Received: 15 September 2017 | Accepted: 13 November 2017

DOI: 10.1002/jcla.22370

RESEARCH ARTICLE

WILEY

Aberrant methylation of mutL homolog 1 is associated with increased risk of non-small cell lung cancer

Haochang Hu[1] | Xiaoying Chen[2] | Cong Zhou[1] | Bin Li[1] | Yong Yang[1] | Xiuru Ying[1] | Yiyi Mao[1] | Yihan Zhang[1] | Jie Zhong[1] | Jie Dai[1] | Hang Yu[1] | Boyi Wu[1] | Xiaodong Li[1] | Tiangong Wang[1] | Shiwei Duan[1]

[1]Medical Genetics Center, School of Medicine, Ningbo University, Ningbo, Zhejiang, China

[2]Department of Medical Record, Women's Hospital, School of Medicine, Zhejiang University, Hangzhou, Zhejiang, China

Correspondence
Shiwei Duan, Medical Genetics Center, School of Medicine, Ningbo University, Ningbo, Zhejiang, China.
Email: duanshiwei@nbu.edu.cn

Funding information
National Natural Science Foundation of China, Grant/Award Number: 81371469; Natural Science Foundation of Zhejiang Province, Grant/Award Number: LY14H160008; Clinical Research Foundation of Zhejiang Province Medical Association, Grant/Award Number: 2015ZYC-A77; K. C. Wong Magna Fund in Ningbo University

Background: Non-small cell lung cancer (NSCLC) is a common malignant tumor. DNA hypermethylation in the promoter region has been served as a potential molecular marker for several tumors. The goal of the current study was to assess the diagnostic ability of mutL homolog 1 (*MLH1*) promoter methylation in NSCLC.

Methods: A total of 111 NSCLC patients' paired tissue samples were obtained to explore the association between *MLH1* promoter methylation and NSCLC by methylation-specific polymerase chain reaction (MSP) method. Public databases including The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO) were used to verify our findings.

Results: Our results showed a significantly higher *MLH1* methylation frequency in tumor tissue samples than their paired adjacent tissues ($P = .008$). ROC curve indicated that *MLH1* MSP assay was a sensitive but not a specific method in the diagnosis for NSCLC (sensitivity = 0.964, specificity = 0.135, AUC = 0.550). And the association between the methylation level and clinical characteristics has no statistical significance. TCGA cohort evinced a higher methylation probability in tumor group compared with nontumor group (the mean β value: −0.449 [−0.467, −0.437] vs −0.466 [−0.472, −0.437], $P = .011$), which was consistent with our results. Meanwhile, an inverse correlation between *MLH1* methylation and *MLH1* expression was detected in TCGA and GEO databases.

Conclusions: The MSP method for *MLH1* methylation was a sensitive but not a specific diagnostic method for NSCLC.

KEYWORDS
diagnosis, DNA methylation, methylation-specific polymerase chain reaction, mutL homolog 1, non-small cell lung cancer

1 | INTRODUCTION

Lung cancer is the most common cancer and the leading cause of death in the world.[1] The global mortality of lung cancer will grow up to 3 million in 2035.[2] Non-small cell lung cancer (NSCLC) is the major subtype of lung cancer, accounting for 85% of lung cancer.[3] The current diagnosis of lung cancer is done by a combination of symptoms, signs, laboratory tests,[4] and auxiliary imaging.[5-7] Although cytological diagnosis could provide physician a clear view for the diagnosis of lung cancer, it also missed up to half of the lung cancer.[8] As the golden standard method, biopsy by bronchoscope, mediastinoscopy, or thoracentesis was unsuitable in the screening for early NSCLC.[9] Most lung cancer patients were diagnosed at the advanced stage,[10] and thus, the 5-year overall survival remains poor.[1]

Non-small cell lung cancer is a complex disease affected by the interactions from genetic, epigenetic, and environmental factors.[11] As the most studied epigenetic modification, DNA methylation is a promising tool for the early detection of lung cancer.[12,13] The mutL homolog 1 (*MLH1*) is one of the main members of mismatch repair (MMR) gene family.[14] The loss of MMR function was reported to be correlated with carcinogenesis.[15] Previous studies demonstrated that aberrant DNA methylation might increase the expression of oncogenes and silence the expression of tumor suppressor genes during tumorigenesis.[12]

Recently, several studies reported aberrant *MLH1* promoter hypermethylation in NSCLC patients.[16-19] However, using *MLH1* methylation in the diagnosis for NSCLC was still debatable. In the present study, we obtained 111 NSCLC to determine whether *MLH1* promoter methylation played a role in NSCLC in Han Chinese. Data mining studies were also performed to elaborate the findings in our study.

2 | METHODS AND MATERIALS

2.1 | Tissue samples

Formalin-fixed, paraffin-embedded (FFPE) tissues were collected from 111 NSCLC patients in Huzhou People's Hospital, China from August 2010 to November 2013. All the patients were diagnosed by pathological examination. Histological classification was defined according to the WHO guidelines, and tumor stage was determined according to the UICC TNM classification. Age, sex, smoking history, disease stage, tumor location, and histological type for all cases were extracted from the medical records. The Ethics Committee of Huzhou People's Hospital approved this study, and written informed consent was obtained from each participant.

2.2 | DNA extraction and bisulfite conversion

Genomic DNA was isolated from FFPE tissues using the E.Z.N.A.TM FFPE Tissue Kit (Omega Bio-Tek, Norcross, GA, USA). DNA concentrations were tested by Nanodrop2000 spectrophotometer (Thermal Scientific Co. Ltd., Wilmington, DE, USA). Bisulfite treatment was conducted subsequently to convert unmethylated cytosine to uracil, while the methylated one remained as cytosine. EZ DNA Methylation-Gold Kit™ (Zymo Research, Orange, CA, USA) was applied to achieve this procedure.

2.3 | Methylation-specific polymerase chain reaction (MSP)

The details of MSP were as described previously.[20] The primer sequences were as follows: *MLH1* methylated alleles, 5′-AACGAATT AATAGGAAGAGCGGATAGCG-3′ (forward) and 5′-CGTCCCTCCC TAAAACGACTACCC-3′ (reverse); *MLH1* unmethylated alleles, 5′-TAAAAATGAATTAATAGGAAGAGTGGATAGTG-3′ (forward) and 5′-AATCTCTTCATCCCTCCCTAAAACA-3′ (reverse). PCR conditions for both methylated (M) and unmethylated (U) primer pairs comprised initial denaturation at 95°C for 10 minutes, followed by 37 cycles of 30 seconds denaturation at 95°C, 45 seconds annealing at 55°C and 30 seconds extension at 72°C. Then, the products were stored at 4°C. PCR products were subject to gel electrophoresis through 2.0% agarose gel stained with ethidium bromide, and then being visualized with UV illumination using a digital imaging system (Bio-Rad, Hercules, CA, USA).

2.4 | Data mining study

DNA methylation profiles (Illumina Human Methylation 450K) of 830 NSCLC tissues (458 adenocarcinoma [AC] and 372 squamous cell carcinoma [SCC]) and 77 nontumor tissues (34 AC and 43 SCC) were downloaded from the Web site of Cancer Genomics Browser of ` (UCSC; https://genome-cancer.ucsc.edu/). The average methylation level of four cytosine-phosphate-guanine (CpG) sites on *MLH1* (cg00893636, cg21490561, cg11600697, and cg23658326) was used to represent *MLH1* methylation. To verify the correlation between *MLH1* DNA methylation level and mRNA expression, *MLH1* mRNA expression in NSCLC (522 AC and 504 SCC) was also downloaded from cBioPortal database (http://www.cbioportal.org/). Furthermore, *MLH1* expression profiles with 5-AZA treatment were collected from the Gene Expression Omnibus (GEO) database (www.ncbi.nlm.nih.gov/geo, accession no. GSE32496).

2.5 | Statistical analysis

Statistical analysis was conducted with the PASW statistics 18.0 software (SPSS, Inc., Somers, NY, USA). Chi-square test was used to detect the methylation differences between tumor tissues and nontumor tissues. The diagnostic value of *MLH1* methylation for NSCLC was evaluated by the receiver operating characteristics (ROC) test. Spearman rank test was used to calculate the correlation between *MLH1* methylation and gene expression. Differences were considered statistically significant if P values were $<.05$.

3 | RESULTS

In the current study, we recruited 111 NSCLC patients (38 females and 73 males) to investigate the role of *MLH1* methylation in the diagnosis for NSCLC. The median age at diagnosis of our patients was 64 years (range: 33-82 years). There were 79% patients with stage I + II and 21% patients with stage III + IV. And 61 patients were smokers, and 50 were nonsmokers. Four Methyl450K CpG sites (cg00893636, cg21490561, cg11600697, and cg23658326) were located in the tested fragment (91 bp, hg19, chr3:37034751-37034841, Figure 1).

As shown in Figure 2, the methylation status of *MLH1* promoter in 111 tumor tissues and corresponding adjacent non-neoplastic lung tissues were tested by MSP method. *MLH1* methylation was detected in 107 of 111 (96%) NSCLC tumor tissues and 96 of 111 (86%) the paired adjacent nontumor tissues, respectively. There was a significant

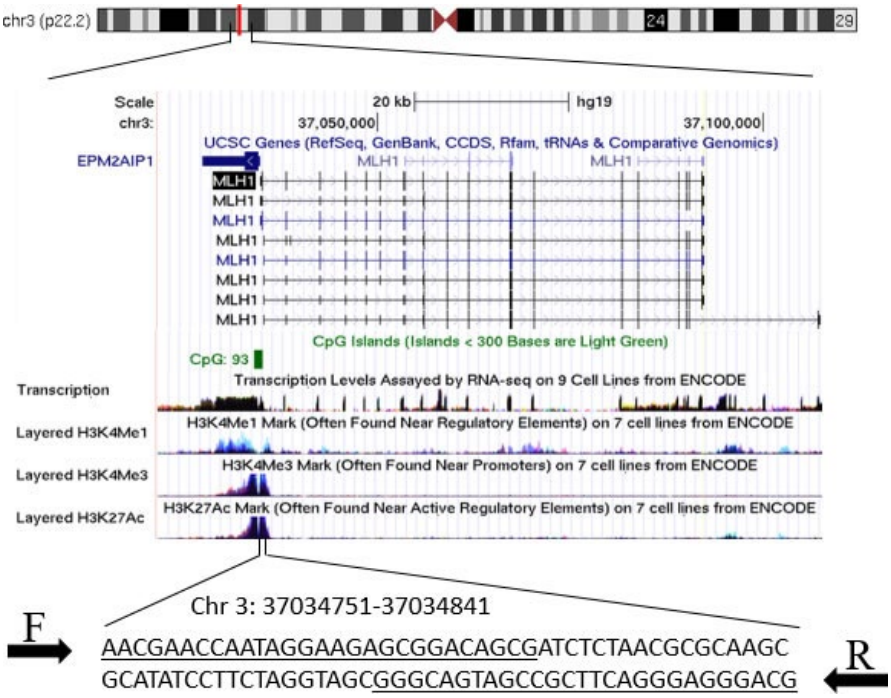

FIGURE 1 Genomic position and functional annotation of the amplified fragment from University of California Santa Cruz genome browser. The MSP primers were underlined. F: forward primer; R: reverse primer

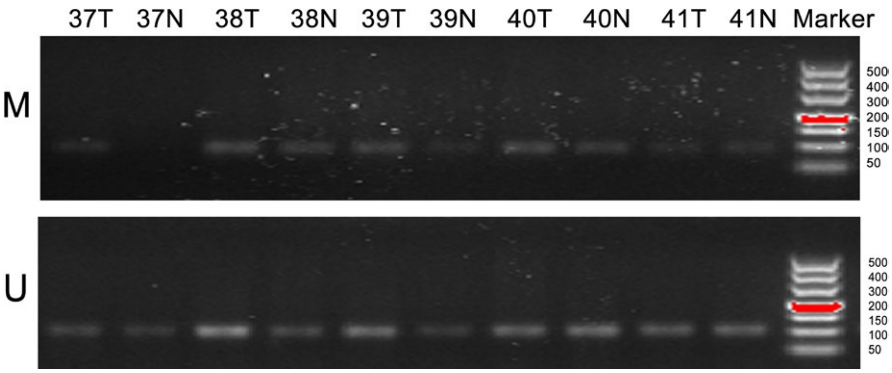

FIGURE 2 Representative methylation-specific polymerase profiles in non-small cell lung cancer tumor tissues and their adjacent non-tumor tissues. T: tumor tissue; N: non-tumor tissue; M: methylation; U: unmethylation

difference of *MLH1* methylation between NSCLC tumor tissues and adjacent lung tissues ($P = .008$).

There was a sensitivity of 0.964, a specificity of 0.135, and an AUC of 0.550 (0.474, 0.625) using *MLH1* methylation in the prediction for NSCLC. This suggested that *MLH1* MSP assay was a sensitive but not specific method in the diagnosis for NSCLC. According to the histological types, methylation of *MLH1* was detected in 67 of 69 (97%) AC samples and 40 of 42 (95%) SCC samples. Therefore, our results did not support *MLH1* methylation as a differential biomarker between AC and SCC.

Subsequently, we examined the correlation between *MLH1* methylation and the clinicopathological features of NSCLC patients. And there was no significant correlation between *MLH1* methylation and clinical phenotypes (including gender, age, smoke history, tumor location, histological type, and clinical stage) in tumor tissues (all $P > .05$, Table 1).

Date extracted from The Cancer Genome Atlas (TCGA) database also validated that *MLH1* methylation levels in tumor tissues were higher than that in nontumor tissues (the mean β value: −0.449 [−0.467, −0.437] vs −0.466 [−0.472, −0.437], $P = .011$, Figure 3). Besides, we analyzed the methylation level in AC and SCC patient, respectively. Interestingly, our results showed a significant association of *MLH1* methylation with SCC risk (the mean β value: −0.468 (−0.470, −0.464) vs −0.471 (−0.474, −0.470), $P < .001$, Figure 3) but not AC risk (the mean β value: −0.439 (−0.445, −0.433) vs −0.436 (−0.441, −0.431), $P = .056$, Figure 3). To noted, there was a negative correlation between *MLH1* expression and *MLH1* methylation (AC: $P = .008$, $r = -.124$; SCC: $P < .0001$, $r = -.296$, Figure 4). Furthermore, GEO data showed that there was a trend of increased *MLH1* expression in NSCLC cell lines (A549, H1993, and H2073) after 5′-aza-deoxycytidine (5AZA) treatment (Fold change >1.03, Figure 5).

4 | DISCUSSION

A number of studies found a handful of genes with aberrant DNA methylation in lung cancers,[21-23] implying a potential role of DNA methylation in the prediction for lung cancer. *MLH1* is a tumor suppressor gene involved in DNA mismatch repair, which could correct the DNA replication.[24] Therefore, genetic and epigenetic alterations

TABLE 1 Association between mutL homolog 1 promoter methylation and clinical characteristics in non-small cell lung cancer patients

Characteristics	N	M/U	Spearman's *r*	*P* value
Gender				
Male	73	71/2	−.064	.503
Female	38	36/2		
Age				
≤ 65	62	61/1	−.12	.29
> 65	49	46/3		
Smoke history				
Smoker	61	58/3	−.078	.416
Nonsmoker	50	49/1		
Tumor location				
Left lung	46	45/1	−.065	.501
Right lung	65	62/3		
Histological type				
Squamous carcinoma	42	40/2	.048	.613
Adenocarcinoma	69	67/2		
Clinical stage				
I + II	88	85/3	−.02	.832
III + IV	23	22/1		

N, numbers; M, methylation; U, unmethylation.

in this process might have wide-ranging biological consequences and even induce the carcinogenesis.[25] Previous studies on *MLH1* methylation were mainly involved in endometrial cancer,[26] colorectal carcinomas,[27] as well as gastric cancer.[28] As for the disease of respiratory system, the deregulation of *MLH1*-associated pathways through promoter hypermethylation was found to be linked to increased cancer cell migration and tumor invasiveness in laryngeal SCC.[29] Additionally, a previous animal experiment demonstrated that DNA mismatch repair deficiency accelerated the development of lung neoplasm in mice.[30] In the present study, we explored the relationship between

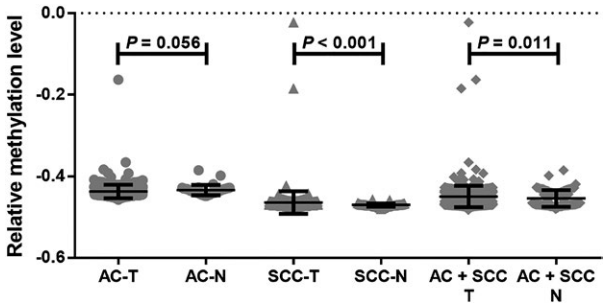

FIGURE 3 Comparisons of mutL homolog 1 methylation levels between non-small cell lung cancer tumor tissues and non-tumor tissues. T, tumor tissues; N, non-tumor tissues; AC, adenocarcinoma; SCC, squamous cell carcinoma. Statistical values and the bar were presented as median with interquartile range

MLH1 methylation and NSCLC in a Chinese cohort in attempt to evaluate it as a diagnostic biomarker for NSCLC.

Our study showed a significant difference of *MLH1* methylation between tumor tissues and adjacent lung tissues. Further bioinformatics analyses confirmed *MLH1* methylation was higher in malignant lung tissues, and found an inverse correlation of *MLH1* methylation with its gene expression. Our study suggested that *MLH1* methylation had a potential diagnosis value for NSCLC. Our ROC test indicated that *MLH1* MSP method is a sensitive but not specific diagnostic method for NSCLC. Previous studies showed a panel of biomarkers for NSCLC.[31-34] Recently, miRNA appeared to be valuable diagnostic candidate biomarkers which were employed for the diagnosis of NSCLC in early stages.[35] A diagnostic test based on miRNA-944 and miRNA-3662 showed 75.7% sensitivity and 82.3% specificity (AUC = 0.898) in distinguishing NSCLC from healthy individuals.[36] In addition, a panel of *CDO1*, HOXA9, *AJAP1*, *PTGDR*, *UNCX*, and *MARCH11* methylation could be potential biomarkers for early detection of NSCLC with high sensitivity and specificity.[37] And frequent simultaneous methylation of *DLEC1*, *ITGA9*, and *MLH1* in more than 50% NSCLC patients indicated the possibility of considering them as a panel of epigenetic markers in NSCLC.[31] Future study might be performed to evaluate aberrant *MLH1* methylation as a supplemental component in the diagnostic panel for NSCLC.

The results of our MSP study and TCGA data analysis about *MLH1* methylation in historic subtypes of NSCLC (AC and SCC) have discrepancy with the literature recordation. Walter et al[38] have demonstrated that members of the DNA-repair pathway, such as *MLH1*, were correlated significantly with lung tumors classification in German. Gomes et al[18] found *MLH1* methylation pattern seemed to vary substantially by histological type, with a higher methylation in SCC in Portuguese. However, Geng et al[39] found no significant differences in *MLH1* methylation between AC and SCC in Chinese population. Coincidentally, in our case-control study in Chinese, *MLH1* methylation difference was not statistical significance between AC and SCC. Additionally, TCGA clinical data showed that *MLH1* methylation was related to SCC risk but not AC risk. Notably, the NSCLC patients in TCGA database were from all over the world. Thus, we speculated that racial disparities may play a role in the mixed results of DNA methylation in NSCLC subtype. Subsequently, more experiments would be repeated in different ethnic groups to define the application range of our biomarker.

Tumor suppressor genes (TSG) methylation in the CpG island has an effect on gene expression silencing.[40] The mRNA expression data showed that *MLH1* mRNA expression was inversely correlated with *MLH1* methylation. Meanwhile, GEO data showed that demethylation with 5AZA treatment caused a trend of increased *MLH1* expression in lung cancer cell lines. Wang et al[41] found that *MLH1* promoter methylation was inversely related to mRNA expression and protein expression in Taiwan population. Also, a meta-analysis found that the decreased protein expression was correlated with *MLH1* promoter hypermethylation.[42] However, *MLH1* methylation did not correlate with *MLH1* expression in Portugal population,[18] Poland population,[31] and Australia population.[16] Hence, population-based differences might exist for the effect of *MLH1* methylation on *MLH1* expression. Further studies in different populations should be performed to verify the findings. In

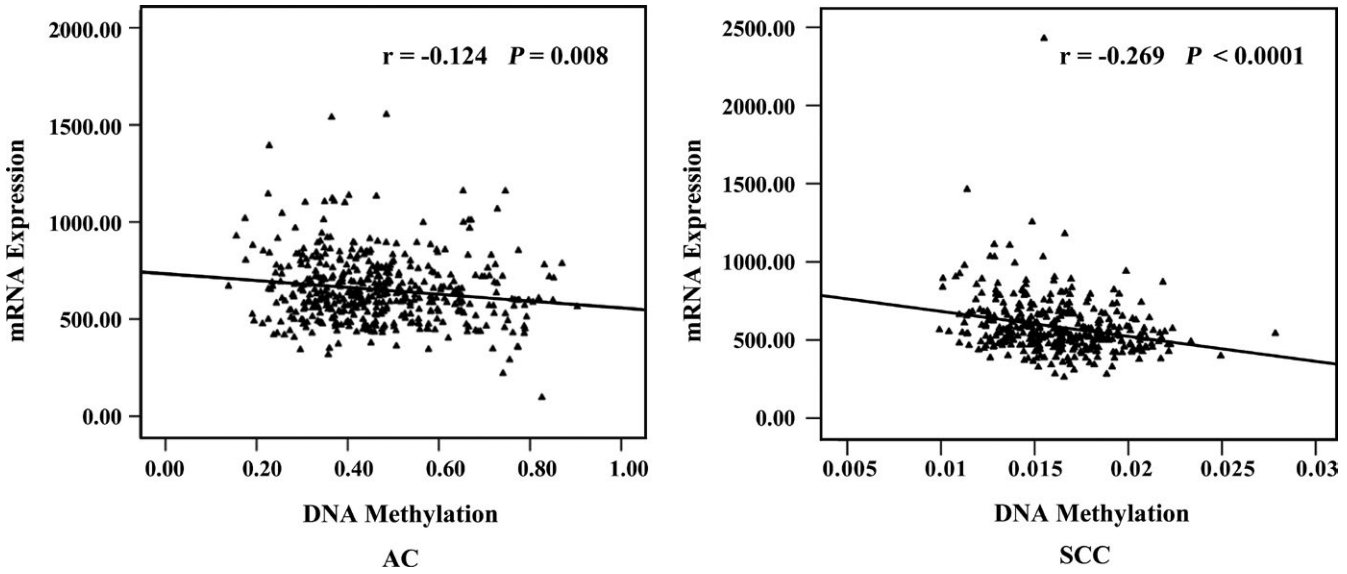

FIGURE 4 Correlation between mutL homolog 1 (*MLH1*) methylation and *MLH1* gene expression in 522 adenocarcinoma and 504 squamous carcinoma from The Cancer Genome Atlas data portal

addition, the methylation evaluation of *MLH1* was based on one region in most studies, which might not stand for the whole gene. According to our analysis of public database (http://mexpress.be/), *MLH1* expression was not always inversely correlated with the methylation levels of all the CpG loci at *MLH1*, especially in lung AC (Table S1). Thus, the diversity in the studied region of *MLH1* gene and the heterogeneity in histological type might also explain the discrepancy between *MLH1* methylation and gene expression among different studies. Besides DNA methylation, other factor (miR-31-5p) could inhibit directly *MLH1* expression in NSCLC cell lines.[43] Thus, the complete mechanism of *MLH1* in NSCLC should be further explored in the future.

There were some limitations in the current study. Due to the insufficient concentration of mRNA and limited tissue samples, we were unable to carry out the correlation analysis of *MLH1* expression with *MLH1* methylation in our patients. Secondly, there was only one positive gene found in the present study, and more candidate genes should be detected and verified in future. Thirdly, limited CpG sites in a certain region of *MLH1* were routinely selected to assess the methylation level which could not represent the methylation level of the whole gene, and it might be the cause of the nonconformity between *MLH1* methylation and *MLH1* expression. Finally, further studies should be performed by quantitative MSP, as MSP is a qualitative approach with a low sensitivity in methylation detection.[42,44]

In conclusion, the MSP method for *MLH1* methylation was a sensitive but not a specific diagnostic method for NSCLC. And further studies should be performed to find more promising diagnostic biomarkers for NSCLC.

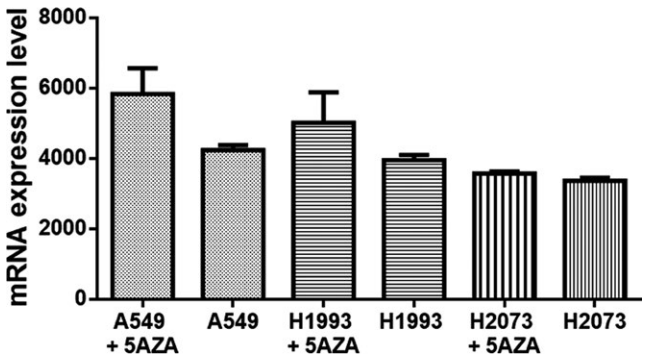

FIGURE 5 mutL homolog 1 expression values with and without 5′-aza-deoxycytidine treatment in lung cancer cell lines (A549, H1993, and H2073) derive from Gene Expression Omnibus database (GSE32496). 5AZA, 5′-aza-deoxycytidine treatment

ACKNOWLEDGMENTS

The research is supported by grants from National Natural Science Foundation of China (81371469), Natural Science Foundation of Zhejiang Province (LY14H160008), Clinical Research Foundation of Zhejiang Province Medical Association (2015ZYC-A77), and K. C. Wong Magna Fund in Ningbo University.

AUTHOR CONTRIBUTIONS STATEMENT

SD and HH contribute to the conception, design, and final approval of the submitted version. BL, CZ, YY, JD, XL, TW, and HH contribute to interpretation of data and completion of figures and tables. JZ, HY, BW, XY, YZ, and YM contribute to performing the experiments and analyzing the data. HH, XC, CZ, and SD contribute to writing the article. All the authors have read and approved the final article.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

ORCID

Shiwei Duan http://orcid.org/0000-0001-7682-2877

REFERENCES

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. *CA Cancer J Clin*. 2016;66:7-30.
2. Didkowska J, Wojciechowska U, Manczuk M, Lobaszewski J. Lung cancer epidemiology: contemporary and future challenges worldwide. *Ann Transl Med*. 2016;4:150.
3. Liu Z, Li W, Lei Z, et al. CpG island methylator phenotype involving chromosome 3p confers an increased risk of non-small cell lung cancer. *J Thorac Oncol*. 2010;5:790-797.
4. Spiro SG, Gould MK, Colice GL; American College of Chest P. Initial evaluation of the patient with lung cancer: symptoms, signs, laboratory tests, and paraneoplastic syndromes: ACCP evidenced-based clinical practice guidelines (2nd edition). *Chest*. 2007;132:149S-160S.
5. Silvestri GA, Gould MK, Margolis ML, et al. Noninvasive staging of non-small cell lung cancer: ACCP evidenced-based clinical practice guidelines (2nd edition). *Chest*. 2007;132:178S-201S.
6. De Wever W, Vankan Y, Stroobants S, Verschakelen J. Detection of extrapulmonary lesions with integrated PET/CT in the staging of lung cancer. *Eur Respir J*. 2007;29:995-1002.
7. Carter D, Vazquez M, Flieder DB, et al. Comparison of pathologic findings of baseline and annual repeat cancers diagnosed on CT screening. *Lung Cancer*. 2007;56:193-199.
8. Nikolaidis G, Raji OY, Markopoulou S, et al. DNA methylation biomarkers offer improved diagnostic efficiency in lung cancer. *Cancer Res*. 2012;72:5692-5701.
9. Sun M, Song J, Zhou Z, et al. Comparison of serum MicroRNA21 and tumor markers in diagnosis of early non-small cell lung cancer. *Dis Markers*. 2016;2016:3823121.
10. Ettinger DS, Akerley W, Borghaei H, et al. Non-small cell lung cancer. *J Natl Compr Canc Netw*. 2012;10:1236-1271.
11. Ansari J, Shackelford RE, El-Osta H. Epigenetics in non-small cell lung cancer: from basics to therapeutics. *Transl Lung Cancer Res*. 2016;5:155-171.
12. Gokul G, Khosla S. DNA methylation and cancer. *Subcell Biochem*. 2013;61:597-625.
13. Fleischhacker M, Dietrich D, Liebenberg V, Field JK, Schmidt B. The role of DNA methylation as biomarkers in the clinical management of lung cancer. *Expert Rev Respir Med*. 2013;7:363-383.
14. Lujan SA, Williams JS, Pursell ZF, et al. Mismatch repair balances leading and lagging strand DNA replication fidelity. *PLoS Genet*. 2012;8:e1003016.
15. Kadyrova LY, Dahal BK, Kadyrov FA. Evidence that the DNA mismatch repair system removes 1-nucleotide Okazaki fragment flaps. *J Biol Chem*. 2015;290:24051-24065.
16. Seng TJ, Currey N, Cooper WA, et al. DLEC1 and MLH1 promoter methylation are associated with poor prognosis in non-small cell lung carcinoma. *Br J Cancer*. 2008;99:375-382.
17. Ali AH, Kondo K, Namura T, et al. Aberrant DNA methylation of some tumor suppressor genes in lung cancers from workers with chromate exposure. *Mol Carcinog*. 2011;50:89-99.
18. Gomes A, Reis-Silva M, Alarcao A, Couceiro P, Sousa V, Carvalho L. Promoter hypermethylation of DNA repair genes MLH1 and MSH2 in adenocarcinomas and squamous cell carcinomas of the lung. *Rev Port Pneumol*. 2014;20:20-30.
19. Wu F, Lu M, Qu L, Li DQ, Hu CH. DNA methylation of hMLH1 correlates with the clinical response to cisplatin after a surgical resection in Non-small cell lung cancer. *Int J Clin Exp Pathol*. 2015;8:5457-5463.
20. Chen C, Wang L, Liao Q, et al. Hypermethylation of EDNRB promoter contributes to the risk of colorectal cancer. *Diagn Pathol*. 2013;8:199.
21. Walter K, Holcomb T, Januario T, et al. Discovery and development of DNA methylation-based biomarkers for lung cancer. *Epigenomics*. 2014;6:59-72.
22. Duppel U, Woenckhaus M, Schulz C, Merk J, Dietmaier W. Quantitative detection of TUSC3 promoter methylation -a potential biomarker for prognosis in lung cancer. *Oncol Lett*. 2016;12:3004-3012.
23. Hulbert A, Jusue-Torres I, Stark A, et al. Early detection of lung cancer using dna promoter hypermethylation in plasma and sputum. *Clin Cancer Res*. 2017;23:1998-2005.
24. Kunkel TA, Erie DA. DNA mismatch repair. *Annu Rev Biochem*. 2005;74:681-710.
25. Landi S, Gemignani F, Canzian F, et al. DNA repair and cell cycle control genes and the risk of young-onset lung cancer. *Cancer Res*. 2006;66:11062-11069.
26. Dowty JG, Win AK, Buchanan DD, et al. Cancer risks for MLH1 and MSH2 mutation carriers. *Hum Mutat*. 2013;34:490-497.
27. Donehower LA, Creighton CJ, Schultz N, et al. MLH1-silenced and non-silenced subgroups of hypermutated colorectal carcinomas have distinct mutational landscapes. *J Pathol*. 2013;229:99-110.
28. Li Y, Yang Y, Lu Y, et al. Predictive value of CHFR and MLH1 methylation in human gastric cancer. *Gastric Cancer*. 2015;18:280-287.
29. Pierini S, Jordanov SH, Mitkova AV, et al. Promoter hypermethylation of CDKN2A, MGMT, MLH1, and DAPK genes in laryngeal squamous cell carcinoma and their associations with clinical profiles of the patients. *Head Neck*. 2014;36:1103-1108.
30. Downey CM, Jirik FR. DNA mismatch repair deficiency accelerates lung neoplasm development in K-ras(LA1/+) mice: a brief report. *Cancer Med*. 2015;4:897-902.
31. Pastuszak-Lewandoska D, Kordiak J, Antczak A, et al. Expression level and methylation status of three tumor suppressor genes, DLEC1, ITGA9 and MLH1, in non-small cell lung cancer. *Med Oncol*. 2016;33:75.
32. Burbee DG, Forgacs E, Zochbauer-Muller S, et al. Epigenetic inactivation of RASSF1A in lung and breast cancers and malignant phenotype suppression. *J Natl Cancer Inst*. 2001;93:691-699.
33. Zochbauer-Muller S, Fong KM, Virmani AK, Geradts J, Gazdar AF, Minna JD. Aberrant promoter methylation of multiple genes in non-small cell lung cancers. *Cancer Res*. 2001;61:249-255.
34. Toyooka S, Suzuki M, Maruyama R, et al. The relationship between aberrant methylation and survival in non-small-cell lung cancers. *Br J Cancer*. 2004;91:771-774.
35. Zandberga E, Kozirovskis V, Abols A, Andrejeva D, Purkalne G, Line A. Cell-free microRNAs as diagnostic, prognostic, and predictive biomarkers for lung cancer. *Genes Chromosom Cancer*. 2013;52:356-369.
36. Powrozek T, Kuznar-Kaminska B, Dziedzic M, et al. The diagnostic role of plasma circulating precursors of miRNA-944 and miRNA-3662 for non-small cell lung cancer detection. *Pathol Res Pract*. 2017;213:1384-1387.
37. Ooki A, Maleki Z, Tsay JJ, et al. A panel of novel detection and prognostic methylated DNA markers in primary non-small cell lung cancer and serum DNA. *Clin Cancer Res*. 2017;23:7141-7152.
38. Walter RF, Mairinger FD, Werner R, et al. Folic-acid metabolism and DNA-repair phenotypes differ between neuroendocrine lung tumors and associate with aggressive subtypes, therapy resistance and outcome. *Oncotarget*. 2016;7:20166-20179.
39. Geng X, Wang F, Zhang L, Zhang WM. Loss of heterozygosity combined with promoter hypermethylation, the main mechanism of human MutL Homolog (hMLH1) gene inactivation in non-small cell lung cancer in a Chinese population. *Tumori*. 2009;95:488-494.
40. Esteller M. Epigenetics in cancer. *N Engl J Med*. 2008;358:1148-1159.

41. Wang YC, Lu YP, Tseng RC, et al. Inactivation of hMLH1 and hMSH2 by promoter methylation in primary non-small cell lung tumors and matched sputum samples. *J Clin Investig*. 2003;111:887-895.
42. Han Y, Shi K, Zhou SJ, Yu DP, Liu ZD. The clinicopathological significance of hMLH1 hypermethylation in non-small-cell lung cancer: a meta-analysis and literature review. *Onco Targets Ther*. 2016;9:5081-5090.
43. Zhong Z, Dong Z, Yang L, Chen X, Gong Z. MicroRNA-31-5p modulates cell cycle by targeting human mutL homolog 1 in human cancer cells. *Tumour Biol*. 2013;34:1959-1965.
44. Malpeli G, Amato E, Dandrea M, et al. Methylation-associated down-regulation of RASSF1A and up-regulation of RASSF1C in pancreatic endocrine tumors. *BMC Cancer*. 2011;11:351.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: Hu H, Chen X, Zhou C, et al. Aberrant methylation of mutL homolog 1 is associated with increased risk of non-small cell lung cancer. *J Clin Lab Anal*. 2018;32:e22370. https://doi.org/10.1002/jcla.22370