Article

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Combined Methylation of *SHOX2* and *RASSF1A* Genes in Diagnosing Malignant Pleural Effusion

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Background: It is a significant challenge to identify pleural effusion (PE) through differential diagnosis in clinical settings. The present study endeavors to devise a strategy to differentiate between malignant pleural effusion (MPE) and benign pleural effusion (BPE) by detecting gene methylation.

Methods: This study recruited 214 patients with PE, among which 104 patients were identified with MPE, while the remaining 110 patients were categorized as having BPE. The methylation levels of short stature homeobox 2 (*SHOX2*) and RAS association domain family 1, isoform A (*RASSF1A*) genes were analyzed through methylation-specific polymerase chain reaction (MS-PCR).

Results: The methylation status of either *SHOX2* or *RASSF1A* genes was significantly elevated in MPE compared to BPE. The sensitivity and specificity of *SHOX2* and *RASSF1A* methylation in diagnosing PE were 66.3% and 90.9%, respectively. The sensitivity of the combined methylation detection intended to diagnose pulmonary MPE was 73.5% and 52.8% in non-pulmonary MPE ($p < 0.05$), suggesting that combined detection of *SHOX2* and *RASSF1A* methylation had high diagnostic value for lung cancer. In comparison to the results of cytology and DNA ploidy detection, methylation detection demonstrated a superior diagnostic efficiency in the diagnosis of lung cancer ($p < 0.05$). Additionally, the combined detection of *SHOX2* and *RASSF1A* methylation was more potent in diagnosing BPE and MPE ($p < 0.05$), while compensating for the limitations of cytology and DNA ploidy detection.

Conclusions: The detection of *SHOX2* and *RASSF1A* methylation can effectively differentiate between BPE and MPE, especially in diagnosing pulmonary MPE.

Keywords: *SHOX2*; *RASSF1A*; methylation; lung cancer diagnosis; pleural effusion

Introduction

Pleural effusion (PE) is a prevalent clinical manifestation that arises from over 50 distinct pathologies, including infections, malignancies, heart failure, and hypoproteinemia. In the United States, the annual incidence of PE exceeds 1,500,000 cases by estimation [1]. Based on its etiology, PE is categorized into malignant pleural effusion (MPE) and benign pleural effusion (BPE) [2]. MPE is primarily attributed to metastatic breast cancer, malignant pleural mesothelioma, lung cancer, lymphoma, and gastrointestinal tumors, and its emergence often portends a deteriorating prognosis for patients [3]. Tuberculous pleurisy is the predominant etiology of BPE, which is a common form of PE in developing nations [4]. Patients diagnosed with MPE typically culminate with an unfavorable outcome, and the presence of MPE also significantly impacts the assessment of tumor stage and the selection of therapeutic intervention. Conversely, patients diagnosed with BPE generally have a favorable prognosis and achieve clinical remission by taking anti-infective and using closed chest drainage as treatment. Although cytological method or pleural biopsy are the recognized gold-standard diagnostic techniques, it is still a significant challenge to differentiate between MPE and BPE in clinical practice [5].

Lung cancer is leading cause of cancer-related mortality globally and has the highest incidence compared to other malignant tumors [6]. Lung cancer is subdivided into small cell lung cancer and non-small cell lung cancer (NSCLC). The main subtypes of NSCLC are lung squamous cell carcinoma (LSCC), lung adenocarcinoma (LUAD), and large cell lung cancer, which constitute approximately 85% of lung cancer cases.

Various diagnostic methods, such as transthoracic needle biopsies, sputum cytology, bronchoscopic techniques, and surgical biopsy, have been extensively employed for diagnosing bronchogenic carcinoma [7,8]. However, the result of conventional morphology-based diagnostic method, which involves cytological and histological examination, may be adversely impacted by the quality of the specimen and the pathologist's diagnostic expertise [9]. Fortunately, the emergence of molecular diagnostic techniques, which are characterized by higher sensitivity and objectivity, can circumvent the inherent limitations of morphological di-

Table 1. Clinic characteristics of patients.

Type of pleural effusion	Classification	Number	Age (Year)		Gender	
			Range	Median	Male (%)	Female (%)
MPE	Lung cancer	68	28–88	63.97	45	23
	Lymphoma	8	6–82	52.13	4	4
	Breast cancer	6	46–88	63.5	0	6
	Gastroenteric carcinomas	10	38–83	60.9	6	4
	Hepatobiliary pancreatic cancer	6	51–71	62.5	6	0
	Other malignancies	6	36–73	61.5	4	2
BPE	Pulmonary pleural edema	49	45–91	67.8	40	9
	Tuberculous pleural effusion	21	17–79	51.2	16	5
	Cardiogenic pleural effusion	13	50–89	69.2	10	3
	Other pleural effusion	27	9–89	67.8	18	9

BPE, benign pleural effusion; MPE, malignant pleural effusion.

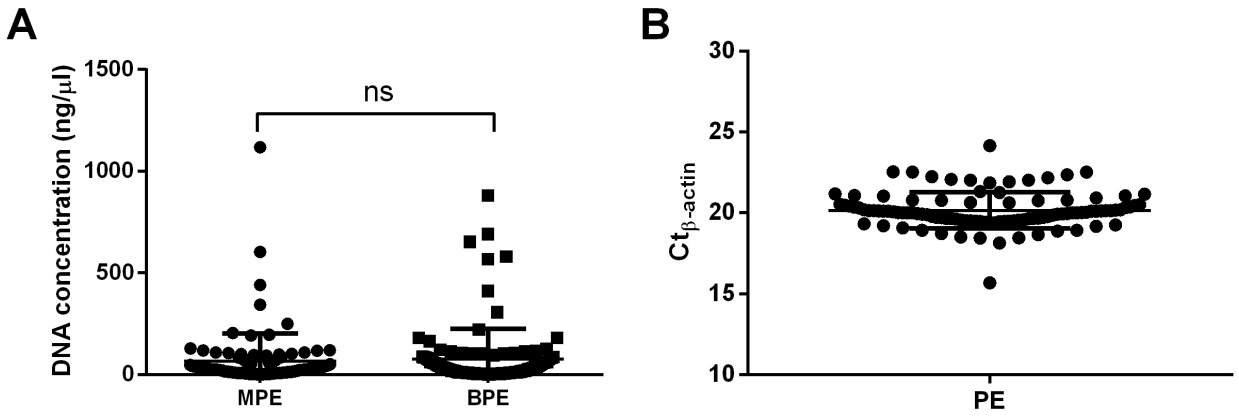

Fig. 1. The DNA concentration of pleural effusion samples. (A) The distribution of DNA concentration of pleural effusion samples. (B) The distribution of Ct values of β-actin (*ACTB*) gene. PE, pleural effusion. ns, no significance; Ct, cycle threshold.

agnosis. Among various biomarkers, alterations in DNA methylation turn out to be a particularly promising candidate [10]. The mediation of epigenetic changes and gene expression, as well as the preservation of cellular identity, are significantly influenced by DNA methylation, which is frequently observed in tumorigenesis [11]. Specifically, previous studies have demonstrated a wide range of DNA methylation abnormalities in lung cancer, including the hypermethylation of promoter CpG islands, which can suppress tumor suppressor genes and consequently influence the progression of carcinogenesis [12,13].

The short stature homeobox 2 (*SHOX2*) gene in humans is situated in the pseudoautosomal region of the X chromosome, while the *SHOX2* homolog is located on chromosome 3. Together, *SHOX* and *SHOX2* constitute the short stature homeobox gene family in humans. RAS association domain family 1, isoform A (*RASSF1A*), a well-established tumor suppressor, is among the most frequently dysregulated genes in human cancers [14]. *RASSF1A* belongs to the C-terminal *RASSF* family as a scaffold protein and is encoded by the *RASSF1* gene [15]. The evidence indicates that the anomalous hypermethylation of the promoter CpG islands of *SHOX2* and *RASSF1A* could serve as a biomarker for the diagnosis and prognosis of lung cancer, breast cancer and colorectal cancer [16–18]. Our results suggested that *SHOX2* and *RASSF1A* may have distinct functions in the progression of cancer. The detection of *SHOX2* and *RASSF1A* methylation significantly enhanced the sensitivity of the diagnostic process, thereby offering a more precise and efficient approach to differential diagnosis.

Methods

Patients and Clinical Specimens

The current study was registered with the National Machinery Registration Standard (20173403354). Patients with pleural effusion who were treated at Zhujiang Hospital, Guangzhou, China, from June 2021 and September 2022 were carefully chosen for this study. The *SHOX2* and *RASSF1A* methylation levels were detected in 104 MPE and 110 BPE samples, and conventional cytopathological tests were also performed in these patients. The inclusion criteria of this study are as follows: (i) patients with complete clinical data; and (ii) patients who provided written informed consent. The exclusion criteria of this study are as follows:

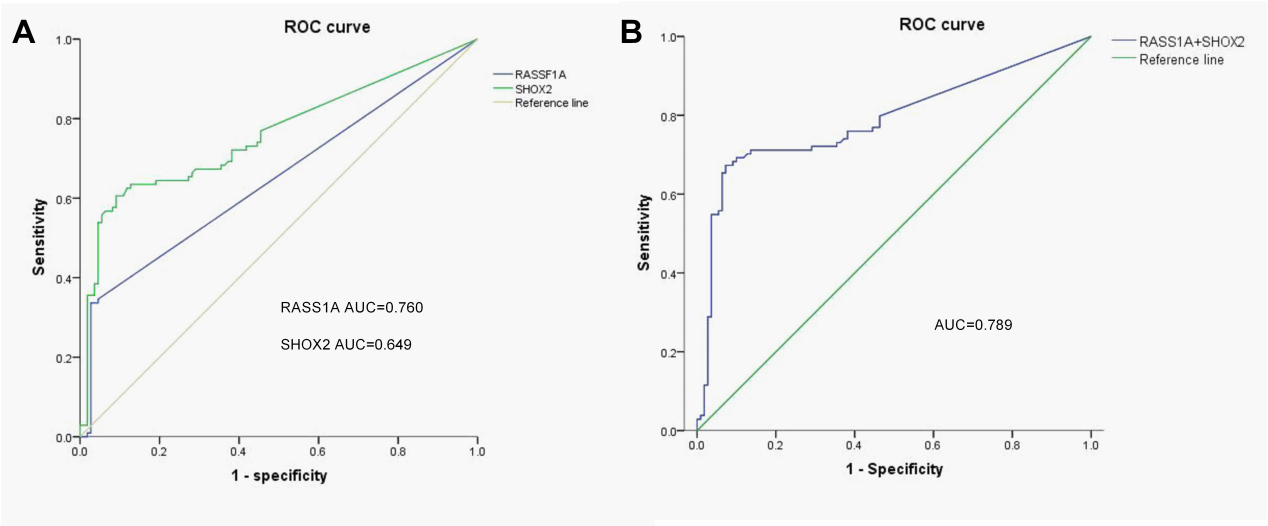

Fig. 2. Diagnostic performance of the DNA methylation test for differentiating between benign pleural effusion (BPE) and malignant pleural effusion (MPE). (A) The receiver operating characteristic (ROC) curves of RAS association domain family 1, isoform A (*RASSF1A*) and short stature homeobox 2 (*SHOX2*) methylation. (B) The ROC curve of combination of *RASSF1A* and *SHOX2* methylation.

(i) patients with other comorbidities, such as hemothorax and empyema; (ii) patients with cognitive impairment and psychiatric disorders; and (iii) patients with systemic infectious diseases. The relevant characteristics of the patients are concisely presented in Table 1. Among the cases examined, 104 cases were identified as MPE and diagnosed as cancer, with 68 cases of lung cancer, 8 cases of lymphoma, 3 cases of colorectal cancer, 4 cases of liver cancer, 7 cases of gastric cancer, 6 cases of breast cancer, and 8 cases of other malignant carcinoma. The other 110 cases were exclusively BPE, encompassing 49 cases of pneumonia, 21 cases of tuberculous, and 40 cases of other benign diseases (Table 1). This study was approved by the Ethics Committee of Zhujiang Hospital (22-031) and all the participants provided written informed consent. The study was conducted in accordance with the Declaration of Helsinki.

Collection and Processing of Pleural Effusion Specimens

Pleural effusion specimens were obtained by clinical physicians who conducted sterile punctures on different regions containing accumulated fluid as required through pleural puncture and chest drainage. After being fixed in 20 mL of cell prevention solution (21A016, Tellgen, Shanghai, China), the pleural effusion specimens were then centrifuged for 10 min at 4000 $\times g$. After discard the supernatant, 1 mL of cell prevention solution was added to dissolve the pellets. The pleural effusion was then stored at room temperature for no more than two weeks.

Cytological and Pathological Analyses

The identification of pathological alterations in pleural biopsy tissue or the cytological analysis of malignant tumors is widely regarded as the definitive method for diagnosing MPE. The supernatant was carefully removed from the tube without dislodging the pellet. Next, the pellet was subjected to liquid-based cytology processing, through which the pellet was made into paraffin blocks. The histopathological examination was performed using hematoxylin and eosin (H&E) staining. All pathologic examinations were conducted by two experienced pathologists.

DNA Extraction, Bisulfite Treatment and Methylation Analysis

Ten milliliters of pleural effusion were used to extract DNA. DNA bisulfite conversion was performed using Methy-All-In-One Kit (22A008, Tellgen, Shanghai, China). The concentration of the extracted DNA was determined using highly sensitive fluorescent dye assays (Hangzhou Allsheng Instruments Co., Ltd., Hangzhou, China). The sodium bisulfite was added to 200 ng of each DNA sample. After purification, methylation-specific polymerase chain reaction (MS-PCR) was performed to amplify the bisulfite-converted DNA. The channels for the amplification of *SHOX2*, *RASSF1A*, and β-actin (*ACTB*) were Vector Incoherent Channe (VIC), carboxyl fluorescein (FAM), and Cyanine 5 (CY5), respectively. The plasmids utilized as positive controls in this study contained methylated DNA sequences of *SHOX2* and *RASSF1A*, which were devoid of any bioactivity. The PCR amplification of methylated *SHOX2*, *RASSF1A*, and *ACTB* genes was conducted

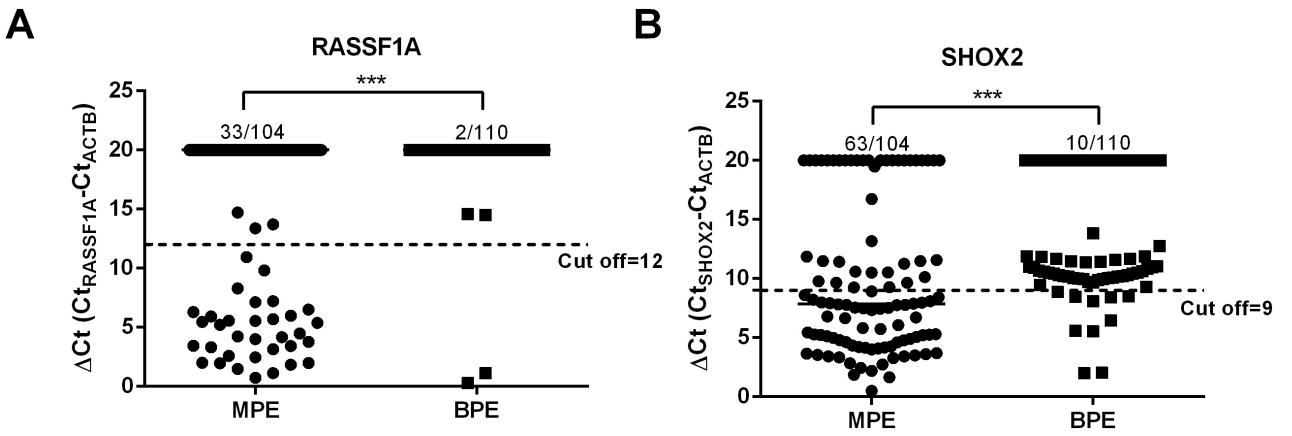

Fig. 3. Methylation frequency and association with clinicopathologic characteristics. (A) The methylation level of *RASSF1A* in the tested specimens. (B) The methylation level of *SHOX2* in the tested specimens. △Ct, delta cycle threshold. ***$p < 0.001$.

using the ABI 7500 Real-Time PCR instrument (4351106, Applied Biosystems, CA, USA). The samples were deemed eligible for analysis when $18 \leq Ct_{ACTB} \leq 30$. The quantification of methylation levels for each gene was determined using the equations as follows:

$$\triangle Ct_{SHOX2} = Ct_{SHOX2} - Ct_{ACTB}$$
$$\triangle Ct_{RASSF1A} = Ct_{RASSF1A} - Ct_{ACTB}$$
$$\triangle Ct, \text{ delta cycle threshold}$$

Statistical Analysis

SPSS 20.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 6.0 (GraphPad, San Diego, CA, USA) were for data analysis. Chi-square test was applied to analyze the levels of methylation in *SHOX2* and *RASSF1A* genes. To assess diagnostic efficacy, the area under the receiver operating characteristic (ROC) curve (AUC) was calculated. Statistical significance was determined by a two-sided p value < 0.05.

Results

DNA Concentration of Pleural Effusion Samples

An MS-PCR assay was performed on 104 MPE and 110 BPE samples to validate whether *SHOX2* and *RASSF1A* genes are cancer-specific. The concentration of DNA in pleural effusion range from 0.14 ng/μL to 880 ng/μL. Fig. 1A illustrates the distribution of DNA concentrations of pleural effusion samples; no significant difference between MPE and BPE in this regard was detected. For each PCR amplification, 50 ng of DNA was used, after the DNA concentration of the samples was determined. The Ct values of the amplified *ACTB* gene, which acts as the internal control in PCR, were remarkably consistent, with very minimal value fluctuation (Fig. 1B). All 104 MPE and 114 BPE specimens yielded a valid measurement.

Diagnostic Performance of Combined Detection of SHOX2 and RASSF1A Methylation Levels for Differentiating between BPE and MPE

An ROC analysis was conducted to calculate the AUC and ascertain the corresponding cutoff value. As shown in Fig. 2. The AUCs of the methylation of *RASSF1A*, *SHOX2* and the combination of *SHOX2* and *RASSF1A* were 0.649, 0.760, and 0.789, respectively. These results indicate the combined detection of *SHOX2* and *RASSF1A* methylation levels has a better ability in differentiating between BPE and MPE.

Methylation Frequency and Association with Clinicopathologic Characteristics

MS-PCR was carried out to evaluate the diagnostic value of *SHOX2* and *RASSF1A* methylation. The delta cycle threshold (△Ct) was measured based on the Ct_{SHOX2} <32 and $Ct_{RASSF1A}$ <35 criteria, with a value of 40 assigned for "NoCt" [19]. The *SHOX2* and *RASSF1A* methylation levels ($\triangle Ct_{SHOX2}$ and $\triangle Ct_{RASSF1A}$) are presented in Fig. 3. The *SHOX2* and *RASSF1A* methylation levels in MPE were significantly higher compared to those in BMP.

To achieve higher sensitivity and specificity, the cutoff values of △Ct for *RASSF1A* and *SHOX2* methylation were set to 12 and 9, respectively. Based on the *SHOX2* and *RASSF1A* methylation cutoff values, the sensitivity and specificity of the combined detection of *SHOX2* and *RASSF1A* methylation level in differentiating between MPE and BPE were 66.3% and 90.9% (Table 2). Moreover, the sensitivity and specificity of methylation detection were also determined for differentiating between pulmonary MPE and non-pulmonary MPE. The sensitivities of methylation detection in diagnosing lung cancer and non-pulmonary MPE were 73.5% and 52.8%, respectively (Table 3). The findings indicate that the determination of the methylation levels of *SHOX2* and *RASSF1A* holds promise as a diagnostic tool for distinguishing between BPE and MPE.

Table 2. Sensitivity and specificity of cytology, DNA ploidy, and methylation detections in diagnosing MPE.

Parameter	N	Cytology	DNA ploidy	Methylation	Cytology + DNA ploidy + Methylation
Sensitivity	104	32 (30.8%)	45 (43.3%)	69 (66.3%)	78 (75.0%)
Specificity	110	110 (100.0%)	109 (99.1%)	100 (90.9%)	100 (90.9%)

MPE, malignant pleural effusion.

Table 3. Sensitivity of cytology, DNA ploidy, and methylation detections in diagnosing pulmonary MPE and non-pulmonary MPE.

Parameter	N	Cytology	DNA ploidy	Methylation	Cytology + DNA ploidy + Methylation
Pulmonary MPE	68	18 (26.5%)	31 (45.6%)	50 (73.5%)	55 (80.9%)
Non-pulmonary MPE	36	14 (38.9%)	14 (38.9%)	19 (52.8%)	23 (63.9%)

Table 4. Sensitivity of cytology, DNA ploidy and methylation detections in PE for the diagnosis of different types of cancer.

Pathological types	N	Cytology	DNA ploidy	Methylation	Cytology + DNA ploidy + Methylation
LSCC	9	1 (11.1%)	0 (00.0%)	3 (33.3%)	3 (33.3%)
LUAD	51	16 (31.4%)	30 (58.8%)	40 (78.4%)	45 (88.2%)
Other lung cancers	8	1 (11.1%)	1 (11.1%)	7 (87.5%)	7 (87.5%)
Lymphoma	8	3 (37.5%)	3 (37.5%)	3 (37.5%)	5 (62.5%)
BC	6	2 (33.3%)	1 (16.7%)	4 (66.7%)	4 (66.7%)
GCA	10	7 (70.0%)	7 (70.0%)	6 (60.0%)	8 (80.0%)
Other cancers	12	2 (16.7%)	3 (25.0%)	6 (50%)	6 (50%)

LSCC, lung squamous cell carcinoma; LUAD, lung adenocarcinoma; BC, breast cancer; GCA, gastrointestinal cancer.

Sensitivity of Methylation Detection in Diagnosing Different Cancers

Additional testing was performed to determine if there was an association between the levels of DNA methylation and the clinicopathologic features. As shown in Table 4 most of the MPE cases were attributed to lung cancer, encompassing NSCLC (*e.g.*, LSCC, LUAD) and undifferentiated carcinoma, and the sensitivity of *SHOX2* and *RASSF1A* methylation detection in distinguishing between different cancers was 73.5%. In LUAD and LSCC, the positive detection rates of *SHOX2* and *RASSF1A* methylation were 78.4% and 33.3%, respectively. Other MPE-related cancers, such as lymphoma, breast cancer and gastrointestinal cancer, had positive detection rates of *SHOX2* and *RASSF1A* methylation at 37.5%, 66.7% and 60.0%, respectively (Table 4).

Comparison of DNA Methylation Detection with Conventional Analysis

Next, we compared the diagnostic effectiveness between the combined detection of *SHOX2* and *RASSF1A* methylation and the conventional methods. The sensitivities of cytological and DNA ploidy detections were 30.8% and 43.3%, and their respective specificities were 100% and 99.1%. In addition, the combined sensitivity and specificity of cytology, DNA ploidy and methylation detections were 75.0% and 90.9%, respectively. Moreover, the sensitivities of cytology, DNA ploidy and methylation detections were also determined for pulmonary MPE and non-pulmonary MPE. As a result, the combined sensitivities of cytology, DNA ploidy and methylation detections for pulmonary MPE and non-pulmonary MPE were 80.9% and 63.9%, respectively (Table 2). The findings revealed that the sensitivity of methylation detection was higher than that of cytology and DNA ploidy detections in differentiating MPE and BPE, while the sensitivity of combined methylation, cytology and DNA ploidy detection in diagnosing MPE is the highest.

The combined sensitivity of cytology, DNA ploidy and methylation detections in diagnosing LUAD and LSCC were 88.2% and 33.3%, respectively. Aside from that, the combined sensitivity of cytology, DNA ploidy and methylation detections in diagnosing other lung cancers was 87.5%. On the other hand, the combined sensitivity of cytology, DNA ploidy and methylation detections in diagnosing lymphoma, breast cancer and gastrointestinal cancer were 66.7%, 80.0% and 50.0%, respectively (Table 4). The combination panel consisting of cytology, DNA ploidy and methylation detections approaches demonstrated the highest diagnostic efficacy. All the results demonstrated that the sensitivity of DNA methylation detection was higher than that of cytology and DNA ploidy detections in diagnosing lung cancer, especially LUAD.

Discussion

MPE is a prevalent manifestation of cancer-related pleural lesions, such as metastatic lung cancer and breast cancer. Its presence often portends a higher tumor grade

and precludes the possibility of primary tumor resection as treatment, indicating poor outcomes. Conversely, patients with BPE can be cured if treated promptly. Therefore, it is crucial to accurately distinguish between MPE and BPE and enhance the prognosis. To date, a specific method for differential diagnosis that possesses high sensitivity and specificity is not available in clinical practice. Thoracoscopy, which is considered a gold-standard diagnostic method, can diagnose more than 95% of malignant pleural lesions. However, its application is limited because this technique demands high expertise of surgical staff and advanced equipment, and the individuals who have been examined with thoracoscopy are susceptible to complications [20]. Cytological analysis, while capable of identifying malignancy in most patients, is prone to causing misdiagnosis [21]. Emerging molecular detection technologies present enhanced sensitivity in diagnosis and yield more objective test results, thereby effectively addressing the limitations of morphological diagnosis.

PE-DNA can be potentially used as the samples for diagnosing thoracic malignancies, but studies on corroborating its utility in this aspect remain scarce. However, in the realm of tumor-targeted therapy, PE-DNA is considered high-quality sample for molecular detection, because it is readily available and the quantity of shed cells does not impact the result of molecular detection [22]. The correlation between aberrant DNA methylation and malignant characteristics of a range of tumors has previously been established [23]. Several genes, including *SHOX2* and *RASSF1A*, have been found to be abnormally methylated, making them promising biomarkers for cancer diagnosis [24,25]. The application of *SHOX2* and *RASSF1A* methylation level detection in cancer diagnosis has been investigated in the case of bronchoalveolar lavage fluid. Detection of *SHOX2* and *RASSF1A* methylation level in bronchoalveolar lavage fluid was found to have high diagnostic sensitivity and specificity in cancer detection [26,27]. In this study, 104 patients with MPE and 110 patients with BPE were examined for promoter methylation of *RASSF1A* and *SHOX2*. The findings revealed significantly elevated levels of methylation in the MPE group compared to the BPE group, consistent with the established association between hypermethylation and malignancy. ROC curves were used to evaluate the ability of *SHOX2* and *RASSF1A* methylation to diagnose PE. The AUCs for *SHOX2*, *RASSF1A*, as well as combination of *SHOX2* and *RASSF1A* methylation detections were 0.760, 0.649, and 0.789, respectively. These results suggest that either *SHOX2* or *RASSF1A* methylation detection exhibits a pronounced diagnostic capacity in discriminating MPE from BPE; instead, the combined detection approach demonstrates the best performance in this regard. According to the established cutoff values, our analysis revealed that the positive detection rates of *SHOX2* and *RASSF1A* methylation were significantly higher in MPE group than in BPE group. Besides, the positive detection rate of both *SHOX2* and *RASSF1A* methylation was 66.3%, accompanied by a remarkably high specificity of 90.9%, exhibiting the superior diagnostic efficacy of the combined detection approach in comparison to that of the established cytological examination and DNA ploidy detection. The sensitivity of the combined detection of *SHOX2* and *RASSF1A* methylation in diagnosing MPE originating from lung cancer, which is the primary etiology, is 73.5%.

Furthermore, the current study also encompassed illustrating the distribution of *SHOX2* and *RASSF1A* methylation levels across various subtypes of lung cancer. According to the findings, the positive detection rates of *SHOX2* methylation in small cell lung cancer (SCLC) and LSCC ranged from 80% to 100%, and from 63% to 96.1%, respectively. Adenocarcinomas, on the other hand, demonstrated the lowest positive detection rate from 39% to 82.9% [28]. *RASSF1A* and *SHOX2* methylation was more prevalent in LUAD as compared to LSCC [29]. In our cohort, LUAD was the leading cause of pulmonary MPE, and the positive detection rates of *RASSF1A* and *SHOX2* methylation for diagnosing LUAD were the highest in the MPE group. *SHOX2* and *RASSF1A* methylation levels across various cancers were also measured. For other MPE patients, the positive detection rate of methylation was lower than that in lung cancer patients. In summary, the findings indicate the possibility of utilizing methylation detection in various cancer-derived pleural effusion samples on a broad scale. Compared to conventional assays, the utilization of *SHOX2* and *RASSF1A* methylation analysis demonstrated superior diagnostic efficacy. As a non-invasive detection method, methylation analysis of the *SHOX2* and *RASSF1A* can be employed in most patients with PE and can help determine PE characteristics.

The present study was limited by the small sample size. Future studies involving larger patient cohorts are necessary to corroborate the findings presented in this study. This study only investigated the efficacy of DNA methylation detection in diagnosing MPE, and did not investigate the efficacy of methylation in different tissue types for the same purpose. Additionally, the correlation between methylation levels of the two genes and patient prognosis remains unclear.

Conclusions

Analysis of *SHOX2* and *RASSF1A* methylation demonstrates high diagnostic capability in distinguishing MPE from BPE and has the potential to improve diagnostic outcomes significantly. In particular, combined detection of *SHOX2* and *RASSF1A* methylation may serve as an effective strategy for diagnosing lung cancer.

Availability of Data and Materials

All data can be obtained from the corresponding author.

Author Contributions

QZ made substantial contributions to conception and designed the research study; YW and CL performed the experiments; FW and BS collected and analyzed the data. YW has been involved in drafting the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

This study was approved by the Ethics Committee of Zhujiang Hospital (22-031) and all the participants provided written informed consent. The study was conducted in accordance with the Declaration of Helsinki.

Acknowledgment

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Conflict of Interest

Yan Wang is a reimbursed Medical Advisor of Shanghai Methyldia Technology Co., Ltd., Bin She was an employee of Shanghai Methyldia Technology Co., Ltd. at the time of investigation. Other authors declare no conflict of interest.

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