Detection of *SHOX2* and *RASSF1A* methylation for early-stage lung adenocarcinoma

YIHAO CHEN[1*], XIAOWEN ZHANG[2*], PEIXIAN LI[1*], ZHUXING CHEN[2], ZHIPENG LIU[1,3], SHU CHEN[1] and YAMING XIONG[1]

[1]Institution of Translational Medicine, The First People's Hospital of Foshan, Foshan, Guangdong 528000, P.R. China; [2]Pulmonary Nodule Surgical Department, The First People's Hospital of Foshan, Foshan, Guangdong 528000, P.R. China; [3]School of Medicine, South China University of Technology, Guangzhou, Guangdong 510640, P.R. China

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Abstract. Lung cancer (LC) is the most common malignant tumor globally, and lung adenocarcinoma (LUAD) represents the most common pathologic subtype. The DNA methylation of Short Stature Homeobox 2 (SHOX2) and Ras Association Domain Family 1 Isoform A (RASSF1A) showed the potential of diagnosing LC. With the aim of investigating the diagnostic value of SHOX2 and RASSF1A methylation in early-stage LUAD, 50 lung tissue specimens from patients suffering from early-stage LUAD were analyzed, including 25 tumor tissue specimens and 25 paired non-tumor lung tissue specimens. The methylation status of SHOX2 and RASSF1A was assessed by using methylation-specific PCR. The results showed significantly elevated methylation status of SHOX2 and RASSF1A in tumor tissues compared with non-tumor tissues. The sensitivity and specificity of SHOX2 methylation were 76.0 and 88.0%, respectively. However, the sensitivity of RASSF1A methylation was only 32.0%, with a specificity of 96.0%. Furthermore, the sensitivity and specificity of SHOX2 and RASSF1A combined methylation were 80.0 and 84.0%, respectively. No significant correlation was observed between the methylation of SHOX2/RASSF1A and clinicopathological characteristics, regardless of sex, age, tumor differentiation degree, TNM stage, or nodal status. These findings suggest that the combined detection of SHOX2 and RASSF1A methylation has a potential diagnostic value for early-stage LUAD.

Correspondence to: Dr Yaming Xiong or Dr Shu Chen, Institution of Translational Medicine, The First People's Hospital of Foshan, 81 Lingnan Avenue North, Foshan, Guangdong 528000, P.R. China
E-mail: xym0214@163.com
E-mail: happylady0512@126.com

*Contributed equally

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Introduction

Lung cancer (LC) is the most common malignant tumor worldwide and represents the leading cause of cancer-related mortality, with ~1.8 million new cases of LC diagnosed globally, resulting in ~1.6 million deaths annually (1). More than 85% of LC cases are classified as non-small cell LC (NSCLC), with lung adenocarcinoma (LUAD) becoming the most prevalent pathologic subtype (2). Late diagnosis remains a major cause of high mortality for LC. Early detection, particularly at stage I, significantly improves patient outcomes, resulting in 81-85% 5-year survival rates, while only 15-19% at stage IV (3,4).

As the prognosis of LUAD remains generally poor, it is becoming increasingly important to investigate the molecular mechanisms of tumor initiation and progression. In this context, DNA methylation biomarkers are widely used for early-stage cancer diagnosis and disease recurrence monitoring. Methylation within CpG islands of DNA gene promoters can induce transcriptional silencing of tumor suppressor genes, thereby playing a critical role in oncogenesis (5). On the basis of these findings, two commercially available assays utilizing plasma cell-free DNA methylation levels have been introduced into clinical practice to detect colorectal cancer and LC: 'Epi proColon', which assesses SEPT9 methylation for colorectal cancer, and 'Epi proLung', which evaluates PTGER4 and *SHOX2* methylation for LC (6,7).

SHOX2 and *RASSF1A* methylation were preliminarily used for LC diagnosis. A study was conducted to compare *SHOX2* methylation in lung tumors vs. normal tissues, and found that 96% of matched pairs had elevated methylation levels in tumor tissues (8). Similarly, the *RASSF1A* gene promoter hypermethylation was found in 63% of NSCLC cell lines, but it remained hypomethylated in normal epithelial cells (9).

Therefore, the present study aimed to investigate the diagnostic value of *SHOX2* and *RASSF1A* methylation in early-stage LUAD. Using methylation-specific PCR (MSP), the methylation status of *SHOX2* and *RASSF1A* in tumor tissues and the corresponding non-tumor lung tissues from early-stage LUAD cases were evaluated. Additionally, the diagnostic value of *SHOX2* and *RASSF1A* methylation was

analyzed and their relationship with clinicopathological characteristics was examined.

Materials and methods

Patients. The present study was approved by the Ethics Committee of Foshan First People's Hospital (approval no. 145; date, 2025; Foshan, China). In total, 25 participants (6 males and 19 females) were included in the present study. The clinical data of these 25 patients, suffering from early-stage LUAD and hospitalized in Foshan First People's Hospital from November 2022 to March 2023, were retrospectively analyzed. The corresponding clinicopathological characteristics are shown in Table I. Informed consent was provided by all patients prior to undergoing surgery. Postoperatively, the tumor tissues and paired non-tumor lung tissues were collected and stored at -80˚C. The diagnosis of LUAD was confirmed by two experienced pathologists.

DNA extraction. According to the manufacturer's instructions, total genomic DNA was isolated from tissue samples using the TIANamp Genomic DNA kit (Tiangen Biotech Co., Ltd.). The DNA quality and integrity were determined by measuring the A260/280 ratios, with a ratio of >1.8 indicating high purity, and total DNA concentrations were calculated by NanoPhotometer N60 Touch (Implen, https://implen.cn/).

Genomic DNA modifications by bisulfite treatment. The genomic DNA, ranging from 200-500 ng per reaction, was modified with sodium bisulfite by using the EpiJET Bisulfite Conversion Kit (Thermo Fisher Scientific, Inc.). The complete conversion of unmethylated cytosine to uracil was carried out at 98˚C for 10 min and 60˚C for 150 min. Then the converted DNA was purified and stored at -80˚C.

MSP. MSP was performed to assess the methylation status of CpG islands in the promoter regions of *RASSF1A* and *SHOX2* using 2 sets of sequence-specific primers, one targeting the methylated DNA sequence, and the other one targeting the unmethylated DNA sequence. PCR-grade water was included as a negative control. A total volume of 20 μl for each reaction, including 1 μl of converted DNA, 10 μl of 2X Rapid Taq Master Mix (Vazyme Biotech Co., Ltd.), 0.5 μl of each primer (10 μM), and 8 μl of PCR-grade water. The PCR program was set for all the methylated and unmethylated primers, consisting of initial denaturation at 95˚C for 5 min, 35 cycles of amplification at 95˚C for 30 sec, 60˚C for 50 sec, and 72˚C for 45 sec, and final extension of 72˚C for 10 min. Subsequently, 8 μl of each PCR product was electrophoresed on a 3% agarose gel and visualized with SYBR Safe (cat. no. S33102; Invitrogen; Thermo Fisher Scientific, Inc.) staining method (Fig. S1), and the images of the bands were captured using Azure 300 (Azure Biosystems, Inc.). *SHOX2* and *RASSF1A* were considered methylated when the MSP amplification products were detected in reactions with methylation-specific primers. The primer sequences were as follows: *RASSF1A* methylation-specific (10) sense, 5'-GGGTTTTGCGAGAGCGCG-3 and antisense, 5'-GCTAACAAACGCGAACCG-3'; *RASSF1A* unmethylation-specific sense, 5'-GGTTTTGTGAGAGTG TGTTTAG-3' and antisense, 5'-CACTAACAAACACAA ACCAAA-3'; *SHOX2* methylation-specific (11) sense, 5'-CGT ACGAGTATAGGCGTTTACG-3' and antisense, 5'-AAAACG ATTACTTTCGCCCG-3'; *SHOX2* unmethylation-specific sense, 5'-TGTATGAGTATAGGTGTTTATG-3' and antisense, 5'-AAAACAATTACTTTCACCCA-3'.

Statistical analysis. The experiments were conducted with three independent replicates. Categorical data are presented as number (percentage) [n (%)]. All the statistical analyses were performed using Fisher's exact test. All the data were analyzed by SPSS Statistics software version 13.0 (IBM Corp.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Methylation status of SHOX2 and RASSF1A. The methylation status of *SHOX2* and *RASSF1A* in lung tumor tissues and paired non-tumor lung tissues from 25 patients diagnosed with early-stage LUAD were examined. Among them, 19 cases were found to have *SHOX2* methylation in tumor tissues, whereas only 3 paired non-tumor lung tissue samples were tested positive ($P<0.001$). Furthermore, *RASSF1A* methylation was detected in 8 tumor tissue samples, compared with only 1 positive paired non-tumor tissue sample ($P=0.023$). Additionally, combined methylation of *SHOX2* and *RASSF1A* was observed in 20 tumor tissue samples, compared with only 4 paired non-tumor lung tissue samples were tested positive ($P<0.001$). Fisher's exact test showed that there was a statistically significant difference in methylation status between lung tumor tissues and paired non-tumor lung tissues (Table II). Moreover, in tumor tissues, *SHOX2* exhibited complete methylation in 9 cases and partial methylation in 10 cases, while *RASSF1A* showed complete methylation in 1 case and partial methylation in 7 cases. In paired non-tumor lung tissues, *SHOX2* showed complete methylation in 1 case and partial methylation in 2 cases, whereas *RASSF1A* exhibited partial methylation in 1 case, and no sample showed complete methylation (Table SI).

Diagnostic performance of SHOX2 and RASSF1A methylation. The diagnostic performance of *SHOX2* and *RASSF1A* methylation in early-stage LUAD was further analyzed. The sensitivity and specificity of *SHOX2* methylation were 76.0 and 88.0%, respectively. However, the sensitivity of *RASSF1A* methylation was only 32.0%, with a specificity of 96.0%. Finally, the specificity and sensitivity of *SHOX2* and *RASSF1A* combined methylation were also analyzed. The result revealed that the specificity and sensitivity were 84.0 and 80.0%, respectively (Table III).

Correlation of methylation status with clinicopathological parameters. The potential associations between *SHOX2* methylation, *RASSF1A* methylation, and the clinicopathological parameters in LUAD cases were also evaluated by using Fisher's test. No statistically significant associations were observed between *SHOX2* methylation, *RASSF1A* methylation and the clinicopathological parameters (Table I).

Discussion

DNA methylation, as the most prevalent epigenetic mechanism that regulates gene expression, plays a critical role in

Table I. Correlation between methylation status and diverse parameters.

Clinicopathological characteristic	Total (n=25)	Methylation level of SHOX2, n (%)	Methylation level of RASSF1A, n (%)
Sex			
Male	6	3 (50.0)	1 (16.7)
Female	19	16 (84.2)	7 (36.8)
P-value		0.125	0.624
Age, years			
<60	11	9 (81.8)	3 (27.3)
≥60	14	10 (71.4)	5 (35.7)
P-value		0.661	>0.999
TNM stage			
I	22	16 (72.7)	6 (27.3)
II	3	3 (100.0)	2 (66.7)
P-value		0.554	0.231
Lymph node metastasis			
Positive	2	2 (100.0)	1 (50.0)
Negative	22	16 (72.7)	6 (27.3)
P-value		>0.999	0.507
Differentiation degree			
Moderately /poorly	11	9 (81.8)	4 (36.4)
Well	6	5 (83.3)	2 (33.3)
P-value		>0.999	>0.999

SHOX2, short stature homeobox 2; RASSF1A, Ras association domain family 1 isoform A.

Table II. Methylation of *SHOX2* and *RASSF1A* in early-stage lung adenocarcinoma.

Group	Total	Methylation level of SHOX2, n (%)	Methylation level of RASSF1A, n (%)	Methylation level of SHOX2 + RASSF1A, n (%)
Lung cancer	25	19 (76)	8 (32)	20 (80)
Normal	25	3 (12)	1 (4)	4 (16)
P-value		<0.001	0.023	<0.001

SHOX2, short stature homeobox 2; RASSF1A, Ras association domain family 1 isoform A.

Table III. Diagnostic value of *SHOX2* and *RASSF1A* methylation.

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
SHOX2 methylation	76.0	88.0	86.4	78.6	82.0
RASSF1A methylation	32.0	96.0	88.9	58.5	64.0
SHOX2 + RASSF1A methylation	80.0	84.0	83.3	80.8	82.0

SHOX2, short stature homeobox 2; RASSF1A, Ras association domain family 1 isoform A; PPV, positive predictive value; NPV, negative predictive value.

tumor initiation and development (12,13). DNA methylation is emerging as a potential biomarker to detect LC (14,15). For instance, the methylation of *SHOX2* and *RASSF1A* were identified as two potential biomarkers for LC diagnosis (16,17).

Nevertheless, the value of *SHOX2* and *RASSF1A* methylation in screening and diagnosis of early-stage LUAD cases require further investigation.

In the present study, MSP was used to detect the methylation status of the *SHOX2* and *RASSF1A* genes in tumor tissues and paired non-tumor lung tissues from patients diagnosed with early-stage LUAD. However, it is noteworthy that the MSP method has inherent limitations. MSP requires two separate primer reactions, can only detect pre-designed CpG sites, and suffers from low throughput and efficiency. Moreover, it provides qualitative rather than quantitative results. A primary limitation of the present study is its reliance on a single methodological approach, MSP, for methylation detection, which inherently constrains the interpretation and scope of the conclusions. Further research employing other techniques is essential for confirmation. Additionally, gene expression levels of SHOX2 and RASSF1A were not evaluated, and only six male patients were included, which represent a limitation of the present study and also highlight a critical direction for future research and refinement. The results showed that methylation levels of *SHOX2* and *RASSF1A* were significantly elevated in tumor tissues. These findings were in line with previous studies (18,19), suggesting that the aberrant methylation of *SHOX2* and *RASSF1A* may play an important role in the initiation and development of LUAD.

SHOX2, a transcriptional regulator whose dysregulation was driven by promoter hypermethylation, has been implicated in LC (12). Functionally, *SHOX2* was a modulator for key cellular processes such as cell proliferation, apoptosis and epithelial-mesenchymal transition (20). With upregulated *SHOX2* expression in LC, it enhanced LC cell viability, migration and invasion *in vitro*, and promoted LC tumorigenesis and pulmonary nodule metastasis *in vivo* (16). Notably, although *SHOX2* methylation was significantly elevated in LC, which appeared contradictory given that DNA methylation was typically associated with gene silencing and considered to downregulate *SHOX2* expression. It was reported that transcriptional activity was closely associated with DNA methylation patterns in different genomic regions: The hypermethylation in promoter and enhancer regions generally restrained gene expression, while the hypermethylation of gene body was positively related to the active gene transcription (21,22). As a matter of fact, the elevated methylation level of *SHOX2* (gene body) in LC cells suggested that the hypermethylation of *SHOX2* (gene body) may promote the mRNA expression of *SHOX2* in this context (16).

As a well-known tumor suppressor gene that regulates the cell cycle and apoptosis (23), the methylation of *RASSF1A* gene promoter region caused *RASSF1A* expression deficiency, which led to the activation of Yes-associated protein (YAP) and contributed to the development of malignant characteristics, including invasiveness, apoptosis resistance, and ultimate transformation into cancer cells (24).

In addition, the diagnostic performance of *SHOX2* and *RASSF1A* methylation was evaluated. The sensitivity and specificity of *SHOX2* methylation were 76.0 and 88.0%, respectively. By contrast, *RASSF1A* methylation exhibited a lower sensitivity of 32.0%, but a higher specificity of 96.0%. Moreover, the sensitivity and specificity of *SHOX2* and *RASSF1A* combined methylation were 80.0 and 84.0%, respectively.

In consistent with the present findings, the positive detection rate of *RASSF1A* in histological specimens of LUAD was 39% (25). *SHOX2* methylation in formalin-fixed paraffin-embedded (FFPE) tissues could distinguish between benign and malignant lung diseases with 60% sensitivity and 90.4% specificity (26). Similarly, Shi *et al* (19) confirmed the diagnostic performance of *SHOX2* methylation in FFPE samples of LUAD, reporting a sensitivity of 64.3% and a specificity of 92.1%. A comprehensive meta-analysis comprising 2,296 participants (including 1,129 LC cases) demonstrated the diagnostic accuracy of *SHOX2* methylation by showing a sensitivity of 70% at a specificity of 96% in various samples, such as lymph nodes, bronchial aspirates, pleural effusion, plasma and tumor tissues from LC (27).

Furthermore, a diagnostic sensitivity of 69.6-81.0% and a specificity of 90-97.4% for the *SHOX2* and *RASSF1A* combined methylation in bronchoalveolar lavage fluid of LC were achieved (28,29). In FFPE samples, 66.2% sensitivity and 94.1% specificity for LUAD were shown for the *SHOX2* and *RASSF1A* combined methylation (18). The combination of *SHOX2* and *RASSF1A* methylation in 251 FFPE LC specimens yielded a diagnostic sensitivity of 89.8% and a specificity of 90.4% (19).

Although no statistically significant association was found between SHOX2 methylation, *RASSF1A* methylation and the clinicopathological parameters, it has been reported that there are associations between them. *RASSF1A* methylation was found to have a higher frequency in male patients (30). In addition, *SHOX2* gene hypermethylation showed a correlation with smoking history, and *RASSF1A* and *SHOX2* methylation were confirmed to serve as promising biomarkers for predicting malignant lung diseases and might potentially help with the distinguishment of aggressive phenotypes in early-stage LUAD (31).

The positive correlation between the invasiveness of LUAD and *SHOX2* and *RASSF1A* combined methylation level was confirmed, with greater invasiveness leads to higher methylation positivity rates (18). A positive association between the combined methylation levels of *SHOX2* and *RASSF1A* and the expression of Ki-67 in early-stage LUAD was demonstrated by further research (32). As inferred, patients who tested positive for *SHOX2* and *RASSF1A* methylation may experience more rapid tumor progression (18). Additional analysis showed that the *SHOX2* methylation level was positively correlated with the stage of LC (19). Moreover, a high correlation was found between high frequency of *RASSF1A* promoter methylation and cancer pathogenesis as well as more aggressive clinical phenotype (33,34). It was also indicated in the research that lower sensitivity in stage I tumors was exhibited for *SHOX2* methylation, and a markedly lower sensitivity of LUAD than that of small cell LC and squamous cell carcinoma (19).

The statistical power of the present study was limited by the relatively small cohort size; and future validation in a larger population is warranted. Further investigations were warranted to validate the clinical value of RASSF1A and SHOX2 methylation assays to improve the diagnosis and treatment of LC. In conclusion, the detection of *RASSF1A* and

SHOX2 methylation might become a complementary approach to improve conventional pathological diagnosis of LUAD and facilitate earlier disease diagnosis and treatment of LUAD.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

YX and SC conceived and designed the study. YC wrote the manuscript. YC, XZ and PL performed the experiments and data analysis. ZC and ZL assisted with sample processing and data analysis. All authors read and approved the final version of the manuscript. YC and YX confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Informed consent was obtained from all participants before sample collection. The present study was approved by the Ethics Committee of Foshan First People's Hospital (approval no. 145; date, 2025; Foshan, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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