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ORIGINAL RESEARCH

The clinicopathological significance of *hMLH1* hypermethylation in non-small-cell lung cancer: a meta-analysis and literature review

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Abstract: The *hMLH1* gene plays an essential role in DNA repair. Methylation of the *hMLH1* gene is common in many types of cancer and can lead to the loss of hMLH1 expression. However, the association and clinicopathological significance between *hMLH1* promoter hypermethylation and non-small-cell lung cancer (NSCLC) is elusive. Here, we investigated the correlation of *hMLH1* promoter hypermethylation and NSCLC using 13 studies by comprising 1,056 lung cancer patients via a meta-analysis. We observed that 1) loss of hMLH1 protein expression was significantly associated with its promoter hypermethylation, 2) *hMLH1* gene inactivation through hypermethylation contributed to the tumorigenesis of NSCLC, which could be a decisive factor for the pathogenesis of NSCLC due to its high occurrence in NSCLC tissues compared to normal lung tissues, 3) a correlation exists between histologic subtypes/disease stages (TNM I+II vs III+IV) and hypermethylation status of *hMLH1* gene, and 4) NSCLC patients with *hMLH1* hypermethylation and subsequent low expression levels of hMLH1 have a short overall survival period than those patients with normal expression of *hMLH1* gene. hMLH1 mRNA predicts patient survival in lung cancer, and this was confirmed by using a public database. We then discussed the tumor suppressor function of *hMLH1* and the clinicopathological significance of *hMLH1* in NSCLC. We concluded that *hMLH1* hypermethylation should be an early diagnostic marker for NSCLC and also a prognostic index for NSCLC. hMLH1 is an interesting therapeutic target in human lung cancers.

Keywords: non-small-cell lung cancer, NSCLC, *hMLH1* gene, methylation, meta-analysis

Introduction

Lung cancer is the major cause of cancer death in males and females, worldwide. Approximately 58 percent of lung cancer cases occur in less developed countries, while the highest incidence of lung cancer is seen in North America and Europe.[1] In the Unites States, it is estimated that lung cancer affects around 226,160 patients, with a death rate of around 160,340 per year.[2] Prognosis remains poor despite advances in diagnosis and treatment. Therefore, early screening markers specific to lung cancer are needed.

In addition to the genetic mutations in cancer, epigenetic modification is a frequently occurring event in human lung cancer. Hypermethylation, a major type of epigenetic alteration, in the promoter areas of tumor suppressor genes (TSGs) has been well confirmed as a mechanism of transcriptional silencing in human malignant cancers, which also show features of genome-wide hypomethylation.[3] The DNA mismatch repair (MMR) system plays a crucial role in the maintenance of genetic and epigenetic stability.[4] Inactivation of MMR[5] by gene mutation[6] and promoter methylation[6–8] in human cells have been associated with human malignant tumors. The *hMLH1* (human

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mutL homologue 1), having sequence homology with the DNA MMR gene, is located at 3p22.3. *hMLH1* gene is composed of 19 exons, encoding a 756 amino acid protein[9] hMLH1, which physically interacts with other components of MMR and cell cycle/signaling/apoptosis molecules.[10–13] Dysfunction of hMLH1 by changes in microsatellite, short tandem repetitive sequences,[14–16] and/or hypermethylation[17] are found to be associated with cancer predisposition.[18]

Although methylation of *hMLH1* gene is found in several human cancer types, there is a difference in the frequency of methylation. It is essential to investigate whether the dysfunction of *hMLH1* is crucial for the tumorigenesis of lung cancer because of the reported high frequency of methylation (greater than 50%)[19–21] and because of its prognostic effects.[22,23] In the present study, we reviewed the publicly available literature and meta-analyzed the inputs with the aim to categorize the clinical importance of *hMLH1* hypermethylation, one of the epigenetic modifications in non-small-cell lung cancer (NSCLC) initiation and development.

Methods

Search methods

We first explored the MEDLINE though PubMed in September 2015 using the following subject words: "human mutL homologue 1", "*hMLH1*", "methylation", "hypermethylation", "lung cancer", and "NSCLC". Then, the same terms were also searched via Scopus, Embase, Biosis Previews, Cochrane Library, and Clinical Topic Database. Irrelevant and duplicate publications were excluded by analyzing titles and abstracts from all the studies identified through the search criteria. Full-text search was performed in the remaining publications to determine if it should be included or excluded. Randomized controlled trials and observational studies such as cohort studies/case series study, and case–control studies were selected, and case reports were excluded. The literature was limited to papers published in English and Chinese. Once all data were collected, the bibliographic references were scrutinized to obtain pertinent studies. The most detailed clinical studies were chosen if several similar studies were reported using the same population of patients.

Inclusion and exclusion criteria

The selected publications were scrutinized for *hMLH1* hypermethylation/expression to clinicopathological consequences in patients of NSCLC in this meta-analysis. The eligibility of the studies was evaluated using the following criteria: 1) full papers published in English or Chinese, 2) *hMLH1* hypermethylation and/or expression evaluated in the NSCLC patients' lung tissues and/or paired normal lung tissues, 3) research that determines the effects of *hMLH1* hypermethylation on NSCLC clinicopathological characteristics and prognosis, 4) a sensitive technique of bisulfate conversion-specific and methylation-specific PCR (MSP) or combined bisulfate restriction analysis (COBRA) or MethyLight assay being used to detect *hMLH1* methylation status, 5) publications with hazard ratio (HR) and probabilities for overall survival (OS) at 95% confidence interval (CI) were available. We excluded literature if 1) studies used cell cultures in vitro and animals in vivo; 2) they were secondary articles (reviews, editorials, letters, and expert opinion), case reports, conference abstracts; 3) literature not written in English and Chinese; and 4) publications lack of OS and HR about OS.

Data extraction

The data extraction followed the methods as described previously.[24] Two authors independently collected the data from the selected literature. Any dispute in opinion raised by the two investigators was resolved after discussion. The basic information of each publication such as the authorship, year, number of patients, sources of tissues, clinical stage, and *hMLH1* hypermethylation and/or expression were recorded and transferred to a table. The eligibility for a meta-analysis was gauged by heterogeneity.

Data analysis

We analyzed the data using Review Manager 5.2 (Cochrane Collaboration, Oxford, UK). Odds ratios (ORs) as well as 95% CIs were measured for dichotomous variables. A P-value of <0.05 was considered to be significantly significant. The heterogeneity of variance, not random effects, between the studies was measured by I^2, with the significance being set at $P<0.10$. If I^2 was less than 50%, heterogeneity is assumed to be low. A fixed-effect model rather than a random model was used if heterogeneity did not exist and vice versa.

Fifty nine publications were obtained by searching the databases of Scopus, Embase, Biosis Previews, Cochrane Library, and Clinical Topic Database. After filtering the titles, abstracts, and references by inclusion and exclusion criteria, 13 publications in full-text were finally included for qualitative analysis in this meta-analysis. The workflow for the selection is shown in Figure 1. The study from Tang et al[25] was excluded because the authors compared the promoter hypermethylation of *hMLH1* between lung primary adenocarcinomas and colorectal metastasis to the lung and did not provide information on noncancerous controls.

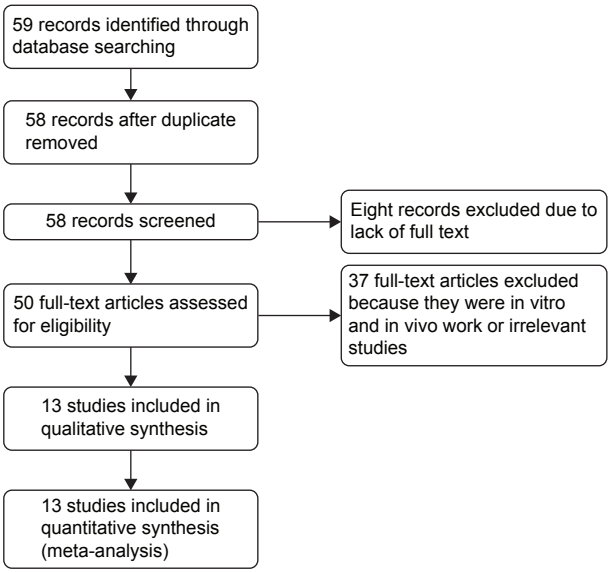

Figure 1 Flow chart of study selection.

In addition, the study from Ali et al[26] separated the NSCLC caused by exposure to chromate in their study, squamous carcinomas with 10 of 33 methylation for *hMLH1* and 23 of 33 unmethylation for *hMLH1*, while adenoma with 0 of 2 methylation of *hMLH1* and 2 of 2 unmethylation of *hMLH1*. However, there were no non-lung cancer controls, and so it was excluded as well.

Patient survival analysis by Kaplan–Meier plotter, a public database

An online database[27] was used to assess the relevance of *hMLH1* expression to OS. The database was established using gene expression data and survival information of 1,928 patients downloaded from Gene Expression Omnibus (GEO) (Affymetrix HGU133A and HGU133+2 microarrays). Briefly, *hMLH1* gene was entered into the database (http://kmplot.com/breast/) to obtain Kaplan–Meier survival plots where the number-at-risk is indicated below the main plot. HR (and 95% CI) and log rank *P* were calculated and displayed on the webpage.

Results

Selection of studies

Fifty-nine articles were recognized following the search criteria set by us. Forty-six of these were not included because they were in vitro and/or in vivo studies, secondary articles, or articles without the appropriate control population. Finally, 13 eligible and relevant studies were included in the current meta-analysis (Figure 1).

These 13 studies were published from 2000 to 2015.[19–23,28–34] The clinical data from a total of 1,069 lung cancer patients and 626 nonmalignant patients from the People's Republic of China, Japan, Korea, Portugal, Australia, and the United States were documented and are shown in Table 1.

hMLH1 methylation and clinicopathological features

Loss of *hMLH1* protein expression was significantly associated with its promoter hypermethylation in NSCLC

To determine whether the *hMLH1* promoter methylation could be linked to the loss of gene expression, protein expression and promoter methylation were examined for the *hMLH1* gene in three included studies comprising 213 lung cancer patients and 136 nonmalignant lung tissues. The pooled data showed that negative protein expression was correlated with promoter hypermethylation of the *hMLH1* gene (Figure 2) with an OR 5.93 (95% CI 2.54–13.84, $P<0.0001$).

The inactivation of *hMLH1* through methylation in NSCLC

In Figure 3, the study ID is shown in the first column; the second column denotes the proportion of *hMLH1* methylation in NSCLC; and the third column represents the proportion of *hMLH1* methylation in normal controls. The weight in the fourth column is proportional to the inverse of the variance of the study – high variance associated with a small study, meaning less weight given to that study and vice versa. The OR is given in the column after the one of weight. In this case, the CI of the pooled OR excludes the number "1" (it is 3.18–42.01) indicating that it is significantly different. The diagram of OR is shown in the final column. The vertical line indicates an OR of 1.0. An OR of 1.00 means that the two groups were equally likely to experience the event. An OR higher than 1 means that the NSCLC group was more likely to experience the event (hypermethylation of *hMLH1*) than the control group. Therefore, if *hMLH1* methylation occurred more frequently in cancer than in nonmalignant tissues, the OR would be greater than 1.0. The horizontal dots and bars represent the relative risk and 95% CI for each study. In summary, our results showed that the lung cancer tissues have significantly high *hMLH1* methylation rates compared to controls. The pooled OR of ten individual studies, which included 912 lung cancer tissues and 666 nonmalignant lung tissues (as controls), is shown in Figure 3 (OR 10.61, 95% CI 2.71–41.54, $P=0.0007$). This implies that inactivated *hMLH1* caused by one of the epigenetic modifications, methylation, significantly contributes to the lung

Table 1 Basic characteristics of the included studies

Study/country	Patients/samples	Methods	Primary aim	Methylation site
Chen et al[28] People's Republic of China	50/tissue, paired with 50/ nonneoplastic lung tissue	MSP	Determine the relationship between *hMLH1* expression and its methylation status	Promoter, CpG islands
Wang et al[32] People's Republic of China	77/tissue	COBRA	Determine the methylation status of the *hMLH1* promoter in resected specimens from patients with primary NSCLC	Promoter, CpG islands
Yanagawa et al[33] Japan	75/tissue, paired with 75/ nonneoplastic lung tissue	MSP	Determine the clinicopathological significance of gene promoter methylation in NSCLC	Promoter, CpG islands
Hsu et al[29] People's Republic of China	105/tissue	MSP	Investigate protein expression and promoter hypermethylation of *hMLH1* in NSCLC	Promoter, CpG islands
Safar et al[21] USA	105/tissue	MSP	Determine the impact of promoter hypermethylation in resected NSCLC	Promoter, CpG islands
Kim et al[30] Korea	99/tissue, 99/controls	MSP	Investigate the aberrant methylation profile of the cancer-related genes in Korean NSCLC patients	Promoter, CpG islands
Feng et al[47] USA	49/tissue, paired with 49/ nonneoplastic tissue	MethyLight		
Liu et al[31] People's Republic of China	60/tissue, paired with 60/ nonneoplastic lung tissue	MSP	Examine methylation profiles for TSG in chromosome 3p in NSCLC	Promoter, CpG islands
Seng et al[22] Australia	239/tissue	MSP	Determine epigenetic effect in chromosome 3p in NSCLC	Promoter, CpG islands
Geng et al[19] People's Republic of China	116/tissue, 116/non-neoplastic lung tissue	COBRA	Study the main mechanism of *hMLH1* gene inactivation in NSCLC samples of Chinese patients	Promoter, CpG islands
Zhang et al[34] People's Republic of China	78/tissue, 78/ nonneoplastic lung tissue	MSP	Investigate the methylation profiles of NSCLC in Chinese population	Promoter, CpG islands
Gomes et al[20] Portugal	40/tissue, paired with 40/ nonneoplastic lung tissue	MSP	Define methylation profile and silencing of DNA repair gene *hMLH1* in NSCLC as well as normal controls	Promoter, CpG islands
Wu et al[23] People's Republic of China	80/tissue	MSP	Determine whether DNA methylation of *hMLH1* affects the prognosis of NSCLC patients	Promoter, CpG islands

Abbreviations: NSCLC, non-small cell lung cancer; MSP, methylation-specific PCR; TSG, tumor suppressor gene; COBRA, combined bisulfate restriction analysis.

cancer tumorigenesis. The high *hMLH1* methylation rates in tissues for lung cancers compared to normal controls, provides the potential of the *hMLH1* gene to be used as a diagnostic index for lung cancer patients by checking its methylation status.

Methylation of hMLH1 in disease stage

We further determined the possible associations between *hMLH1* hypermethylation and clinicopathologic features. To determine whether or not the methylation of the promoter region of the *hMLH1* gene is independently associated with tumor stage, a meta-analysis was performed. When the data were stratified by tumor stage (TNM I+II vs TNM III+IV), methylation of *hMLH1* increased with advanced stage as shown by the results of two studies with 94 NSCLC lung cancer tissues and 215 nonmalignant lung tissues (OR 2.14, 95% CI 1.30–3.52; Figure 4).[22,23] By observing the OR

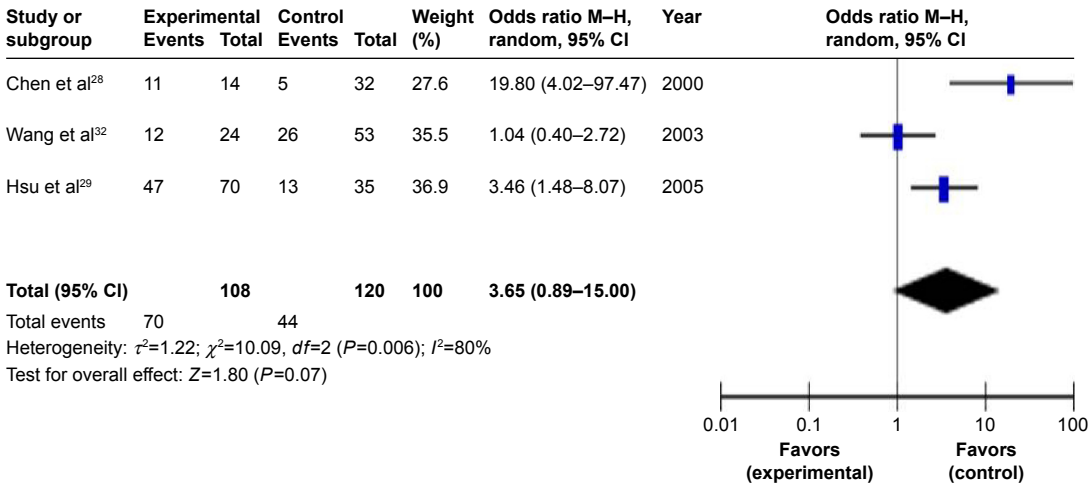

Figure 2 The studies included to examine the relationship between the protein expression of *hMLH1* and its promoter hypermethylation in 213 NSCLC patients and 136 normal lung tissues.
Abbreviations: NSCLC, non-small-cell lung cancer; M–H, Mantel–Haenszel; OR, odds ratio; CI, confidence interval.

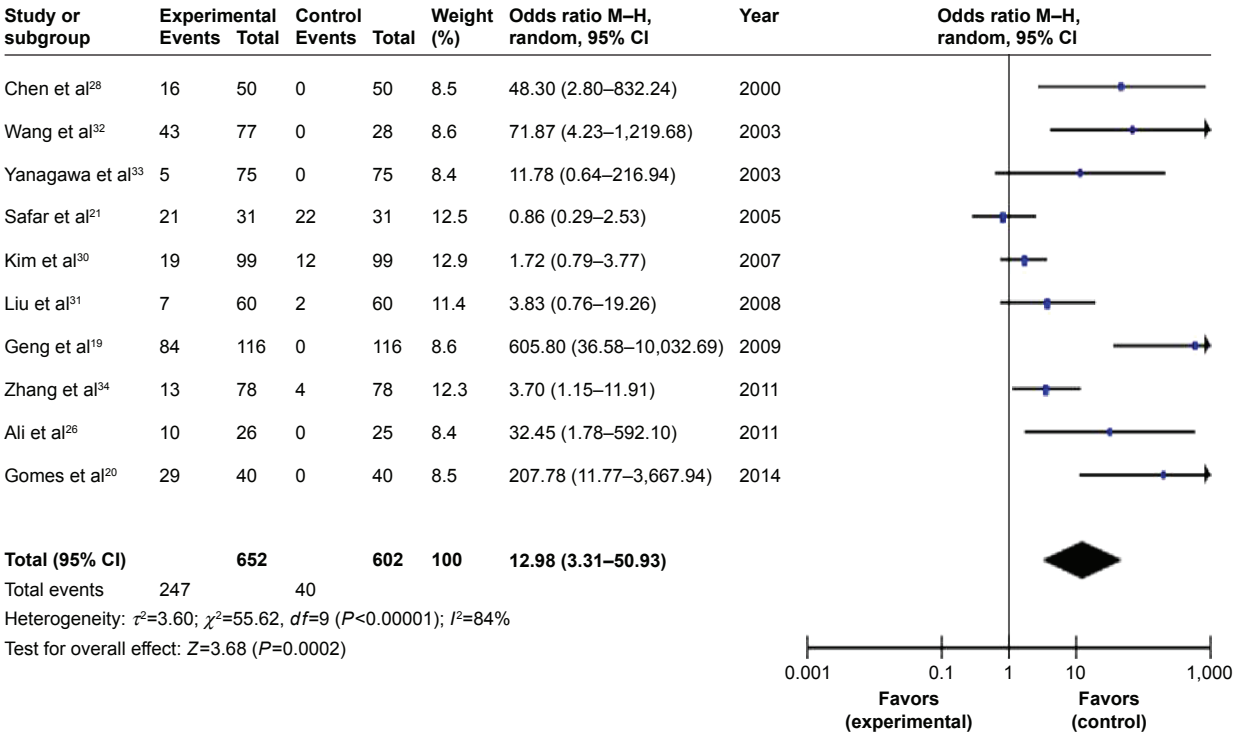

Figure 3 Comparison of *hMLH1* methylation status in 912 NSCLC patients and 666 individuals with nonmalignant lesions.
Note: The summarized ORs is 10.61 with a 95% CI between 2.71 and 41.54 (*Z*=3.39, *P*=0.0007).
Abbreviations: NSCLC, non-small-cell lung cancer; M–H, Mantel–Haenszel; ORs, odds ratios; CI, confidence interval.

shown in the fifth column in Figure 4, where the CI of the summarized data excludes 1.0 (it is 1.30–3.52), we concluded that the *hMLH1* gene methylation status is associated with lung cancer stage, with the advanced stage exhibiting high level of *hMLH1* gene methylation.

Prognostic impact of *hMLH1* gene hypermethylation in NSCLC

We analyzed the relationship of hypermethylation of the *hMLH1* gene with patient survival. The relationship between OS and *hMLH1* promoter hypermethylation was detected in NSCLC by two studies.[22,23] The pooled data (Figure 5) indicated the prognostic impact of *hMLH1* gene methylation in NSCLC patients (OR =0.54, 95% CI =0.35–0.82, *Z*=2.86, *P*=0.004). As we know, the exclusion of number "1" from summarized HR (0.35–0.82) indicated that NSCLC patients with *hMLH1* gene methylation showed poor prognosis.

Quality controls

The quality control of the study was performed through testing the result stability by removing one study at a time.

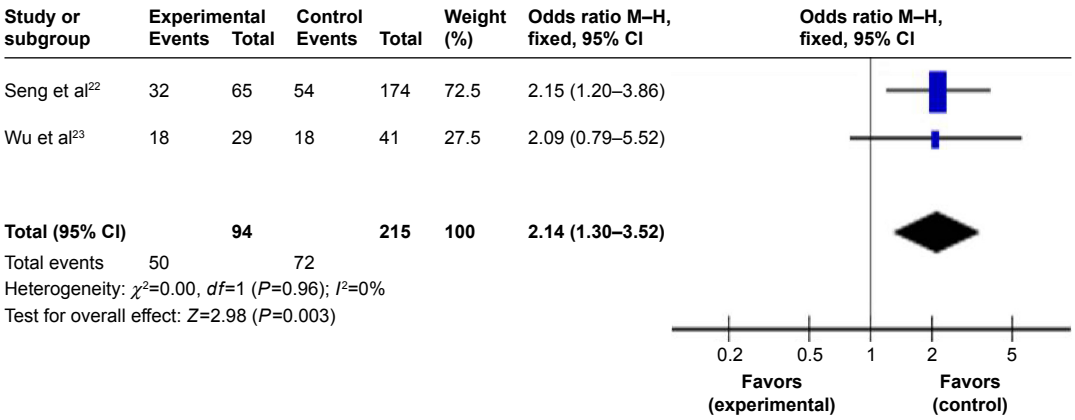

Figure 4 Summarized analysis results of hypermethylation of *hMLH1* gene in various disease stages (TNM I+II vs TNM III+IV) in NSCLC. The pooled OR 2.14, 95% CI 1.30–3.52, *Z*=0.16, *P*=0.003).
Abbreviations: NSCLC, non-small-cell lung cancer; M–H, Mantel–Haenszel; OR, odds ratio; CI, confidence interval.

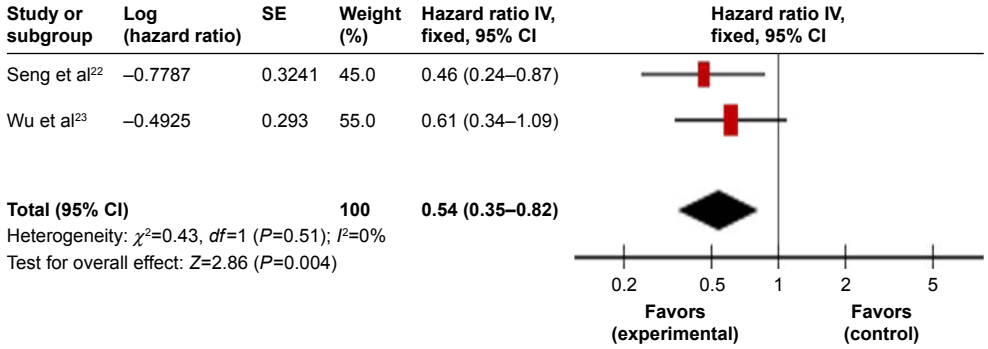

Figure 5 The relation of OS to *hMLH1* methylation obtained by two eligible studies.
Note: The summarized HR for OS demonstrated that NSCLC patients exhibiting *hMLH1* hypermethylation had worse survival in NSCLC, HR 0.54, 95% CI 0.35–0.82, *P*=0.004.
Abbreviations: NSCLC, non-small-cell lung cancer; OS, overall survival; HR, hazard ratio; CI, confidence interval.

The pooled ORs were not altered, indicating the stability of the study is acceptable. For instance, in Figure 3, after removing one weighted study,[30] OR 14.73 (95% CI 2.96–73.17), I^2=82%, Z=3.29 (P=0.001). In funnel plot, the studies were distributed largely symmetrically suggesting the absence of publication biases in the present meta-analysis (Figure 6).

hMLH1 mRNA predicting patient survival was confirmed by publicly available database

This assessment of clinical relevance was further corroborated in a patient survival analysis using an online database containing the expression of 22,277 genes and 20-year survival information of 1,928 lung cancer patients

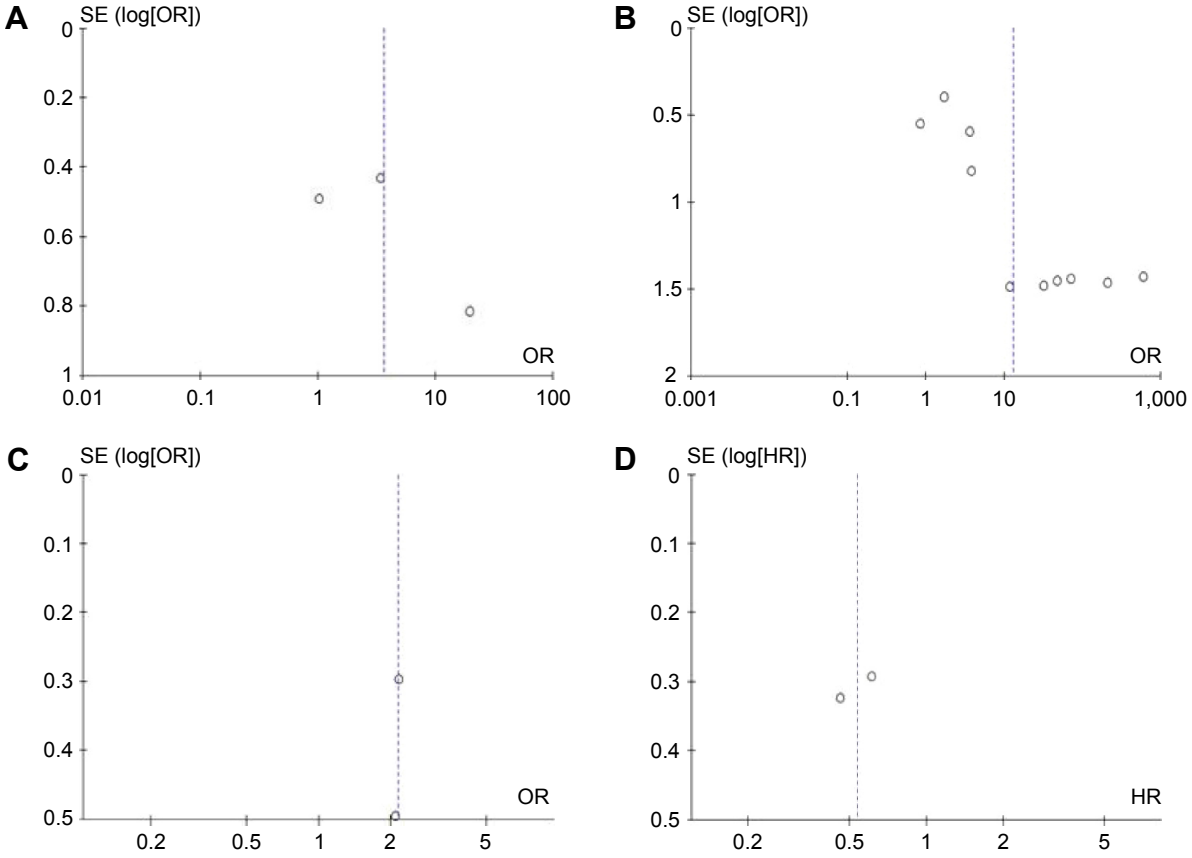

Figure 6 In funnel plots, the studies were distributed largely symmetrically suggesting the absence of publication biases in the present meta-analysis.
Notes: *hMLH1* methylation in lung cancer (**A**), subtypes of histology (**B**), disease stages, TNM I+II vs III+IV (**C**) and overall survival (**D**).
Abbreviations: OR, Odds ratio; SE, standard error; HR, hazard ratio.

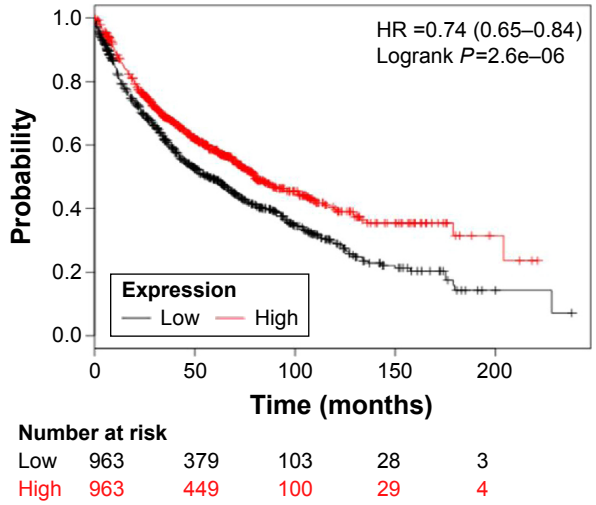

Figure 7 *hMLH1* mRNA prediction of patient survival was confirmed using publicly available database.

Notes: This assessment of clinical relevance was further corroborated in a patient survival analysis using an online database containing the expression of 22,277 genes and 20-year survival information of 1,928 lung cancer patients (http://www.kmplot.com/analysis/).[27] *hMLH1* downregulation was found to correlate strongly with poor OS for all lung cancer patients followed for 20 years (HR 0.74 [95% CI =0.65–0.84], *P*=2.6e–06).

Abbreviations: OS, overall survival; HR, hazard ratio.

(http://www.kmplot.com/analysis/).[27] *hMLH1* downregulation was found to correlated strongly with poor OS for all lung cancer patients followed for 20 years (Figure 7, HR 0.74 [95% CI 0.65–0.84], *P*=2.6e–06).

Discussion

From a review of the literature on the loss of 3p in different types of solid tumors, it appears that specific genes in this region act as tumor suppressor genes. RB1, VHL, and *hMLH1* are the examples that have been applied to localize tumor suppressor genes on 3p.[35] The *hMLH1* gene has been found to contribute to the development of specific MMR-deficient cancers.[35] The most rigorous and methodologically complicated review article is a meta-analysis with comparison and systematic review. A meta-analysis quantitatively analyzes the public available data from previous studies and reaches a summary estimate with statistical power, therefore, effectively directing the field of studies more efficiently. In the present meta-analysis study, we concluded that 1) loss of hMLH1 protein expression was significantly associated with its promoter hypermethylation, 2) *hMLH1* gene inactivation through hypermethylation contributes to the tumorigenesis of NSCLC, and it could be a decisive factor for the tumorigenesis of NSCLC due to its high occurrence in NSCLC tissues compared to normal lung tissues, 3) a correlation exists between histologic subtypes/disease stages (TNM I+II vs TNM III+IV) and hypermethylation status of *hMLH1* gene, and 4) NSCLC patients with *hMLH1* hypermethylation and low expression levels of *hMLH1* have a short OS period than those patients with normal expression of *hMLH1* gene.

Traditionally, the TNM classification has been used for NSCLC. During the period when the included studies were performed, TNM classification was changed from the 6th to 7th edition. The main changes in staging classification are reflected in the T staging. These changes are largely related to the reclassification of the size and location of the primary tumor and satellite nodules.[36] As we know, up- or downgrading of TNM has occurred in some cases during conversion of 6th TNM to 7th TNM. It is impossible to exactly convert the 6th TNM case scores to 7th TNM ones, although most case scores were the same in both editions. This is a limitation of the study.

Furthermore, the conclusion that *hMLH1* promoter hypermethylation is a clinical prognosticator (Figure 5) in lung cancer patients is further supported by a patient survival analysis to correlate *hMLH1* gene expression and OS for 1,928 lung cancer patients (www.kmplot.com) where loss or reduced levels of *hMLH1* is strongly predictive of worse disease outcome for all lung cancer patients in general (Figure 7). Based on these data, we believe that hMLH1 protein may have a great potential to be a new biomarker for prognosis in lung cancer patients.

DNA methylation is influenced by other factors such as aging, infection, inflammation, and food intake.[37] Aging and tumorigenesis have some common features such as accumulation of epigenetic alterations and shortened telomerase, although they are two different biological processes.[37] A genome-wide hypomethylation occurs during the aging process, which is accompanied by a de novo hypermethylation at specific sites.[38] Methylation of RASSF1A promoter is associated with age in primary NSCLC.[39] An age-related association of hypermethylation of *hMLH1* promoter with the loss of hMLH1 protein expression has been found in gastric and colorectal carcinomas.[37,40–43] The proportion of gastric and colorectal carcinomas with hypermethylation of the *hMLH1* promoter increases with age, with 25%–30% of all carcinomas of the stomach and large intestine in elderly patients demonstrating hypermethylation.[37] However, the proportion of lung cancers with hypermethylation of the *hMLH1* promoter has not been related to increases with aging.

The role of *hMLH1* in lung cancer has partly been attributed to promoter hypermethylation, which leads to gene silencing. However, studies indicate that *hMLH1* expression is under regulation by different methods. Homozygous deletions, allelic deletion, and point mutations

have also been found to attenuate *hMLH1* expression.[44–46] Specifically, hMLH1 protein acting as a part of DNA repair pathways undergoes hypoxia regulation via epigenetic modulation as well as transcriptional and translational regulation.[45]

Given that smoking plays a central role in lung cancer development and that DNA methylation is an early event in tumorigenesis,[47] some biomarkers such as CCND2 and APC were shown to be frequently hypermethylated in both cancerous and noncancerous lung tissues of smokers with NSCLC, indicating that hypermethylation of these genes may be associated with chronic smoking status.[47] Antczak et al[44] reported that reduced *hMLH1* expression due to allelic imbalance combined with epigenetic alteration was more frequently associated with heavy smokers; however, a correlation could not be determined between *hMLH1* methylation and the smoking characteristic (smoking history) of the individuals with NSCLC due to lack of quantitative data.

All the authors in the included studies used PCR-based DNA methylation analysis techniques, where the first step in all protocols is bisulfite conversion of the DNA sequence of interest. MSP, combined bisulfate restriction analysis (COBRA), and MethyLight were used in the included studies. Each approach has its own strengths and weaknesses. The design of primers for MSP favors the amplification of unconverted DNA. 1) MSP is commonly used for methylation screening in cancer; it is important to ensure that only the methylated and converted amplification product is being detected. 2) It is strongly recommend that all MSP-based assessments of methylation include a control for amplification of unconverted DNA. For small-scale applications, analyzing MSP products by restriction digestion is the simplest approach, but this is limited to the restriction site. 3) The TaqMan®-based assay (MethyLight) provides the most quantitative information regarding the extent of bisulfite conversion.

Taken together, the data from each included study are comparable.

Based on the meta-analysis results, we suggest that *hMLH1* hypermethylation should be an early diagnostic marker for NSCLC and also a prognostic index for NSCLC. *hMLH1* is an interesting therapeutic target in human lung cancers. Re-expression of *hMLH1* by demethylation in tumors may be likely to bring clinical benefits. The development of specific compounds for *hMLH1* activation will be a promising strategy to target *hMLH1* in clinical application. SGI-110, a dinucleotide combining 5-azaC and deoxyguanosine (Astex Pharmaceuticals, Inc., Cambridge, UK), is less likely to undergo deamination and more stable compared to DNA methyltransferase inhibitors (DNMTIs). Pretreatment with SGI-110 resensitizes ovarian cancer cells to cisplatin in vitro and in vivo by demethylation and reactivation of numerous chemotherapy response-related genes including *hMLH1*.[48] The *hMLH1* inhibitors that are still in the early stages of evaluation need to be further explored and developed in lung cancer patients.

Author contributions

YH and ZL were responsible for designing of the study; YH, KS, SZ, and ZL performed experiments; YH, KS, and DY analyzed data; ZL wrote the manuscript; and all authors reviewed the manuscript. All authors contributed toward data analysis, drafting and critically 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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