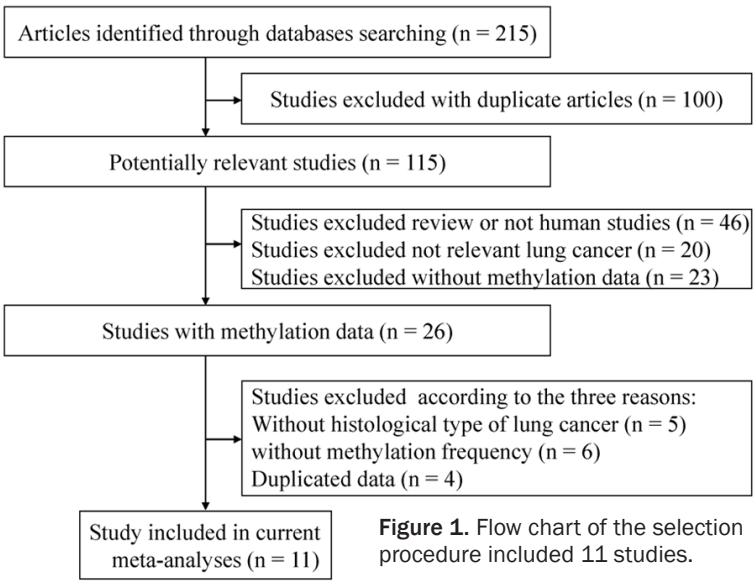

Figure 1. Flow chart of the selection procedure included 11 studies.

Methods and materials

Identification of eligible studies for meta-analysis

A literature search was conducted, using the combined keywords listed below, to identify studies published prior to July of 2015 in the following online libraries: PubMed, Embase, Cochrane library, Web of Science, CNKI, and Wanfang. The keywords were "(mutL homolog 1 OR hMLH1 OR MLH1) AND (lung cancer OR lung neoplasm OR lung carcinoma OR pulmonary cancer) AND (methylation OR epigene*)".

From the identified studies, we used the following criteria to select eligible studies for meta-analysis: 1) all the studies should be case-control based studies of *hMLH1* promoter methylation; 2) all the cancer tissues in the cases were diagnosed with a clear NSCLC histology type; 3) the control tissues were the adjacent or remote normal tissues of NSCLC patients; and 4) the studies should contain sufficient information to infer the methylation frequency of *hMLH1* promoter.

Data extraction

Among the retrieved full-text articles, we extracted the first author's name, published year, country, ethnicity, method, types of samples, the number of NSCLC patients, the number of control samples, clinicopathological parameters, gender, tumor stage and tumor histology, the frequency of promoter methylation, among other data.

Statistical analysis

All the statistical analyses were performed using Stata 12.0 software (Stata Corporation, College Station, TX, USA). The overall odds ratio (OR) and corresponding 95% confidence interval (95% CI) were calculated to evaluate the association of *hMLH1* methylation and NSCLC. Heterogeneity of the meta-analysis was measured by the Cochran's Q statistic and I^2 test [15]. We defined a significant heterogeneity in the meta-analysis if it had a $P < 0.1$ in the Q statistic test or $I^2 > 50\%$, to which we applied a random-effect model for the meta-analysis. For the meta-analysis with moderate or minimal heterogeneity, we applied a fixed-effect model [16]. Publication bias was evaluated using the Egger linear regression test [17]. Subgroup and meta-regression analyses were conducted to find the sources of heterogeneity. A sensitivity analysis by deleting a single study was also performed to assess the stability of the overall OR [18], and $P < 0.05$ was considered to be statistically significant.

Results

Study characteristics

As shown in **Figure 1**, the initial literature search identified a total of 215 articles from the online databases including PubMed, CNKI, among others. First, we excluded 100 overlapping articles among the various databases. From the remaining 115 articles, a further check filtered out 46 reviews or non-human studies, 20 irrelevant studies, and 23 studies without methylation data. Among the remaining 26 methylation studies, there were 6 studies without histological types of lung cancer, 6 studies without methylation frequency and 3 studies with duplicated data. Finally, we identified 11 eligible studies reporting promoter methylation to use in the current meta-analyses [13, 14, 19-27]. The main characteristics of

Table 1. The main characteristics and data of all eligible articles

First author	Country	Ethnicity	Method	Control sample	Case	Control	Male	Female	AC	SCC	Stage 1-2	Stage 3-4
					M/Total	M/Total	M/Total	M/Total	M/Total	M/Total	M/Total	M/Total
Chen 2000 [26]	China	Asians	PCR	NT	15/44	0/50	13/39	3/11	6/19	9/25	12/31	4/19
Yanagawa 2003 [24]	Japan	Asians	MSP	ANT	5/75	0/75	4/54	1/21	2/43	3/29	3/56	2/19
Wang 2003 [25]	Taiwan	Asians	MSP	NT	43/77	0/28	34/63	9/14	17/28	21/42	22/40	19/35
Safar 2005 [23]	USA	Mix	MSP	ANT	22/32	23/32	-	-	-	-	33/48	29/57
Kim 2007 [22]	Korea	Asians	MSP	ANT	18/99	12/99	14/80	4/19	7/38	11/61	-	-
Yang 2007 [27]	China	Asians	MSP	ANT	27/49	7/49	-	-	11/23	16/26	12/34	15/15
Feng 2008 [13]	USA	Mix	MethyLight	ANT	1/49	0/49	-	-	-	-	-	-
Wang 2008 [21]	China	Asians	3DPCR	NMT	8/28	2/12	5/17	3/11	6/15	1/7	-	-
Liu 2008 [20]	China	Asians	MSP	NT	7/60	0/60	-	-	-	-	-	-
Geng 2009 [14]	China	Asians	PCR	NT	84/116	0/116	66/92	18/24	23/32	61/84	36/52	48/64
Zhang 2011 [19]	China	Asians	MSP	ANT	13/78	4/78	-	-	-	-	-	-

M+: Methylation, AC: Adenocarcinoma, SCC: Squamous cell carcinoma, Mix: a mixed population, NT: Normal tissue, ANT: Adjacent normal tissue, NMT: nonmalignant tissue, "-" indicates data not available, MSP: Methylation-specific polymerase chain reaction, PCR: polymerase chain reaction, 3DPCR: 3-dimensional (3-D), polyacrylamide gel-based DNA microarray coupled with linker-polymerase chain reaction (PCR).

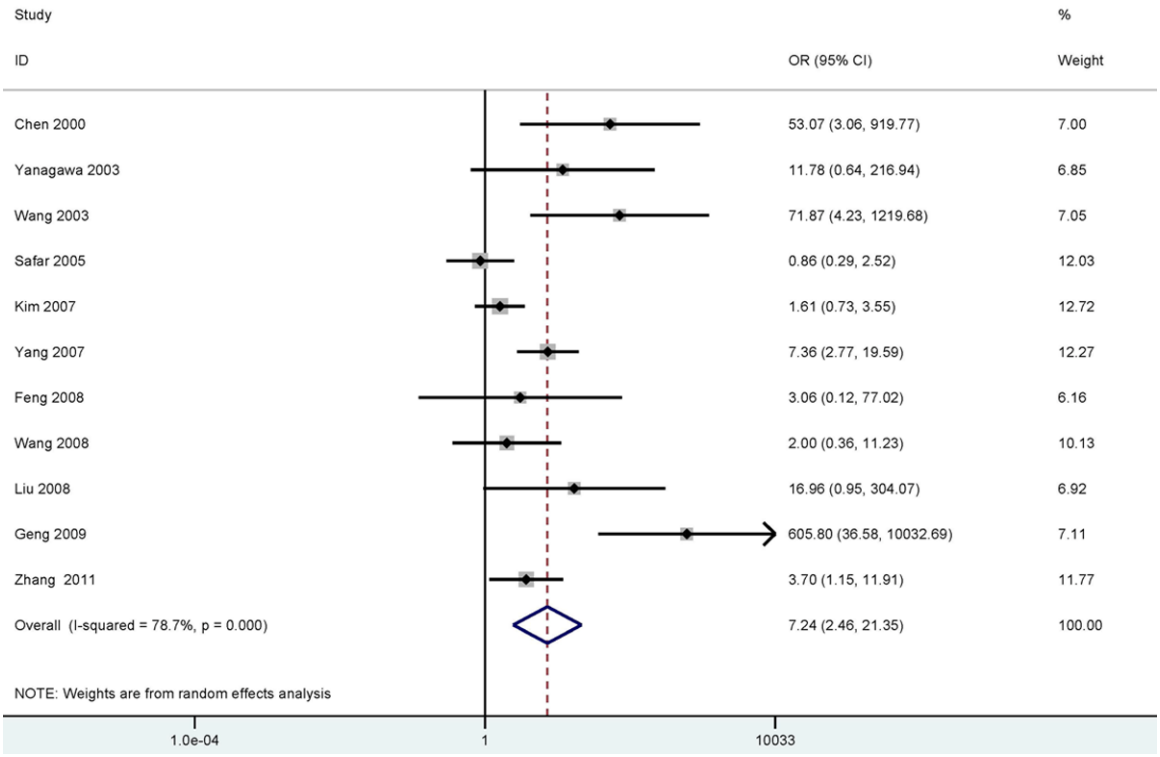

Figure 2. Forest plot of the association between methylated hMLH1 and NSCLC.

hMLH1 promoter methylation were included in **Table 1**.

Meta-analysis of hMLH1 methylation in NSCLC patients

There were a total of 707 NSCLC and 648 control samples with associated *hMLH1* promoter methylation data in the 11 studies. Further analysis showed there was a large heterogeneity in the current meta-analysis (I^2 = 78.7%, P < 0.001); therefore, a random-effect model was applied. Our results showed there was a significant association of *hMLH1* promoter methylation with the risk of NSCLC (OR = 7.24, 95% CI = 2.46-21.35, P < 0.001, **Figure 2**). Further, Egger's test suggested there was publication bias in the current meta-analysis (P = 0.027, **Figure 3**).

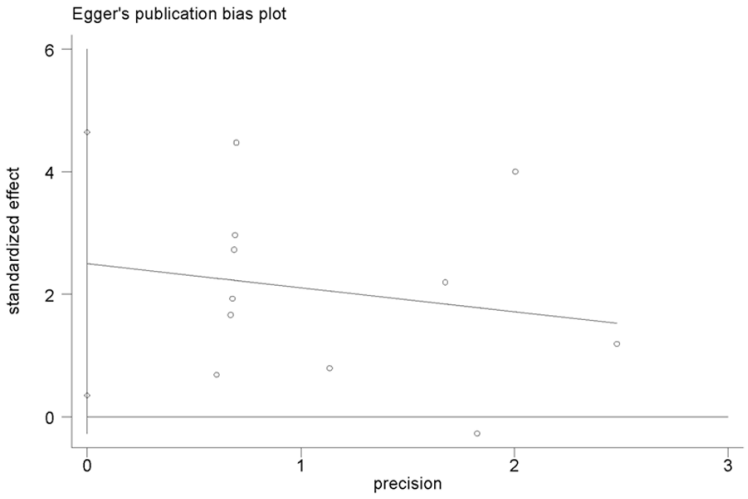

Figure 3. Egger's test showing publication bias between methylated hMLH1 and NSCLC.

Among the 11 studies, 9 represented Asian patient populations and 2 included mixed population samples. Moreover, among the 11 studies, the *hMLH1* methylation methods consisted of MSP (n = 7), MethyLight (n = 1), 3DPCR (n = 1), and PCR (n = 2) (**Table 2**). Therefore, we further performed subgroup meta-analyses by the ethnicity and detection method used in the studies, and our results showed a significant association of *hMLH1* methylation with NSCLC risk in Asians (OR = 10.67, 95% CI = 3.18-35.84, P < 0.001) but not in mixed populations (P = 0.964). In addition, we found a significant difference in the frequency of *hMLH1* methylation observed in NSCLC samples analyzed by MSP, PCR, 3DPCR and MethyLight (OR = 4.18, 95% = 1.60-10.94, P = 0.001; OR = 181.71, 95% CI = 16.15-2044.92, P < 0.001; OR = 2.00, 95% CI = 0.36-11.23, P = 0.431; OR = 3.06, 95% CI = 0.12-77.02, P = 0.496; respectively).

Meta-regression and sensitivity analyses

Further meta-regression was performed to find the sources of heterogeneity in the meta-analysis. The results showed that the detection method was the major source of heterogeneity (coefficient = -1.566, P = 0.014). The differences in the ethnicity subgroups failed to explain the heterogeneity (P = 0.059) (**Table 3**). Additionally, a sensitivity analysis was conducted by deleting a single study at a time to assess the stability of the result. The heterogeneity was significantly decreased when we sequentially removed three studies (Geng 2009 et al., Safar 2005 et al. and Kim 2007 et al.). The *P* values of heterogeneity were 0.004, 0.024 and 0.224, respectively. The overall OR did not significantly change, with a range from 7.24 (95% CI = 2.46-21.35) to 7.09 (95% CI = 3.22-15.61).

The associations of hMLH1 methylation and clinicopathological features

The associations of *hMLH1* methylation and clinicopathological features were further analyzed in the current meta-analysis (**Table 2**). To examine the relationships of *hMLH1* promoter methylation with gender status (Male vs. Female) (**Figure 4**) and tumor histology (AS vs. SCC) (**Figure 5**), we used the fixed effects model, while a random effects model was used to compare tumor stages (stage I-II vs. stage III-IV) (**Figure 6**). Significant associations were not observed between *hMLH1* methylation and gender, tumor histology or tumor stage (*P* values were 0.721, 0.966 and 0.85, respectively). There was not obvious publication bias detected by Egger's test (all P > 0.05).

Discussion

The associations of gene methylation and NSCLC have been evaluated in many studies, and gene methylation patterns may become potential biomarkers for the detection and prognosis of NSLCL, such as the relationships of CpG island methylation and smoking status [28] and *RASSFIA* promoter methylation as a prognostic biomarker [29]. The *hMLH1* gene has been reported as a candidate tumor suppressor gene (TSG) in breast cancer [30], and aberrantly methylated *hMLH1* has been reported in some cancers [31-33]. However, the screening role of the *hMLH1* methylation status in NSCLC lacked assessment. Thus, we performed a meta-analysis to evaluate the ability to screen NSCLC via *hMLH1* methylation.

The results showed that *hMLH1* promoter methylation was significantly associated with risk of NSCLC (OR = 7.24, P < 0.001) among the

Table 2. Meta-analysis of *hMLH1* promoter methylation

	Studies	Overall OR 95 CI %	I^2; P	*P* value	Cases	Controls	P (Egger's test)
Total	11	7.24 (2.46-21.35)	78.7%; < 0.001	< 0.001	707	648	0.027
Method							
MSP	7	4.18 (1.60-10.94)	69.0%; 0.004	0.004	470	421	
PCR	2	181.71 (16.15-2044.92)	31.7%; 0.226	< 0.001	160	166	
3DPCR	1	2.00 (0.36-11.23)	NA; NA	0.431	28	12	
MethyLight	1	3.06 (0.12-77.02)	NA; NA	0.496	49	49	
Race							
Mix	2	0.98 (0.35-2.71)	0.0%; 0.464	0.964	81	81	
Asians	9	10.67 (3.18-35.84)	78.7%; < 0.001	< 0.001	626	567	
					Patients		
Gender					Male	Female	
	6	0.91 (0.53-1.56)	0.0%; 0.969	0.721	345	100	0.076
Histology					AC	SCC	
	7	0.99 (0.64-1.53)	0.0%; 0.684	0.966	198	274	0.91
Stage					Stage 1-2	Stage 3-4	
	6	0.93 (0.41-2.08)	64.5%; 0.015	0.85	261	209	0.168

AC: Adenocarcinoma, SCC: Squamous cell carcinoma, Mix: a mixed population, MSP: Methylation-specific polymerase chain reaction, PCR: polymerase chain reaction, 3DPCR: 3-dimensional (3-D), polyacrylamide gel-based DNA microarray coupled with linker-polymerase chain reaction (PCR).

Table 3. Meta-regression analysis

Subgroup	Coefficient (95% CI)	t	*P* value
Ethnicity	2.005 (-0.100, 4.110)	2.20	0.059
Method	-1.566 (-2.727, -0.404)	-3.11	0.014

11 studies analyzed with a random-effect model. The subgroup analyses were performed according to the ethnicity (Asians and mixed population) and detection methods. In subgroup analysis of the ethnicity, the significant OR value of the *hMLH1* methylation status in the Asian population (OR = 10.67, P < 0.001) was only found when comparing to a mixed population (P = 0.964). In the subgroup analysis of detection methods, the OR values of *hMLH1* methylation were significant in the MSP and PCR subgroups, but not in 3DPCR and MethyLight subgroups (P = 0.431 and P = 0.496, respectively). However, the results should be carefully considered as only a few studies with small sample sizes were included in the mixed population, PCR, 3DPCR and MethyLight subgroups.

Assessment of heterogeneity is an essential requirement in meta-analysis [34]. In our study, there was a substantial heterogeneity between *hMLH1* methylation and NSCLC (I^2 = 78.7%, P < 0.001). Subgroup analyses could not explain the sources of heterogeneity in our meta-analysis; therefore, further meta-regression was performed to find the sources of heterogeneity. Meta-regression analysis revealed that the methylation frequency detection method was the source of the most heterogeneity (P = 0.014), while the ethnicity subgroups could not explain the heterogeneity (P = 0.059). A sensitivity analysis by deleting a single study at a time was also conducted to evaluate the stability of the pooled OR and the heterogeneity of the overall studies. When we sequentially removed the three studies (Geng 2009 et al., Safar 2005 et al. and Kim 2007 et al.), the overall OR did not significantly change, upon eliminating heterogeneity (P = 0.224). Therefore, the sensitivity analysis showed that our results are stable and reliable.

The examination of relationships between *hMLH1* promoter methylation and clinicopathological features of NSCLC were performed in our study. Significant associations were not observed between the *hMLH1* methylation sta-

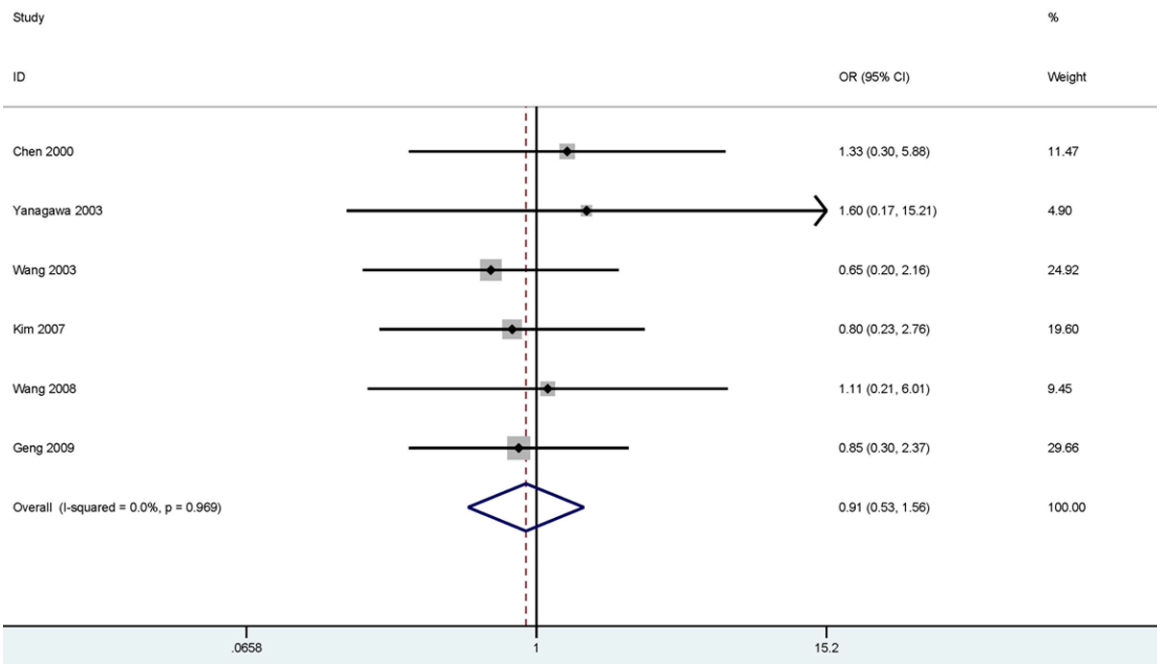

Figure 4. Forest plot of the association between methylated hMLH1 and gender status.

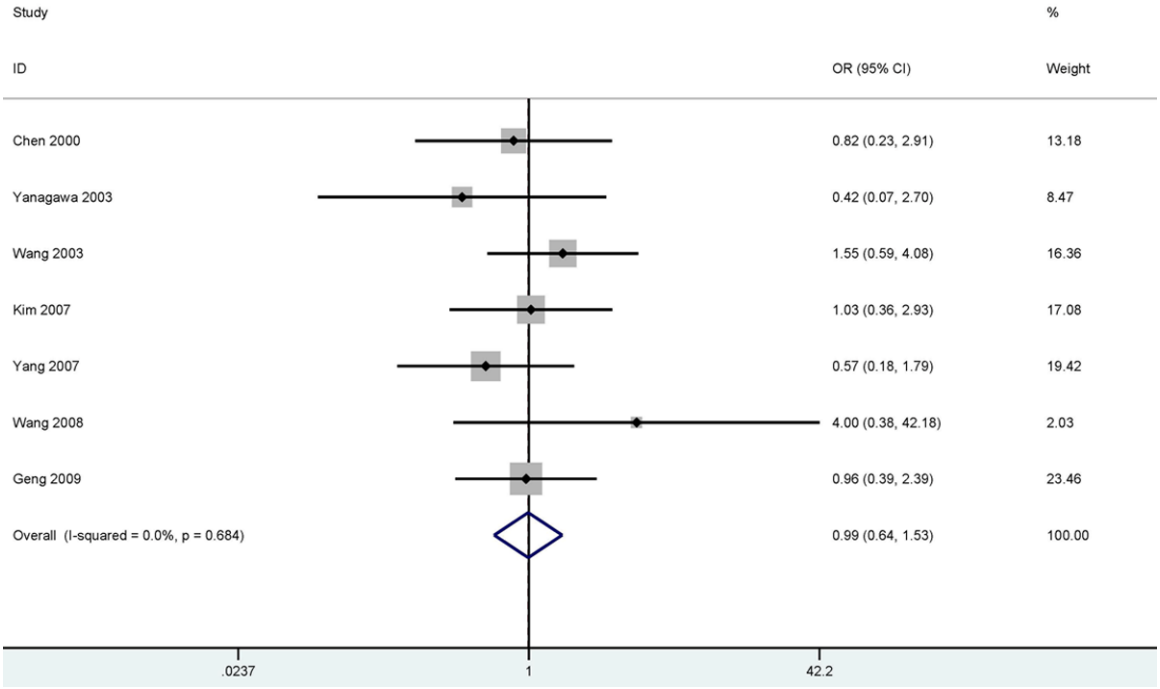

Figure 5. Forest plot of the association between methylated hMLH1 and tumor histology.

tus and gender, tumor histology or tumor stage. Egger's test showed that there was no evidence of publication bias.

There was a substantial publication bias in comparison of NSCLC tissue samples and normal tissue samples (P = 0.027). We tried to minimize publication bias by searching PubMed, Embase, the Cochrane library, Web of Science, CNKI, and Wanfang databases as completely as possible. However, studies with positive results are more readily published than the

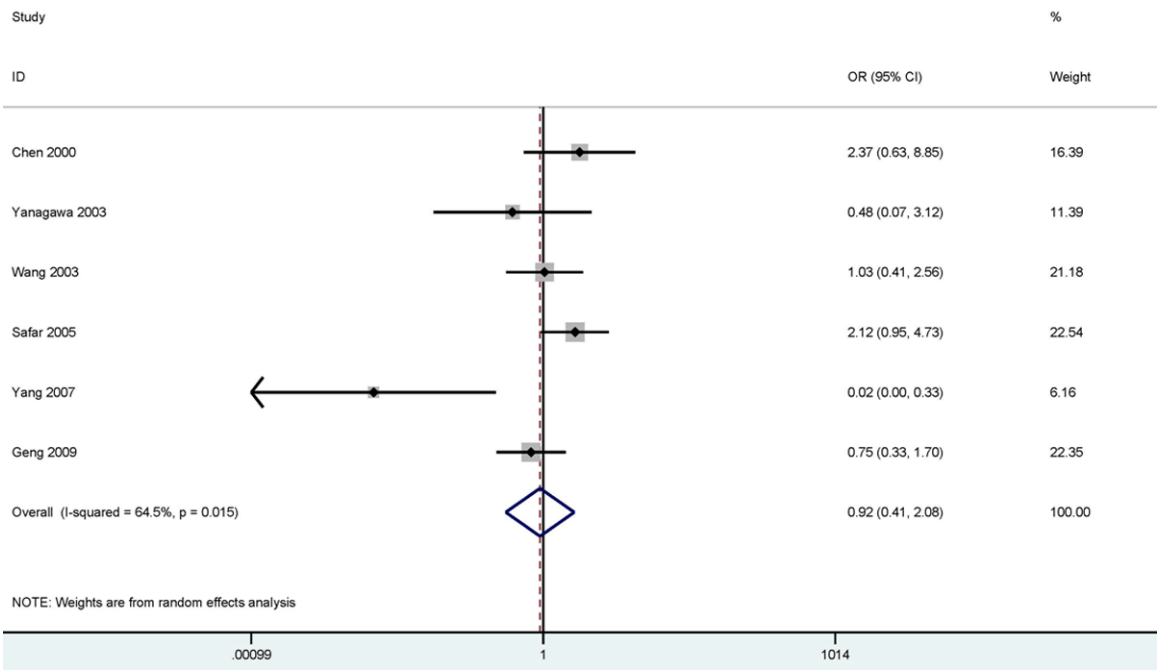

Figure 6. Forest plot of the association between methylated hMLH1 and tumor stage.

studies with negative results. There was a main existence of limitation in our study. The samples from bodily fluids such as blood and sputum, which lacked the associations between *hMLH1* methylation and NSCLC, were very important for their ease of collection in the clinic and might contribute to the feasible diagnosis and screening of NSCLC. More studies with larger sample sizes are still needed in the future.

In conclusion, our study suggests that *hMLH1* promoter methylation may play an important role in initiation and progression of NSCLC, especially among Asians.

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Disclosure of conflict of interest

None.

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