Original Article

Enhancing the precision of auxiliary diagnosis for lung cancer through use of *SHOX2* and *RASSF1A* methylation status in lung biopsy and lymph node biopsy specimens

Ping Gu[1#], Yue Wang[1#], Chunlei Shi[1], Yuqing Chen[1], Jialin Qian[1], Yanjie Niu[1], Hai Zhang[1], Yunxia Bao[2], Bin She[3], Xiaosha Ren[3], Iyare Izevbaye[4], Baohui Han[1], Hua Zhong[1], Yu Dong[1]

[1]Department of Respiratory and Critical Care Medicine, Shanghai Chest Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China; [2]Department of Laboratory Medicine, Shanghai Chest Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China; [3]Department of Academic Development, Shanghai Methyldia Technology Co., Ltd., Shanghai, China; [4]Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Grady Health System, Atlanta, GA, USA

Contributions: (I) Conception and design: Y Dong, H Zhong, B Han; (II) Administrative support: H Zhong, B Han; (III) Provision of study materials or patients: P Gu, C Shi, Y Chen, J Qian, Y Niu, H Zhong; (IV) Collection and assembly of data: P Gu, Y Wang; (V) Data analysis and interpretation: P Gu, Y Bao, B She, X Ren; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

[#]These authors contributed equally to this work.

Correspondence to: Yu Dong, PhD; Hua Zhong, PhD; Baohui Han, PhD. Department of Respiratory and Critical Care Medicine, Shanghai Chest Hospital, Shanghai Jiao Tong University School of Medicine, 241 Huaihai West Road, Changning District, Shanghai 200030, China. Email: yudchest@163.com or dryudong@163.com; eddiedong8@hotmail.com; hanxkyy@aliyun.com.

Background: Exfoliated cells in biopsy fluid are commonly utilized for cytological testing to complement histological examination. Nevertheless, the sensitivity of cytological testing is relatively low. Therefore, there is an urgent need to identify more sensitive biomarkers that can substitute for cytology and improve the overall diagnostic accuracy of lung cancer biopsies. The study aims to find biomarkers with greater sensitivity to replace cytology and enhance the overall diagnostic accuracy of lung cancer biopsies by evaluating the complementary roles of cytology and methylation in relation to histology.

Methods: A cohort of 173 patients, including 127 individuals diagnosed with lung cancer and 46 individuals with benign lesions, was successively recruited from Shanghai Chest Hospital. These patients underwent cell smear, ThinPrep cytologic test (TCT), methylation analysis, and histological examination to assess the complementary roles of cytology and methylation in relation to histology could be evaluated.

Results: The cutoff values of *SHOX2* and *RASSF1A*, as determined by the receiver operating characteristic (ROC) curve and scatter plot, were 9 and 12, respectively. The combination of *SHOX2* and *RASSF1A* (LungMe) yielded areas under the curve (AUCs) of 0.967 and 0.999 in lung biopsy and lymph node biopsy liquids, respectively. The sensitivity and specificity of LungMe were 95.3% (121/127) and 97.8% (45/46), respectively. The combination of cytology and histology in the diagnosis of lung cancer did not lead to an increase in diagnostic sensitivity or negative predictive value (NPV), while the combination of LungMe methylation and histology achieved a diagnostic sensitivity and NPV of 100%. The sensitivity of cytology was influenced by the biopsy method, but methylation effectively supplemented cytology in the diagnosis of lung cancer, with a combined diagnostic efficiency of 87.5% to 100%. Furthermore, positive methylation was identified in 87.5% of patients with negative cytology results from lung biopsy and in 100% of patients with negative cytology results in lymph node biopsy. Among samples tested as histologically negative, five cases were observed in which the methylation tests were positive. Among them, four cases were confirmed to be malignant after a second biopsy, surgery, or clinical follow-up, with one case determination pending further evaluation.

Conclusions: The findings suggest that LungMe methylation could replace cytology as an adjunctive diagnostic tool for histology. Moreover, a positive methylation test result could indicate a potential

malignancy, necessitating further confirmation by the hospital and thereby reducing the likelihood of missed diagnoses.

Keywords: *SHOX2*; *RASSF1A*; methylation; biopsy liquid; lung cancer

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Introduction

Lung biopsy, an invasive procedure, represents the final opportunity for diagnosis and thus should be as accurate as possible. The tissue samples obtained from the biopsy, despite being scarce, are used for morphological pathological diagnosis, immunohistochemistry (IHC), and even gene mutation detection. Sensitivity of biopsy can reach as high as 96.0%, but its negative predictive value (NPV) remains unsatisfactory. The additional analysis of biopsy fluid supposed to have the potential for improving the NPV. The exfoliated cells in biopsy fluid are generally used for ThinPrep cytologic test (TCT) or cell smear test, which can supplement histological examination. However, the sensitivity of cytological testing is relatively low, at only approximately 30.8% (1), and the supplementary value of cytology for biopsy samples remains negligible. Consequently, there is a critical need for supplementary diagnostic indicators that offer higher sensitivity to that of cytology and enhance the overall diagnostic accuracy of lung cancer biopsy.

Methylation plays a significant role in tumorigenesis and is extensively considered in the auxiliary diagnosis of various cancers, such as colorectal cancer, gastric cancer, and cervical cancer (2-5). In the context of lung cancer, studies have highlighted the diagnostic potential of methylation markers such as *SHOX2* and *RASSF1A* (6,7). The sensitivity of *SHOX2* and *RASSF1A* methylation exceeds 70%, and the specificity can reach up to 97.4% in bronchoalveolar lavage fluid (BALF) samples, which is considerably higher than that of traditional cytological methods (8,9). For the diagnosis of malignant pleural effusion (MPE), the reported diagnostic sensitivity of *SHOX2* and *RASSF1A* methylation ranges from 66.3% to 76.5%, while the specificity ranges from 90.9% to 96.0% (1,10). In this study, we assessed the feasibility of using the *SHOX2* and *RASSF1A* methylation levels derived from biopsy liquids to diagnose lung cancer. Specifically, we categorized the biopsy specimens into two types: lung biopsy and lymph node biopsy. We further examined various biopsy methods, including transbronchial lung biopsy (TBLB), percutaneous lung biopsy, and lymph node biopsy, comparing the sensitivity of using methylation levels for diagnose across these different methods. Ultimately, we found that fresh exfoliated cells require a substantially shorter reporting time in methylation testing, and provide more stable results. Meanwhile, methylation testing is less influenced by the experience of the testers than cytology, thus constituting a valuable reference for subsequent histological diagnoses. We present this article in accordance with the STARD reporting checklist (available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-2024-1082/rc).

Highlight box

Key findings

- LungMe methylation can serve as an adjunctive diagnostic alternative to cytology for histology. A positive methylation test may imply potential malignancy, prompting further verification and minimizing missed diagnoses.

What is known and what is new?

- The sensitivity of cytological testing is relatively low, and the supplementary value of cytology with biopsy samples is negligible. Moreover, the diagnostic efficiency of *SHOX2* and *RASSF1A* methylation status for the diagnosis of lung cancer in biopsy liquids is unknown.
- *SHOX2* and *RASSF1A* methylation could replace cytology as an adjunctive diagnostic tool for histology.

What is the implication, and what should change now?

- Histology could be effectively complemented by *SHOX2* and *RASSF1A* methylation status, thus rendering cytology obsolete in this regard. Application of methylation status could alert hospitals to possible cases of malignancy and decrease the frequency of missed cases.

Methods

Patient inclusion

A cohort of 173 patients who underwent either lung

biopsy or lymph node biopsy at Shanghai Chest Hospital from February to July 2024 were enrolled in this study, comprising 127 patients with lung cancer and 46 with benign lung diseases. Of the 127 patients with malignancy, 123 patients were found to have cancer cells through histological examination, while 4 patients were diagnosed with positive methylation. After a second biopsy and subsequent follow-up, confirmation was obtained through histology or during later visits. Therefore, in the calculation of the positive rate, the number of positive cases determined by histology was 123. In contrast, the lymph node biopsy group consisted of 78 patients, with 61 diagnosed with malignancy and 17 with a benign condition. This study was approved by the ethics committee of Shanghai Chest Hospital (No. LS1866). All patients provided their written informed consent to participate in this study. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

Cell smear

After biopsy, the exfoliated cells were immediately transferred from the biopsy needle onto a glass slide. In brief, the bevel or tip of the needle gently touched the slide to transfer the cells. To prevent cell degradation and preserve the morphology, the smear was immediately fixed by being allowed to air dry or being sprayed by a fixative. Once fixed and dried, the smear was stained using a suitable cytological stain to enhance the contrast and visibility of the cellular structures.

Biopsy liquid collection

After the biopsy, the biopsy needle was aspirated serially in TCT preservative fluid, into which the biopsy tissue and exfoliated cells were placed. After a period of standing, the tissue was put into a preservative fluid containing formalin for histological examination. The remaining fluid was the biopsy fluid, which was divided into two parts: one part was used for TCT, and the other part was used for methylation analysis. The routine procedure in hospitals is to perform TCT and cytology smears to complement histology.

Histological examination

After fixation with formalin, the tissue was embedded in paraffin to create formalin-fixed paraffin-embedded (FFPE) tissue. Subsequently, the tissue was sectioned into 4-micrometer thin slices using a microtome. The slices were then stained with hematoxylin and eosin (HE) and observed under a microscope.

TCT

The biopsy samples were carefully positioned into containers filled with liquid-based cell preservation liquids to ensure cell viability and preservation of cellular morphology. The specimens were subjected to agitation using a vortex mixer to achieve a uniform dispersion of cells within the liquid. For samples with high mucus content, the duration of agitation was prolonged to ensure complete dispersion of the mucus. Subsequently, the specimen liquid was transferred into a centrifuge tube and subjected to centrifugation at 2,000 RPM for 5 minutes. This step effectively separated the cells from impurities. After centrifugation, the supernatant was carefully removed, with 1.5 to 4.0 milliliters of the cell precipitate remaining. The liquid containing the cell precipitate was spread onto a slide in a thin layer. After smear preparation, the smears were dried and stained, and finally, the cell morphology was observed under a microscope.

Methylation-specific quantitative polymerase chain reaction (PCR)

The methylation and TCT preservation liquids are compatible and rich in alcohol. Therefore, the exfoliated cells from the TCT preservative liquid could be directly used for methylation testing without the need for any intermediate transfer or treatment. The biopsy liquid underwent centrifugation at 10,000 RPM for 5 minutes so that the cellular components were precipitated for DNA extraction. DNA extraction and bisulfite conversion were performed with the Methy-All-In-One Kit (Tellgen Co., Shanghai, China). A quantity of 200 nanograms of DNA per specimen underwent sodium bisulfite conversion, with unmethylated cytosines being selectively transformed to uracil via a DNA purification kit (Tellgen Co.). *SHOX2* and *RASSF1A* methylation levels were then detected using the LungMe real-time PCR kit (Tellgen Co.). The methylation status of the *SHOX2* and *RASSF1A* genes was assessed using 5 μL of the modified DNA, with β-actin serving as an internal control for PCR analysis. The positive control samples included plasmids containing inactive methylated DNA of *SHOX2* and *RASSF1A*. The PCR amplification was carried out using the SLAN-48 S real-time PCR

instrument (Shanghai Hongshi Medical Technology Co., Ltd., Shanghai, China), and the analysis of the results was performed via SDS software (Shanghai Hongshi Medical Technology Co., Ltd.).

Statistical analysis

Statistical analyses were conducted using SPSS 20.0 software (IBM Corp., Armonk, NY, USA) and GraphPad Prism 10 (GraphPad Software, Boston, MA, USA). The prevalence of methylation in the *SHOX2* and *RASSF1A* genes, as well as cytological examination results, was analyzed using the Chi-squared test. The receiver operating characteristic (ROC) curve was constructed to measure the diagnostic performance, with the area under the ROC curve (AUC) providing a comprehensive measure of the test's ability to discriminate between positive and negative outcomes. Statistical significance was set at a P value of less than 0.05, indicating that the results are unlikely to have occurred by chance.

Results

Basic patient information

Among patients with malignancies, a significantly higher number of males compared to females was observed, while no significant difference was observed between males and females with benign conditions. No significant statistical differences were observed in age between the groups, with the average age ranging from 61.1 to 68.2 years. Regarding patient smoking, there were more nonsmokers than smokers in both malignant (61.4% *vs.* 38.6%) and benign (69.6% *vs.* 30.4%) patients. Notably, the majority of patients were diagnosed with lung adenocarcinoma (LUAD) in both the lung biopsy and lymph node biopsy groups, accounting for 43.1% to 62.9% of the cases, respectively. In particular, the proportion of lung squamous cell carcinoma (LUSC) in lung biopsy group was 29.2%, while it was only 11.3% in lymph node biopsy group. Meanwhile, the main pathological subtypes of benign cases were interstitial lung disease, lymphadenosis, granulomatous lesions and inflammation. In patients who underwent lung biopsy, the majority subtype was interstitial lung disease (44.8%), while lymphadenosis was more common in patients underwent lymph node biopsy (47.1%).

Among all samples, in addition to 81.1% of patients who had a biopsy before treatment, 18.9% of patients underwent a second biopsy after treatment (*Table 1*).

DNA concentrations and quality control for methylation detection

Methylation-specific PCR was used to examine the diagnostic efficiency of *SHOX2* and *RASSF1A* methylation in biopsy liquids. The amount of biopsy samples was minimal. To ensure that each sample met the requirements for methylation testing, DNA quantification was initially performed. For the test, the minimum detection quantity for DNA is required to be greater than or equal to 1 ng/μL. In this study, 91.7% of the samples met the concentration requirements (*Figure 1A*).

The volume of reagent added can also be adjusted through DNA quantification to ensure a sufficient amount of DNA. The cycle threshold (Ct) value of internal reference must not exceed 28 to meet the conditions, with the optimal range being 18–23. In this study, 100% of the patients' internal references were within 28, so all samples met the testing requirements. Furthermore, the internal control β-actin in the PCR assays exhibited high stability, with 91.9% of the samples (159 out of 173) having a Ct value within the range of 18–23 (*Figure 1B*). The quantity of biopsy material was limited, yet methylation detection required only a small quantity of the sample to meet the testing requirements, demonstrating high sensitivity.

Determination of SHOX2 and RASSF1A methylation cutoff values

The determination of the optimal cutoff values for *SHOX2* and *RASSF1A* methylation in lung and lymph node biopsy fluids was achieved through the application of ROC curves. In lung biopsy fluid, the areas AUCs for *SHOX2* and *RASSF1A* methylation were calculated to be 0.944 and 0.785, respectively. Additionally, the AUC for diagnosing using combination of *SHOX2* and *RASSF1A* methylation levels (LungMe) is 0.967 (*Figure 2A*). In lymph node biopsy fluid, the AUCs for *SHOX2* and *RASSF1A* methylation were even more pronounced, at 0.982 and 0.792, respectively, with the LungMe assay exhibiting a near-perfect AUC of 0.999 (*Figure 2B*).

For the ROC curve, the cutoff value was identified as the value at which the Youden index was maximized. Thus, the cutoff values for *RASSF1A* methylation in lung biopsy and lymph node biopsy liquids were determined to be 19.0 and 18.92. However, in our previous research (11),

Table 1 Basic patient information

Indicator	Lung biopsy (n=94)		Lymph node biopsy (n=79)		Total (n=173)	
	Malignant (n=65)	Benign (n=29)	Malignant (n=62)	Benign (n=17)	Malignant (n=127)	Benign (n=46)
Gender						
Male	48 (73.8)	12 (41.4)	42 (67.7)	10 (58.8)	90 (70.9)	22 (47.8)
Female	17 (26.2)	17 (58.6)	20 (32.3)	7 (41.2)	37 (29.1)	24 (52.2)
Age (years)	67.8±8.8	62.0±17.7	63.3±9.3	62.2±9.5	65.5±9.4	62.5±11.1
Smoking						
Yes	27 (41.5)	10 (34.5)	22 (35.5)	4 (23.5)	49 (38.6)	14 (30.4)
No	38 (58.5)	19 (65.5)	40 (64.5)	13 (76.5)	78 (61.4)	32 (69.6)
Pathological subtypes						
LUAD	28 (43.1)	–	39 (62.9)	–	67 (52.8)	–
LUSC	19 (29.2)	–	7 (11.3)	–	26 (20.5)	–
SCLC	8 (12.3)	–	10 (16.1)	–	18 (14.2)	–
Interstitial lung disease	–	13 (44.8)	–	0 (0)	–	13 (28.3)
Lymphadenosis	–	4 (13.8)	–	8 (47.1)	–	12 (26.1)
Granulomatous lesions	–	4 (13.8)	–	1 (5.9)	–	5 (10.9)
Inflammation	–	4 (13.8)	–	1 (5.9)	–	5 (10.9)
Others	10 (15.4)	4 (13.8)	6 (9.7)	7 (41.2)	16 (12.6)	11 (23.9)
TNM stage						
I–II	7 (10.8)	–	1 (1.6)	–	8 (6.3)	–
III	19 (29.2)	–	18 (29.0)	–	37 (29.1)	–
IV	39 (60.0)	–	43 (69.4)	–	82 (64.6)	–
Biopsy time						
Before treatment	53 (81.5)	–	50 (80.6)	–	103 (81.1)	–
After treatment	12 (18.5)	–	12 (19.4)	–	24 (18.9)	–

Data are presented as n (%) or mean ± SD. LungMe is a combination of *SHOX2* and *RASSF1A* methylation. LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; SCLC, small-cell lung cancer; TNM, tumor-node-metastasis; SD, standard deviation.

which indicated that a cutoff value above 12 could lead to decreased PCR assay stability, we opted to adjust the cutoff value to 12 for better performance and reliability. To determine the *SHOX2* methylation cutoff, the computed Ct values from the lung biopsy and lymph node biopsy were 8.96 and 9.07, respectively. Thus, we designated the rounded figure of 9 as the final cutoff value for *SHOX2* methylation. The scatter plot of the ΔCt values in *Figure 3* provides a more intuitive representation of the methylation levels of the *SHOX2* and *RASSF1A* genes. In *Figure 3*, lower ΔCt values correspond to higher methylation levels, and it can be observed that the methylation levels of *SHOX2* and *RASSF1A* were higher in the lung cancer group than in the benign group both for both lung biopsy and lymph node biopsy liquids (P<0.001).

Based on the established cutoff values, the sensitivity of *SHOX2* methylation was determined to be 86.4% for lung biopsy liquid and 91.9% for lymph node biopsy liquid; in contrast, the sensitivity of *RASSF1A* methylation was relatively lower, at 54.5% and 54.8% for the two specimen types, respectively. However, *RASSF1A* methylation exhibited complete specificity, with a 100% rate for both specimen types. Meanwhile, the combination of *SHOX2* and *RASSF1A* (LungMe) showed high sensitivity and

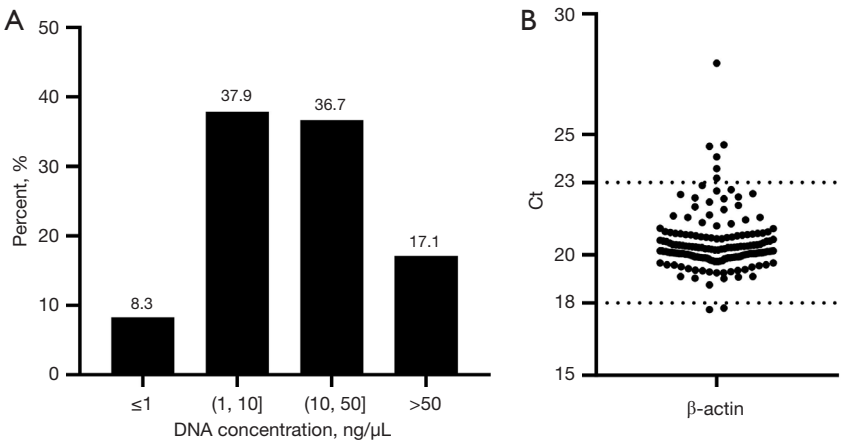

Figure 1 Frequency distribution of sample concentrations and Ct distribution of internal reference for methylation detection. (A) Frequency distribution of sample concentration. (B) Ct distribution of the internal reference for methylation detection. Ct, cycle threshold.

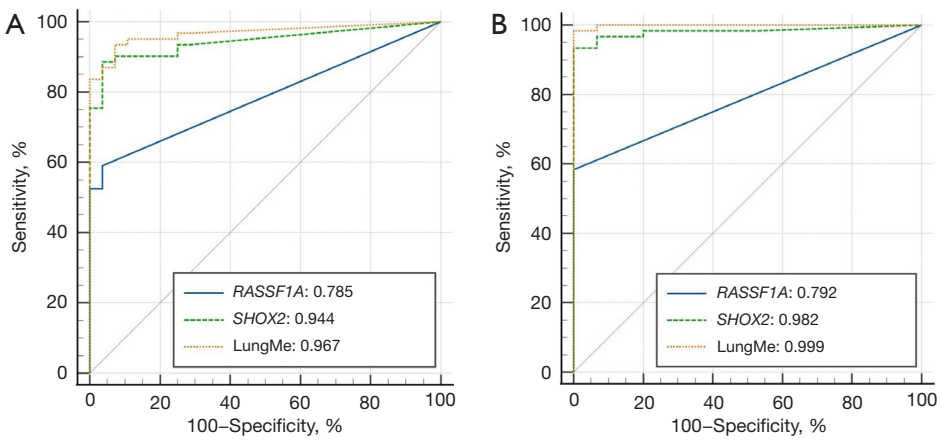

Figure 2 ROC curve of *SHOX2* and *RASSF1A* methylation in diagnosing lung cancer. (A) ROC curve of *SHOX2*, *RASSF1A*, and LungMe methylation from lung biopsy liquid. (B) ROC curve of *SHOX2*, *RASSF1A*, and LungMe methylation from lymph node biopsy liquid. LungMe is a combination of *SHOX2* and *RASSF1A* methylation. ROC, receiver operating characteristic.

specificity, with sensitivities ranging from 93.8% to 96.8% and specificities from 96.6% to 100%, respectively. The sensitivity and specificity of *SHOX2* and *RASSF1A*, as well those of LungMe, did not exhibit statistically significant differences in the comparison of lung biopsy liquid with lymph node biopsy liquid (*Table 2*). These findings underscore the potential of these biomarkers in accurately identifying lung cancer from biopsy liquids.

Comparison between methylation and clinicopathological characteristics

We conducted a comparative assessment of the sensitivity between methylation markers for lung cancer diagnosis. The positive rate of methylation exhibited no significant difference between individuals over 65 years old and those under 65 years old. Our results indicated that the sensitivity of *SHOX2* methylation was higher in male than in female patients, although this difference did not reach statistical significance. Similarly, the methylation sensitivity in smokers was higher than in nonsmokers, but the difference did not achieve statistical significance (P>0.05). The sensitivity of *SHOX2* in diagnosing any pathological subtype was particularly high; however, its sensitivity for diagnosing LUAD was slightly lower. In this context, *RASSF1A* emerged as a valuable complement to *SHOX2* for LUAD diagnosis, enhancing the sensitivity from 85.1% to 92.5%. Furthermore, due to *RASSF1A* being an

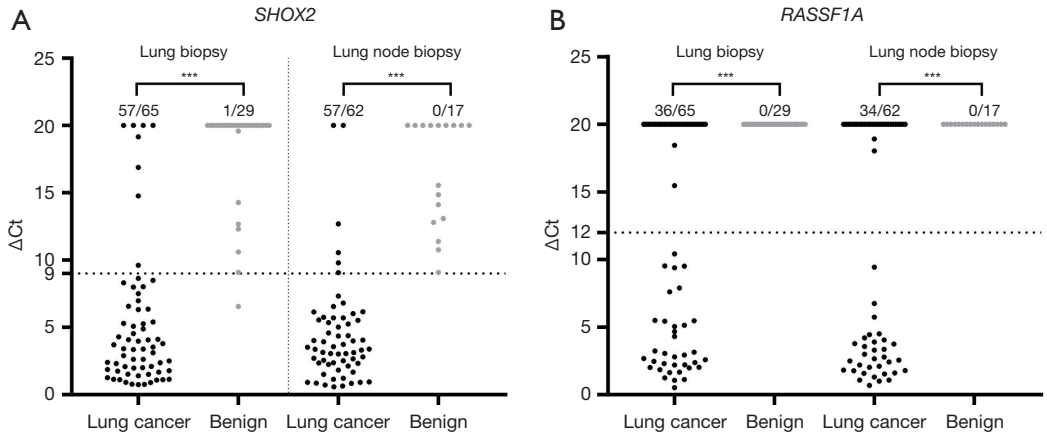

Figure 3 Scatter plot of methylation level from lung biopsy liquid and lymph node biopsy liquid. (A) The methylation levels of *SHOX2* from lung biopsy liquid and lymph node biopsy liquid. (B) The methylation levels of *RASSF1A* from lung biopsy liquid and lymph node biopsy liquid. ***, P<0.001. Ct, cycle threshold.

Table 2 The sensitivity and specificity of *SHOX2* and *RASSF1A* methylation from lung biopsy and lymph node biopsy liquid

Index	Lung biopsy	Lymph node biopsy	P
SHOX2			
Cutoff value	$\triangle Ct_{SHOX2} \leq 9$	$\triangle Ct_{SHOX2} \leq 9$	
Sensitivity	86.4%	91.9%	0.57
Specificity	96.4%	100.0%	0.44
RASSF1A			
Cutoff value	$\triangle Ct_{RASSF1A} \leq 12$	$\triangle Ct_{RASSF1A} \leq 12$	
Sensitivity	54.5%	54.8%	0.80
Specificity	100.0%	100.0%	0.44
LungMe			
Cutoff value	$\triangle Ct_{SHOX2} \leq 9$/ $\triangle Ct_{RASSF1A} \leq 12$	$\triangle Ct_{SHOX2} \leq 9$/ $\triangle Ct_{RASSF1A} \leq 12$	
Sensitivity	92.4%	96.8%	0.68
Specificity	96.4%	100.0%	0.27

LungMe is a combination of *SHOX2* and *RASSF1A* methylation. Ct, cycle threshold.

indicator of strong invasiveness, the sensitivity of *RASSF1A* methylation in diagnosing lung small cell carcinoma (SCLC) was the highest (100%) followed by LUAD (53.7%) and was lowest for LUSC (30.8%). The diagnostic sensitivity of *SHOX2* was consistently high across all stages, with the sensitivity in each stage ranging from 86.2% to 100%. The specificity of *RASSF1A* was 100% and did not increase with higher staging. Interestingly, the sensitivity of LungMe for diagnosing patients before treatment was higher than that for diagnosing those after treatment (P<0.05). For *TP53* mutation, the methylation levels of *SHOX2* and LungMe were significantly higher in patients with *TP53* mutation compared to those without (P<0.05). However, there was no significant difference in *RASSF1A* methylation between the two groups (P>0.05). For *EGFR*, *RB1*, *KRAS* and *ATM* mutations, the methylation levels of *SHOX2*, *RASSF1A* and LungMe were comparable to those without mutation (P>0.05). The methylation analysis of LungMe exhibited the most robust diagnostic capability, achieving a combined positive detection rate of 95.3%. Even in the early stages, the diagnostic sensitivity of methylation was exceptionally high (*Table 3*). Overall, the data suggest that *SHOX2* and *RASSF1A* methylation status from biopsy liquid is a viable tool for diagnosing lung cancer.

Comparison of diagnostic values between methylation and morphological examination

The sensitivity of biopsy histopathology in diagnosing lung cancer reached 96.9%. Despite the high diagnostic sensitivity of biopsy histopathology, missed diagnoses can still occur, necessitating the use of cytological testing with biopsy fluids. However, the sensitivity of routine cytological detection in this study was comparatively low, with the respective sensitivities of cell smear and TCT being 59.8% and 44.1% and the NPV being just 47.4% and 39.3%. When the combination of cytology and histology was applied for diagnosis, neither diagnostic sensitivity nor NPV

Table 3 Clinicopathological characteristics by methylation status

Classification	N	*SHOX2*		*RASSF1A*		LungMe	
		n (%)	P value	n (%)	P value	n (%)	P value
Age			0.64		0.75		0.61
<65 years	51	45 (88.2)		29 (56.9)		48 (94.1)	
≥65 years	76	69 (90.8)		41 (53.9)		73 (96.1)	
Gender			0.15		0.58		0.82
Male	90	83 (92.2)		51 (56.7)		86 (95.6)	
Female	37	31 (83.8)		19 (51.4)		35 (94.6)	
Smoking			0.18		0.43		0.23
Yes	49	46 (93.9)		28 (57.1)		48 (98.0)	
No	78	69 (88.5)		42 (53.8)		74 (94.9)	
Pathologic subtype			0.33		<0.001*		0.34
LUAD	67	57 (85.1)		36 (53.7)		62 (92.5)	
LUSC	26	25 (96.2)		8 (30.8)		26 (100.0)	
SCLC	18	17 (94.4)		18 (100.0)		18 (100.0)	
Others	16	15 (93.8)		8 (50.0)		15 (93.8)	
TNM stage			0.24		0.51		0.18
I–II	8	8 (100.0)		6 (75.0)		8 (100.0)	
III	37	35 (94.6)		21 (56.8)		37 (100.0)	
IV	82	71 (86.6)		43 (52.4)		76 (92.7)	
Biopsy time			0.69		0.92		0.046*
Before treatment	103	93 (90.3)		57 (55.3)		100 (97.1)	
After treatment	24	21 (87.5)		13 (54.2)		21 (87.5)	
TP53 mutation			0.03*		0.34		0.03*
Yes	73	69 (94.5)		43 (58.9)		72 (98.6)	
No	45	36 (80.0)		22 (48.9)		40 (88.9)	
EGFR mutation			0.06		0.69		0.37
Yes	36	29 (80.6)		21 (58.3)		33 (91.7)	
No	82	76 (92.7)		43 (52.4)		79 (96.3)	
RB1 mutation			0.46		0.09		0.69
Yes	22	21 (95.5)		16 (72.7)		21 (95.5)	
No	96	84 (87.5)		49 (51.0)		91 (94.8)	
KRAS mutation			0.65		0.39		0.46
Yes	14	13 (92.9)		6 (42.9)		14 (100.0)	
No	103	91 (88.3)		59 (57.3)		97 (94.2)	
ATM mutation			0.52		0.78		0.54
Yes	14	13 (92.9)		7 (50.0)		13 (92.9)	
No	103	92 (89.3)		57 (55.3)		98 (95.1)	
Total	127	114 (89.8)		70 (55.1)		121 (95.3)	

*, P<0.05, statistically significant. LungMe is a combination of *SHOX2* and *RASSF1A* methylation. LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; SCLC, small-cell lung cancer; TNM, tumor-node-metastasis.

Table 4 Comparison of diagnostic efficacy between methylation and morphological examination

Testing item	Sensitivity, %	PPV (95% CI), %	NPV (95% CI), %
Histology			
FFPE	96.9	100 (96.2, 100)	92.0 (79.9, 97.4)
Cytology			
Cell smear	59.8	100 (94.0, 100)	47.4 (37.3, 57.8)
TCT	44.1	100 (92.0, 100)	39.3 (30.5, 48.8)
Methylation			
LungMe	95.3	99.2 (94.8, 100)	88.2 (75.4, 95.1)
Histology + cytology	96.9	100 (96.2, 100)	92.0 (79.9, 97.4)
Histology+ LungMe	100	99.2 (95.1, 100)	100 (90.2, 100)
Histology + cytology+ LungMe	100	99.2 (95.1, 100)	100 (90.2, 100)

LungMe is a combination of *SHOX2* and *RASSF1A* methylation. PPV, positive predictive value; NPV, negative predictive value; CI, confidence interval; FFPE, formalin-fixed paraffin-embedded; TCT, ThinPrep cytology test.

improved; however, the integration of LungMe methylation with histology yielded a diagnostic sensitivity and NPV of 100%. Furthermore, the diagnostic efficiency and NPV when combined with histology, cytology, and LungMe were not significantly different from those obtained without cytology (*Table 4*). The findings consistently indicated that cytology does not add supplementary value to histological examination and that methylation could replace cytology as a complementary detection method to histology.

Comparison of methylation diagnostic values across different lung biopsy methods

This study examined four different lung biopsy techniques: transbronchial biopsy (TBB), TBLB, endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA), and percutaneous transthoracic lung biopsy (PTLB). The distribution of patients with lung cancer undergoing these procedures was 13.6% for TBB, 31.8% for TBLB, 12.1% for EBUS-TBNA, and 42.4% for PTLB (*Figure 4A*). Following the biopsies, cytological assessments were conducted, including cytology smear and TCT. The sensitivities of the cell smear and TCT across varied biopsy methods ranged from 35.7% to 66.7% for cell smear and 17.9% to 55.6% for TCT. Notably, the results indicated that regardless of the biopsy method used to obtain the specimen, the sensitivity of methylation was relatively high, ranging from 85.7% to 100%. In particular, the specimens obtained through TBB exhibited a methylation sensitivity of 100%. From examining specimens obtained from four types of lung biopsy, we could surmise that methylation provides significant supplementary diagnostic value in the diagnosis of lung cancer for both cytology smear and TCT, with a combined diagnostic efficiency of cell smear, TCT and LungMe methylation ranging from 87.5% to 100% (*Figure 4B*). For all patients undergoing lung biopsy, the positive rate of cytology diagnosis (cell smear and TCT combined) for lung cancer was only 51.5%. However, among patients with a negative cytological diagnosis, 87.5% of patients tested positive for LungMe methylation, representing a significant improvement in the diagnostic efficiency of lung cancer (*Figure 4C*).

Comparison of the diagnostic efficacy of methylation status across different lymph node biopsy methods

We compared two distinct lymph node biopsy techniques: EBUS-TBNA and supraclavicular node biopsy (SCNB). The distribution of patients with lung cancer who underwent lymph node biopsy was 29.0% for EBUS-TBNA and 71.0% for SCNB, as illustrated in *Figure 5A*. The sensitivities of the cytological methods exhibited variation, ranging from 55.6% to 77.3% for cell smears and 27.8% to 61.4% for TCT. Notably, the results indicated that regardless of the biopsy method used to obtain the specimen, the sensitivity of methylation was relatively high, ranging from 95.5% to 100%. In particular, the specimens obtained through TBB exhibited a methylation sensitivity of 100%. Upon

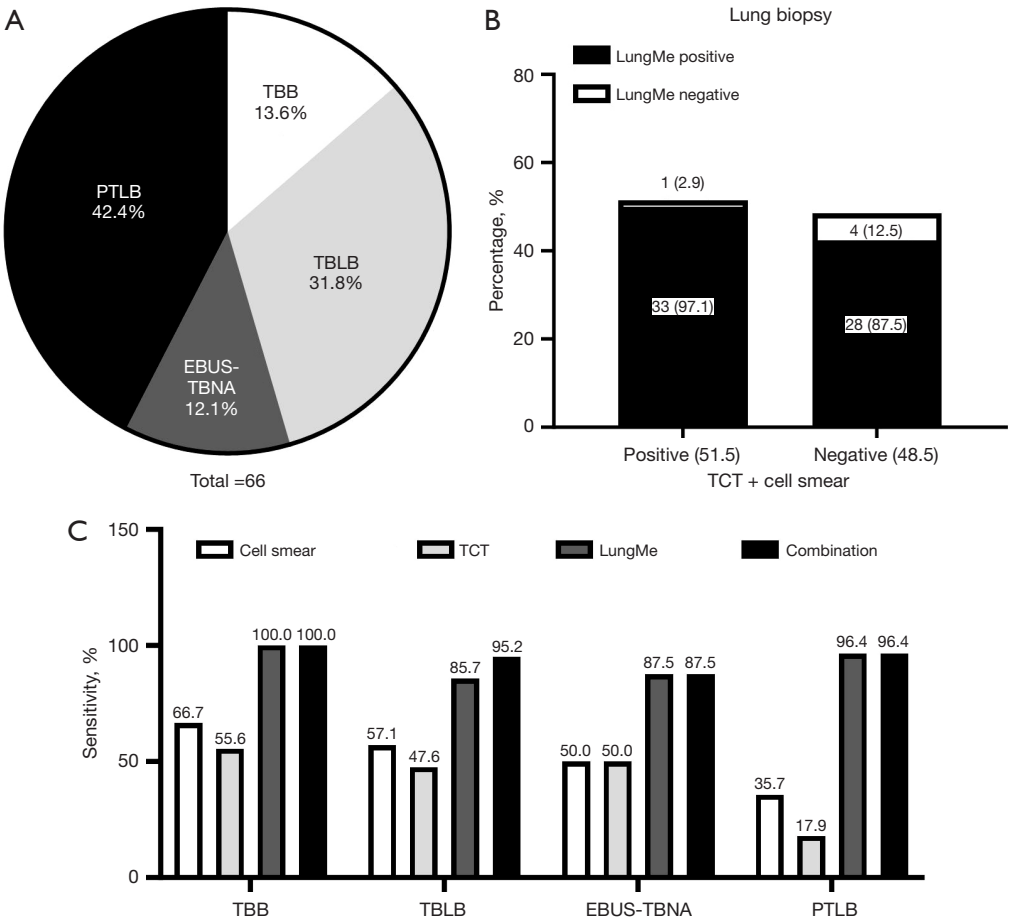

Figure 4 Sensitivity of methylation and cytological diagnosis from lung biopsy liquid. (A) The proportion of different biopsy methods. (B) Sensitivity of methylation and cytological diagnosis across the different biopsy methods. Combination, the combination of cell smear, TCT and LungMe methylation. (C) Value of methylation analysis from lung biopsy liquid in the cytologic diagnosis of lung cancer. LungMe is a combination of *SHOX2* and *RASSF1A* methylation. TBB, transbronchial biopsy; PTLB, percutaneous transthoracic lung biopsy; TBLB, transbronchial lung biopsy; EBUS-TBNA, endobronchial ultrasound-guided transbronchial needle aspiration; TCT, ThinPrep cytologic test.

examination of specimens from both lymph node biopsy types, it was found that methylation significantly enhanced the diagnostic accuracy for both cell smears and TCT, providing a combined diagnostic efficiency ranging from 95.5% to 100%, as shown in *Figure 5B*. Among all patients who underwent lymph node biopsy, the positive cytology diagnosis rate for lung cancer was 71.0%. Among patients with a negative cytological diagnosis, 100% exhibited positive results for LungMe methylation, representing a substantial improvement in the diagnostic efficiency of lung cancer (*Figure 5C*). Biopsy samples are frequently limited, while methylation detection, characterized by high sensitivity, requires minimal sample volumes and thus may be a promising alternative to cytology.

Assessment of SHOX2 and RASSF1A methylation status in biopsy specimens

We first examined two biopsy specimen types: lung biopsy and lymph node biopsy. Second, four examinations were performed for each biopsy specimen: (I) cell smear; (II) TCT test with one-half the biopsy liquid; (III) methylation test with one-half the biopsy liquid; and (IV) histology test. Third, regarding the report time, the cytology required 1 to 2 days, methylation test 2 days, and the histology 5 to 10 days. Fourth, related to the results, of the

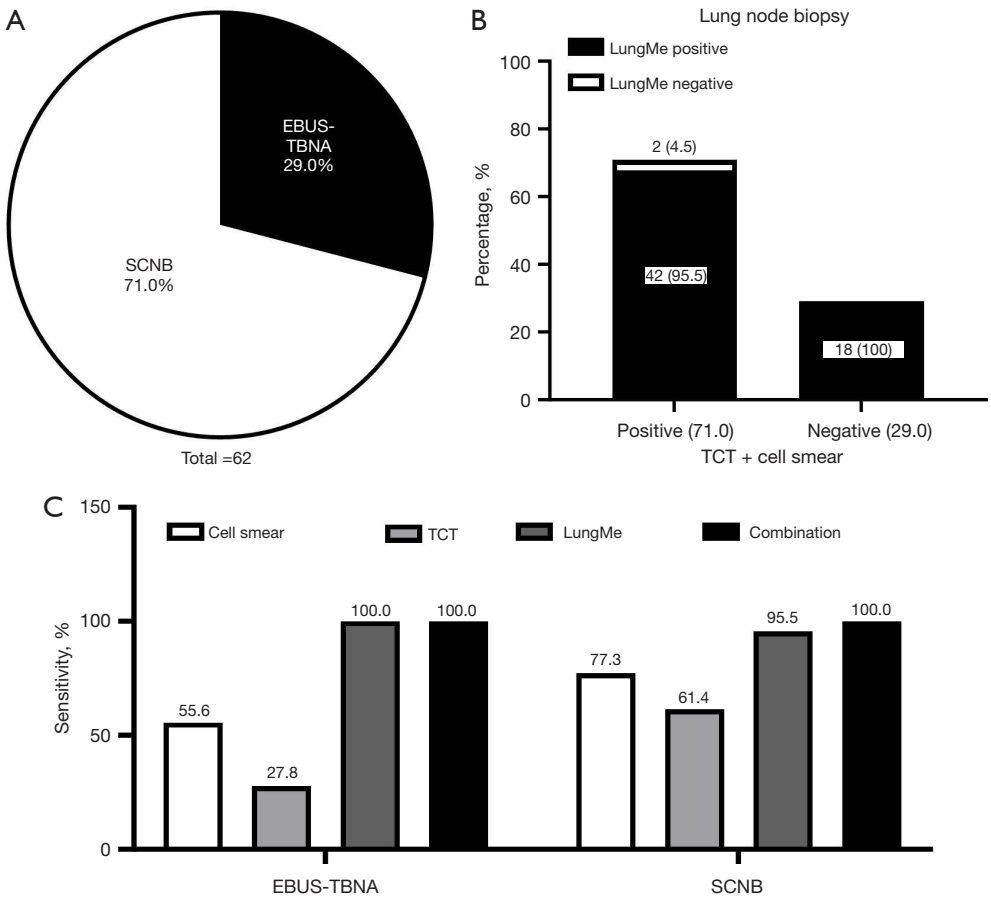

Figure 5 Sensitivity of methylation and cytological diagnosis from lymph node biopsy liquid. (A) The proportion of different biopsy methods. (B) Sensitivity of methylation and cytological diagnosis across different biopsy methods. "Combination" means the combination of cell smear, TCT and LungMe methylation. (C) Value of methylation analysis from lymph node biopsy liquid in the cytologic diagnosis of lung cancer. SCNB, supraclavicular node biopsy; EBUS-TBNA, endobronchial ultrasound-guided transbronchial needle aspiration; TCT, ThinPrep cytologic test.

123 cases diagnosed as malignant by histology, there were 117 positive cases in the methylation tests (95.1%), while the positive rates of cell smear and TCT were 61.8% and 45.5%, respectively. In 50 benign samples tested negative by histology, 5 cases had positive methylation tests. Among them, 4 cases were confirmed as malignant after a second biopsy, surgery, or clinical follow-up, and 1 remained in follow-up and undetermined. Overall, as can be seen from the flow diagram (*Figure 6*), it is clear that cytology did not adequately supplement histology, while methylation demonstrated excellent supplementary and confirmatory value. Moreover, the results from methylation were available earlier and could serve as a reference for the subsequent histology. Methylation yielded a specificity of 97.8% and a sensitivity of 95.3%, which was relatively close to the sensitivity of histology (96.9%) (*Figure 6*).

Discussion

Biopsy remains the gold standard for lung cancer diagnosis but is still subject to missed diagnoses. The positive predictive value (PPV) is 100%, yet the NPV is relatively unsatisfactory. Hospitals have traditionally employed cytological analysis of exfoliated cells postbiopsy to complement histology; however, the outcomes are suboptimal. In this study, a highly sensitive methylation assay was primarily used to improve the NPV.

In this study, the samples were collected continuously, with the patients with lung cancer being predominantly in the middle and late stages, and there was a notably

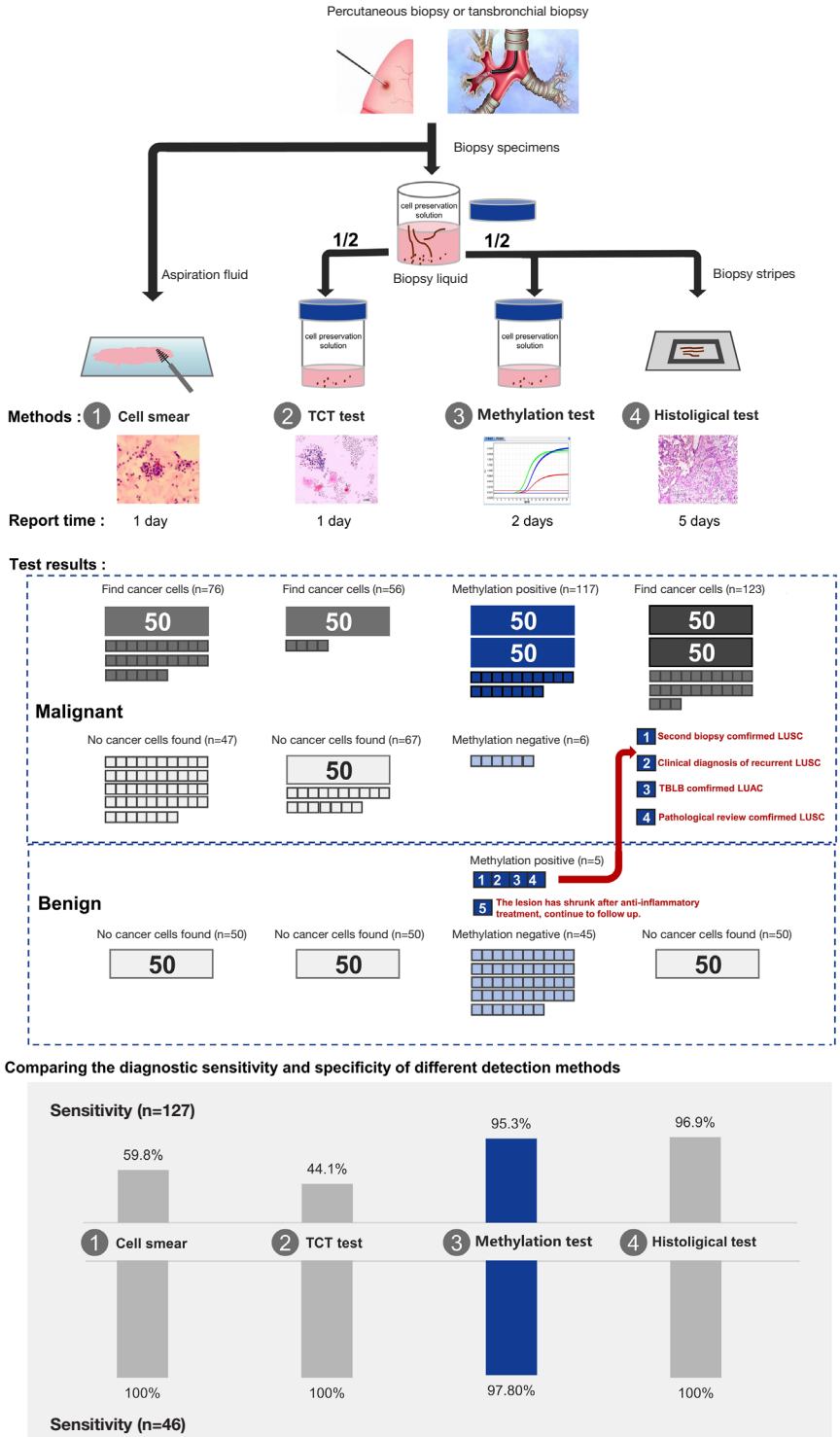

Figure 6 Assessment of *SHOX2* and *RASSF1A* methylation status in biopsy specimens. Cell smear and TCT test were stained with Wright-Giemsa staining, while histological test was stained with HE staining. All the above-mentioned results were observed and interpreted under a microscope at a magnification of 100×. TCT, ThinPrep cytologic test; LUSC, lung squamous cell carcinoma; TBLB, transbronchial lung biopsy; LUAC, lung adenocarcinoma; HE, hematoxylin and eosin.

higher incidence (2.4 times) in males compared to females. Generally, early-stage lung cancer is more prevalent in females than in males; however, the incidence is higher in males during the middle and late stages (12). This discrepancy may stem from distinct etiological factors influencing the types and localization of lung cancer between males and females. Early LUAD identified through low-dose chest computed tomography (LDCT) was predominantly observed in nonsmoking females, indicating that LDCT has limited sensitivity in screening for central lung cancer associated with smokers. LUSC's occurrence is significantly lower in lymph node biopsies compared to lung biopsies, aligning with the hypothesis that LUSC is less aggressive and less prone to lymph node metastasis (13).

Even with the use of exfoliated cells postbiopsy, methylation detection requires smaller sample volume, and there was a sufficient cell count to fulfill the experimental requirements in this study, with optimal quality control in 91.9% of cases. The biopsy sample size in this study was minimal; hence, "nearly-discarded" exfoliated cell samples for methylation detection can economize the use of biopsy material. In addition, using fresh samples for immediate methylation analysis can expedite the diagnostic process and can provide a reference for subsequent tests. Naturally, fresh tissues, as well as paraffin-embedded blocks, are also amenable to methylation analysis.

Lung biopsy and lymph node tissues represent two distinct sample types. The calculation results indicated that the cutoff values of *SHOX2* and *RASSF1A* in this study are identical to those of alveolar lavage fluid (6). The findings indicate that methylation signals predominantly originate from cancer cells, with minimal presence in normal and stromal cells across sample types, underscoring the high tumor specificity of methylation. *SHOX2* exhibited a high sensitivity of 87.7% and 91.9% in lung biopsy and lymph node biopsy liquid, respectively. Despite having a relatively low sensitivity, *RASSF1A* demonstrated a specificity of 100%, aligning with prior findings in MPE (14). *RASSF1A* exhibits low sensitivity, which might be due to its location at 3p21, a region frequently deleted in cases of lung cancer.

The combination of *SHOX2* and *RASSF1A* was implemented for lung cancer diagnosis, with *SHOX2* providing the bulk of sensitivity, which ranged from 85.1% to 100%; meanwhile, *RASSF1A* provided complementary utility, particularly in LUAD. Moreover, *RASSF1A* may be a key biomarker for poor prognosis, high tumor invasiveness, and metastatic potential. We found that the diagnostic sensitivity of *RASSF1A* in diagnosing SCLC was 100%, which correlates with the high invasiveness of SCLC (15,16). In contrast, *RASSF1A* exhibited lower sensitivity (30.8%) in diagnosing LUSC, potentially due to the reduced invasiveness of LUSC (13). General methylation markers exhibit increased positive rates with greater cancer staging, whereas the sensitivity of *RASSF1A* remained relatively constant across the different stages. The overall sensitivity of LungMe in diagnosing lung cancer was exceptionally high, achieving 100% sensitivity for stage I disease. However, the small sample size limits the representativeness of these findings. Additionally, we observed that the posttreatment biopsy positive rate was lower than the pretreatment rate, potentially due to the reduction of the posttreatment tumor mass, fibrotic development, and the presence of cross-reactions, complicating accurate biopsy site targeting (17). However, it is not excluded that some treatments may also affect the methylation status of one or both genes in the LungMe assay, which may also affect the methylation levels. Despite this decrease, the sensitivity of LungMe posttreatment remained at 87.5%, significantly exceeding that of cytology.

In this study, the PPV of histology was determined to be 100%, suggesting a high specificity of histological diagnosis. Meanwhile, the NPV was 92.0%, suggesting potential missed diagnoses. The incorporation of methylation analysis improved the NPV to 100%, whereas cytology did not provide any additional benefit. The combination of negative morphology and methylation results rendered the negative test findings more compelling. Methylation analysis can thus replace the other conventional cytological methods and enable excellent histological diagnosis. Furthermore, positive methylation results may indicate potential malignancy, and further verification is needed, which could reduce missed diagnoses (14).

Additionally, we examined the effects of various biopsy methods on diagnostic sensitivity. For patients who underwent lung biopsy, PTLB and bronchoscopic biopsy were chosen to identify peripheral and central lung cancer according to their anatomical locations, respectively. The results indicated a significant variability in the sensitivity of cytological diagnoses across different biopsy methods, suggesting a correlation between cytological precision and the experience and skill level of the practitioners. Methylation of the same sample demonstrated a high positive rate across the different sampling methods, indicating that the methylation diagnostic method is more

sensitive and slightly influenced by the sampling type. Regardless, methylation analysis had high sensitivity and has the potential to significantly augment diagnostic sensitivity. As a highly sensitive and objective technique, it further can mitigate the variability induced by diverse sampling methodologies and the spectrum of clinicians' proficiency levels. Moreover, among patients with negative cytological diagnoses, 87.5% returned positive for methylation, suggesting that methylation confers a significantly enhanced supplementary diagnostic efficiency to cytology. Additionally, of the patients undergoing lymph node biopsy, 71.0% underwent SCNB. The sensitivity of cytology was improved compared to EBUS-TBNA, although it remained inferior to that of methylation detection.

This study was conducted at a premier specialty hospital, with the highest level of expertise in China, but the sensitivity of cytological analysis from biopsies is still influenced by the biopsy methodology. This suggests that a significant number of hospitals are similarly subject to this effect, underscoring the urgent need for more objective and sensitive methylation indicators.

This study used "nearly-discarded" samples, thereby optimizing the utilization of biopsy specimens for methylation detection. Cells from the TCT preservation solution were directly applied for methylation detection, obviating the need for additional sample collection. Methylation analysis is distinguished by its rapid delivery of results, objectivity, and heightened sensitivity, represents a highly valuable reference for subsequent evaluations. The innovative application of lymph node biopsy fluids for methylation analysis constitutes a novel contribution of our study. However, given the modest sample size employed, the generalizability of our findings may be limited.

Conclusions

This study effectively utilized "nearly-discarded" samples for methylation detection, thereby optimizing the employment of biopsy samples for diagnosis. The application of objective, highly sensitive methylation detection can improve the diagnostic performance associated with diverse sampling methods and the clinicians' proficiency. The integration of methylation analysis improved the NPV to 100%, while cytology offered no supplementary benefit. Methylation analysis may be considered an alternative to cytological diagnosis, complementing histological assessments.

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None.

Footnote

Reporting Checklist: The authors have completed the STARD reporting checklist. Available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-2024-1082/rc

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-2024-1082/coif). B.S. and X.R. are from Shanghai Methyldia Technology Co. Ltd., Shanghai, China. The other authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was approved by the ethics committee of Shanghai Chest Hospital (No. LS1866). All patients provided their written informed consent to participate in this study. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

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