Identification and validation of *PCDHGA12* and *PRRX1* methylation for detecting lung cancer in bronchial washing sample

TAE JEONG OH[1], SEUNGHYUN JANG[1], SU JI KIM[1], MIN A WOO[1], JI WOONG SON[2], IN BEOM JEONG[2], MIN HYEOK LEE[2] and SUNGWHAN AN[1]

[1]Genomictree, Inc., Daejeon 34027; [2]Department of Internal Medicine, Konyang University Hospital, Daejeon 35365, Republic of Korea

Received December 1, 2023; Accepted March 15, 2024

DOI: 10.3892/ol.2024.14379

Abstract. Bronchoscopy is a frequently used initial diagnostic procedure for patients with suspected lung cancer (LC). Cytological examinations of bronchial washing (BW) samples obtained during bronchoscopy often yield inconclusive results regarding LC diagnosis. The present study aimed to identify molecular biomarkers as a non-invasive method for LC diagnosis. Aberrant DNA methylation is used as a useful biomarker for LC. Therefore, microarray-based methylation profiling analyses on 13 patient-matched tumor tissues at stages I-III vs. non-tumor tissues were performed, and a group of highly differentially methylated genes was identified. A subsequent analysis using bisulfite-pyrosequencing with additional tissues and cell lines revealed six methylated genes [ADAM metallopeptidase with thrombospondin type 1 motif 20, forkhead box C2 (mesenchyme forkhead 1), NK2 transcription factor related, locus 5 (*Drosophila*), oligodendrocyte transcription factor 3, protocadherin γ subfamily A 12 (*PCDHGA12*) and paired related homeobox 1 (*PRRX1*)] associated with LC. Next, a highly sensitive and accurate detection method, linear target enrichment-quantitative methylation-specific PCR in a single closed tube, was applied for clinical validation using BW samples from patients with LC (n=68) and individuals with benign diseases (n=33). *PCDHGA12* and *PRRX1* methylation were identified as the best-performing biomarkers to detect LC. The two-marker combination showed a sensitivity of 82.4% and a specificity of 87.9%, with an area under the curve of 0.891. Notably, the sensitivity for small cell LC was 100%. The two-marker combination had a positive predictive value of 93.3% and a negative predictive value of 70.7%. The sensitivity was higher than that of cytology, which only had a sensitivity of 50%. The methylation status of the two-marker combination showed no association with sex, age or stage, but was associated with tumor location and histology. In conclusion, the present study showed that the regulatory regions of *PCDHGA12* and *PRRX1* are highly methylated in LC and can be used to detect LC in BW specimens as a diagnostic adjunct to cytology in clinical practice.

Introduction

Lung cancer (LC) is the leading cause of cancer-related mortality and morbidity, with 1.8 million deaths accounting for 18% of the global cancer-associated mortality rate (1,2). The estimated 5-year survival rate of patients with LC is 68-92% when diagnosed at an early stage. However, if detected at late stages, this drops to 10% (3). Patients with LC have a poor prognosis because the disease is frequently detected at advanced stages without curative treatment options. One of the key reasons for such a poor prognosis is the lack of efficient diagnostic tools for early detection (4).

Screening with low-dose computed tomography (CT) scans of the chest has been introduced as a potential tool for early detection of LC (5). Although low-dose CT allows the detection of early-stage LC with a high sensitivity, which can reduce LC-associated mortality, it has numerous drawbacks including radiation exposure, a high false positive rate and a low diagnostic accuracy (6-8). Furthermore, LC screening by chest x-ray and sputum cytology has failed to overcome early detection and risk assessment limitations, thus failing to improve overall survival (9). Because there is currently no early detection method in clinical practice, a patient with suspected LC is typically subjected to a full clinical workup that includes CT scanning followed by bronchoscopy (7).

Correspondence to: Dr Sungwhan An, Genomictree, Inc., 44-6 10-ro Techno, Yuseong, Daejeon 34027, Republic of Korea
E-mail: sungwhan@genomictree.com

Abbreviations: ADC, adenocarcinoma; AUC, area under the curve; BW, bronchial washing; LTE, linear target enrichment; MtI, methylation index; NPV, negative predictive value; NSCLC, non-small cell lung cancer; *PCDHGA12*, protocadherin γ subfamily A, 12; PPV, positive predictive value; *PRRX1*, paired-related homeobox 1; qMSP, quantitative methylation-specific PCR; ROC, receiver operating characteristic; SCC, squamous cell carcinoma

Key words: biomarker, bronchial washing, lung cancer, methylation, *PCDHGA12*, *PRRX1*

Bronchoscopy is the most common invasive procedure used to diagnose LC. However, the diagnostic yield of bronchoscopy is unsatisfactory, with ambiguous results in half of patients suspected of having LC, especially for peripheral tumors (10,11). If bronchoscopy fails to detect LC, further invasive diagnostic procedures, such as transthoracic needle aspiration or surgical lung biopsy, may be required, posing a significant risk of complications, such as pneumothorax, bleeding and infection (12). Bronchoscopy is the preferred method for confirming suspected lung lesions through pathological assessment of a tissue biopsy or cytological specimen obtained during bronchoscopy (11,13). Cytology examinations include bronchial brushing, bronchial washing (BW) and bronchoalveolar lavage samples (14). Cytology often results in an equivocal or inconclusive result, even when performed by experienced professionals. In addition, the sensitivity range of cytology is low (23.5-32.1%) (15). Nevertheless, cytological sampling is a minimally invasive, safe and well-tolerated method performed during bronchoscopy for LC diagnosis (10,11,14). Biomarkers may also be used as a diagnostic adjunct to resolve equivocal cytology results (16). Therefore, development of molecular biomarker tests with higher sensitivity for routine cytology specimens represents an effective approach to improving the diagnostic yield of bronchoscopy (17).

DNA methylation, a key epigenetic phenomenon, plays a fundamental role in various biological processes, including development, cell differentiation, aging, tumorigenesis and other disease (18). Abnormal DNA methylation is involved in tumor development; it is one of the earliest and most frequent genomic alterations during carcinogenesis (9,19). The analysis of DNA methylation biomarkers provides potential for early detection of LC (10,14,20,21). A number of genes, such as adenomatous polyposis coli, ras-association (RalGDS/AF-6) domain family member 1 (*RASSF1A*), *p16*, short-stature homeobox 2 (*SHOX2*) and various homeobox genes, have been extensively investigated to diagnose LC (22,23). Nonetheless, no established biomarker test with clinical application for LC detection exists.

In the present study, CpG methylation microarray analysis was performed to investigate a subset of differentially hypermethylated genes in primary lung tumors compared with paired adjacent non-tumor tissues. The aim of the study was to identify methylation biomarkers for the early detection of LC and to validate them clinically using BW samples.

Materials and methods

Cell lines and clinical samples. The human LC cell lines A549 (cat. no. CCL-185), NCI-H358 (cat. no. CRL-5807), SK-MES-1 (cat. no. HTB-58) and NCI-H146 (cat. no. HTB-173) were obtained from the American Type Culture Collection and cultured in RPMI-1640 (cat. no. LM011-06; Welgene, Inc.) in a humidified 5% CO_2 incubator at 37˚C, supplemented with 10% fetal bovine serum (cat. no. S001-04, Welgene, Inc.). Normal human bronchial epithelial (NHBE) cells were purchased from Cambrex Bio Science Rockland, Ltd. (cat. no. CC2540) and cultured in BEBM (cat. no. cc-3171; Cambrex Bio Science Rockland, Ltd.) in a humidified 5% CO_2 incubator at 37˚C. The cultured cells were tested for mycoplasma every month to ensure that they were not contaminated using a MycoStrip kit (cat. no. rep-mys-20; InvivoGen). Cells were authenticated by short tandem repeat analysis using *GenePrint*® 10 System (cat. no. B9510; Promega Corporation).

Fresh-frozen primary tumors and paired adjacent non-tumor tissue from 13 patients with non-small cell LC (NSCLC) at various stages (I, n=5; II, n=5; III, n=3) were obtained from the Biobank of Chungnam National University Hospital (Daejeon, South Korea), which participates in the Korea Biobank Project (KBP). Tissue specimens were collected at the time of surgery between February 2014 and December 2019. Every tumor specimen was histologically verified by a board-certified pathologist.

All BW samples were provided by Konyang University Hospital (Daejeon, South Korea). All individuals donating BW samples for the present study were investigated for suspected LC. All BW samples were collected during flexible fiberoptic bronchoscopy (Olympus Corporation) by aspiration with a flexible bronchoscope from the region of the suspicious lesion between April 2022 and March 2023. Cytological diagnostics was performed by a board-certified pathologist. Briefly, 5-10 ml sterile normal saline was instilled two or three times. Fluid (≥10 ml) was then retrieved into a preservative buffer (Genomictree, Inc.). A total of 101 BW samples were obtained from 68 patients with LC (49 NSCLC and 19 SCLC) and 33 individuals with benign diseases and were used for methylation analysis. The patient clinicopathological and demographical information is shown in Table I.

The present study adhered to local ethics guidelines and was approved by the Institutional Review Board of the Chungnam National University Hospital (approval no. 2022-02-061-002, Dajeon, South Korea) and Konyang University Hospital (approval no. 2022-03-025, Dajeon, South Korea). Written informed consent was obtained from all patients.

CpG methylation microarray analysis. To identify differentially methylated genes in primary lung tumors and paired adjacent non-tumor tissues, CpG methylation microarray analyses were conducted using 0.5 μg genomic DNA isolated from 13 patients with LC. CpG methylation microarray analysis was performed as described previously (24) using human CpG island microarray kit, 244k (Agilent Technologies, Inc.) according to the manufacturer's instructions. Raw CpG methylation microarray data were submitted to Gene Expression Omnibus (accession no. GSE246510; ncbi.nlm.nih.gov/geo). The hybridized images were analyzed and quantified using Agilent Feature Extraction (version 9.3.2.1; Agilent Technologies, Inc.) and GeneSpring (version 7.3.1; Agilent Technologies, Inc.).

To determine differentially hypermethylated candidate genes in primary tumor compared with paired adjacent non-tumor tissue samples, statistical analysis was performed using a parametric ANOVA test with Benjamini and Hochberg multiple testing correction ($P<0.05$), followed by fold-change analysis. Mean fold change was calculated by dividing mean methylation levels in tumor tissue by mean methylation levels in non-tumor tissue. Multiple-probe enriched genes were selected as methylation candidate genes if their probes yielded a positive call for methylation in the lung primary tumor

Table I. Clinicopathological features of tissue and bronchial washing samples.

Characteristic	Tissue	Bronchial washing
Sex (%)		
Non-LC (benign)	-	33 (100.0)
Male	-	16 (48.5)
Female	-	17 (51.5)
LC	13	68 (100.0)
Male	12 (77.8)	53 (77.9)
Female	1 (22.2)	15 (22.1)
Mean age (range), years		
Non-LC (benign)	-	67.0 (47-87)
LC	52.4 (58-81)	71.7 (49-93)
Pathological stage (%)		
I	5 (38.5)	13 (19.1)
II	5 (38.5)	6 (8.8)
III	3 (23.1)	14 (20.6)
IV	-	35 (51.5)
Histology (%)		
NSCLC		49 (72.1)
ADC	6 (46.2)	27 (39.7)
SCC	7 (53.8)	20 (29.4)
Other[a]	-	2 (2.9)
SCLC	-	19 (27.9)
Tumor location (%)		
Central	-	34 (50.0)
Peripheral	-	34 (50.0)

[a]Phleomorphic carcinoma and neuroendocrine carcinoma. ADC, adenocarcinoma; SCC, squamous cell carcinoma; NSCLC, non-small cell lung cancer.

compared with non-tumor tissue with at least two adjacent probes, allowing for a one-gap probe within CpG islands.

DNA isolation and bisulfite treatment. Genomic DNA was isolated from cell lines and tissues using the QIAmp DNA Mini kit (cat. no. 51304; Qiagen GmbH) according to the manufacturer's instructions. Genomic DNA was isolated from BW samples using a solid phase magnetic bead-based GT NUCLEIC ACID PREP kit (cat. no. GT-PREP-1; Genomictree, Inc.) according to the manufacturer's instructions. Genomic DNA was chemically modified with sodium bisulfite using an EZ DNA Methylation Gold kit (cat. no. D5006; Zymo Research Corp.) according to the manufacturer's instructions. Bisulfite-converted DNA was purified and eluted with an elution buffer using a Zymo-Spin IC column (Zymo Research Corp.).

Methylation analysis by bisulfite-pyrosequencing. Candidate methylation targets were analyzed for methylation levels using bisulfite-pyrosequencing, as previously described, with slight modifications (24). Bisulfite-treated genomic DNA underwent PCR amplification targeting the region of interest, employing a specific primer set. Either the forward or reverse primer was biotinylated, facilitating generation of a single-stranded DNA template for subsequent pyrosequencing on a PyroMark Q48 Autoprep (Qiagen GmbH). Pyrosequencing primers were designed to detect methylated cytosine by analyzing CpG dinucleotide sites within the target sequences of bisulfite-treated DNA. To facilitate primer design, nucleotide sequences of the candidate genes were retrieved from the NCBI Reference Sequence database (ncbi.nlm.nih.gov/refseq/) and converted into bisulfite-treated sequences. Both PCR primers and sequencing primers were designed to complementarily bind to the bisulfite-converted sequences within the regions of the interest, while avoiding CpGs within the primer sequences, using PyroMark Assay Design Software V 2.0 (Qiagen GmbH). Primer sequences are listed in Table SI. Briefly, 20 ng bisulfite-modified DNA was amplified in a 20 μl reaction with a gene-specific primer set and TOPsimple PCR DryMix-HOT (cat. no. P581H; Enzynomics, Inc.). PCR amplification was conducted under the following thermocycling conditions: 95˚C for 10 min, followed by 40 cycles of 95˚C for 45 sec, optimal annealing temperature for 45 sec, and 70˚C for 45 sec for each target. Pyrosequencing was performed using a PyroMark Gold Q48 reagent cartridge and a PyroMark Q48 instrument (Qiagen GmbH) according to the manufacturer's instructions.

The methylation index (MtI) of every gene in every sample was calculated as the mean value of methylated cytosine/(methylated cytosine + unmethylated cytosine) for all examined CpGs in target regions. All pyrosequencing reactions included a negative control without DNA template. Methylation-positive was considered if the MtI of the primary tumor was greater than that of the corresponding non-tumor tissue.

Assessment of methylation status in BW samples using linear target enrichment (LTE)-quantitative methylation-specific PCR (qMSP) assay. A 3-plex LTE-qMSP assay was developed, integrating two methylation targets from candidate genes, and the control gene collagen type II α1 (*COL2A1*) within a closed single-tube system to assess the methylation status of candidate targets in BW-derived DNA samples. This method involves two sequential rounds of PCR. LTE first employs one-direction PCR targeting to linearly enrich DNA of methylation candidates, followed by qMSP for exponential amplification of both the methylated target and the control gene.

In the first round of PCR, high annealing temperature (70˚C) was applied to facilitate unidirectional DNA synthesis while preventing other primers with regular melting temperatures from initiating DNA synthesis. Two specific primers with a universal tag sequence (UTS) at the 5' end were designed to anneal to two distinct methylation target genes. The template DNA synthesis occurred in one direction only, replicating with each cycle of PCR.

In the subsequent PCR, the reaction was conducted at a reduced temperature (60˚C). A total of three sets of primers and probes were utilized targeting amplification of the two methylation targets and the control gene. The forward primers for the methylation targets were designed to bind specifically to the respective methylation target sites and UTS was used as reverse primer. The control target utilized a primer set specific to a DNA region of *COL2A1* gene lacking CpG dinucleotides,

with annealing at 60˚C. Probes were designed to bind internal sites of each PCR product, thereby generating signals indicated of PCR product formation.

To design primers and probes for LTE-qMSP, nucleotide sequences corresponding to the genes of interest were obtained from the NCBI Reference Sequence database (ncbi.nlm.nih.gov/refseq/). The primer and probe sequences were designed to bind complementarily to the bisulfite-converted sequences of the methylation target regions using MethPrimer program (version 2.0; urogene.org/cgi-bin/methprimer/methprimer.cgi). The primer and probe sequences are provided in Table SII.

For every reaction, 20 ng BW-derived DNA underwent bisulfite conversion. The bisulfite-converted DNA was purified and eluted with 12 μl elution buffer, serving as input for the LTE-qMSP assay. The reaction mixture (25 μl) comprised 10 μl input DNA and 15 μl reagent containing two methylation target methylation-specific reverse primer with a 5' UTS, two methylation-specific forward primers, UTS as reverse primer, two probes for specific methylation sites of targets, *COL2A1*-specific forward and reverse primers, *COL2A1* probe and 5 μl of the master mix, TOPreal™ Fast qPCR 5X PreMIX TaqMan-Probe (Enzynomics, Inc.). LTE-qMSP reaction was performed on an AB7500 FAST Real-Time PCR system (Thermo Fisher Scientific, Inc.) under the following thermocycling conditions: 95˚C for 5 min, 5 cycles of 95˚C for 15 sec and 70˚C for 45 sec, followed by 35 cycles of 95˚C for 15 sec and 60˚C for 45 sec.

The relative methylation in each sample was calculated as 35-ΔC_T [C_T of the amplified target gene-C_T of *COL2A1* (human reference gene)] (25). A higher value indicates a greater level of methylation. If the C_T of the target gene was undetectable, the value was set to 25, the closest value to the lowest 35-ΔC_T among all test results. The assay was conducted by trained personnel blinded to the bronchoscopy or the histopathology results.

Statistical analysis. All statistical analyses were performed using MedCalc (version 9.3.2.0, medcalc.org/). Receiver operating characteristic (ROC) curves were constructed to evaluate the test performance, with calculation of the area under ROC (AUC) and 95% confidence intervals (CIs). Methylation tests were performed once for each sample, and the data are presented as the mean and standard deviations of all test results for the samples. $P<0.05$ was considered to indicate a statistically significant difference.

To calculate sensitivity and specificity in BW samples, test results were categorized as follows: Methylation-positive as '1' and methylation-negative as '0'. A binary logistic regression analysis was used to determine the best performing marker combination for detecting LC in BW samples. To describe demographic and other clinical characteristics, frequency and percent were used. The negative predictive value (NPV) and positive predictive value (PPV) were also calculated.

The paired t test was conducted to compare differences in methylation levels of each gene between tumor and paired adjacent non-tumor tissue. The Kruskal Wallis test was used to analyze differences in methylation levels between patients with LC and individuals with benign diseases in BW samples. Spearman's correlation analysis was performed to investigate the correlation between methylation levels of genes in BW specimens. The difference in the sensitivity and specificity between the methylation testing and cytology examination for LC diagnosis was analyzed using McNemar test. Fisher's exact test was utilized to examine the relationship between clinicopathological parameters and methylation status in BW samples.

Results

Identification of candidate genes hypermethylated in primary lung tumor tissue. To investigate a subset of hypermethylated candidate genes for detecting LC, CpG methylation patterns were compared between primary lung tumors and corresponding adjacent non-tumor tissues using CpG methylation microarray analyses (Fig. S1). The initial hypothesis was that candidate genes should be unmethylated in non-tumor tissues and frequently hypermethylated in tumor tissues. A total of 18,585 unmethylated CpG probes across all 13 non-tumor tissues were selected. Subsequent statistical analysis (ANOVA) identified 2,844 CpG probes differentially hypermethylated in primary tumor tissue. Methylation candidate numbers were then narrowed to 516 CpG probes representing 65 annotated genes that showed consistent hypermethylation in at least two adjacent CpG probes (Table SIII). Among the 65 candidate genes, the analysis focused on 10 hypermethylated genes, apoptosis antagonizing transcription factor, ATP-binding cassette subfamily C member 9, ADAM metallopeptidase with thrombospondin type 1 motif 20 (*ADAMTS20*), forkhead box C2 (mesenchyme forkhead 1) (*FOXC2*), hey-like transcriptional repressor, NK2 transcription factor-related locus 5 (*Drosophila*) (*NKX2-5*), oligodendrocyte transcription factor 3 (*OLIG3*), one cut domain family member 1, protocadherin γ subfamily A 12 (*PCDHGA12*) and paired-related homeobox 1 (*PRRX1*), exhibiting a positive call for methylation in their 5' regulatory regions (promoter or 5' untranslated region) and had not been previously reported as aberrantly hypermethylated in primary lung tumor tissue.

Verification of methylation candidate genes in LC cell lines and tissues using bisulfite-pyrosequencing. To verify the methylation status of 10 candidate genes, a pyrosequencing-based methylation assessment was performed in four representative LC cell lines, A549, NCI-H358, SK-MES-1 and NCI-H146, and their status was compared with that of NHBE cells. Results revealed that all 10 genes were hypermethylated in LC cell lines but methylated at a low level in NHBE cells (Fig. 1). In verification analysis using a pyrosequencing assay to confirm whether these candidate genes were aberrantly hypermethylated in primary lung tumors examined in CpG microarray analysis, all candidate genes except *HELT* exhibited a significantly high level of methylation in primary tumor tissues compared with their corresponding non-tumor tissues (Fig. 2). The mean MtIs of all candidate genes were high in lung tumors (range, 25.1-60.4%), but relatively low in non-tumor tissues (range, 5.6-40.7%; Table II). Among these genes, six (*ADAMTS20*, *FOXC2*, *NKX2-5*, *OLIG3*, *PCDHGA12* and *PRRX1*) were chosen for further validation because of their consistently high (100%) methylation positivity across all tumor tissues.

Table II. Methylation status of 10 candidate genes in 13 paired lung tissues used in CpG methylation microarray analysis.

Gene	Mean MtI, %		Methylation positivity, % (positive samples/total samples)	P-value
	Paired adjacent non-tumor tissue	Primary tumor tissue		
AATF	14.4±2.8	45.9±15.3	92.3 (12/13)	<0.001
ABCC9	5.6±1.2	39.0±25.3	92.3 (12/13)	<0.001
ADAMTS20	8.0±1.9	31.5±13.9	100 (13/13)	<0.001
FOXC2	11.5±1.2	33.4±12.5	100 (13/13)	<0.001
HELT	11.6±1.0	25.1±18.2	76.9 (10/13)	0.081
NKX2-5	40.7±4.9	64.6±11.0	100 (13/13)	<0.001
OLIG3	8.7±2.2	39.1±15.5	100 (13/13)	<0.001
ONECUT1	29.8±4.8	49.8±11.7	76.9 (10/13)	<0.001
PCDHGA12	34.0±4.2	60.4±8.2	100 (13/13)	<0.001
PRRX1	10.6±0.7	43.8±12.6	100 (13/13)	<0.001

Methylation positivity was defined as MtI of primary tumor was greater than that of the corresponding non-tumor tissue. P-value was calculated by paired t-test. MtI, methylation index; AATF, apoptosis antagonizing transcription factor; ABCC9, ATP-binding cassette subfamily C member 9; ADAMTS20, ADAM metallopeptidase with thrombospondin type 1 motif 20; FOXC2, forkhead box C2 (mesenchyme forkhead 1); HELT, hey-like transcriptional repressor; NKX2-5, NK2 transcription factor-related locus 5 (*Drosophila*); OLIG3, oligodendrocyte transcription factor 3; ONECUT1, one cut domain, family member 1; PCDHGA12, protocadherin γ subfamily A 12; PRRX1, paired-related homeobox 1.

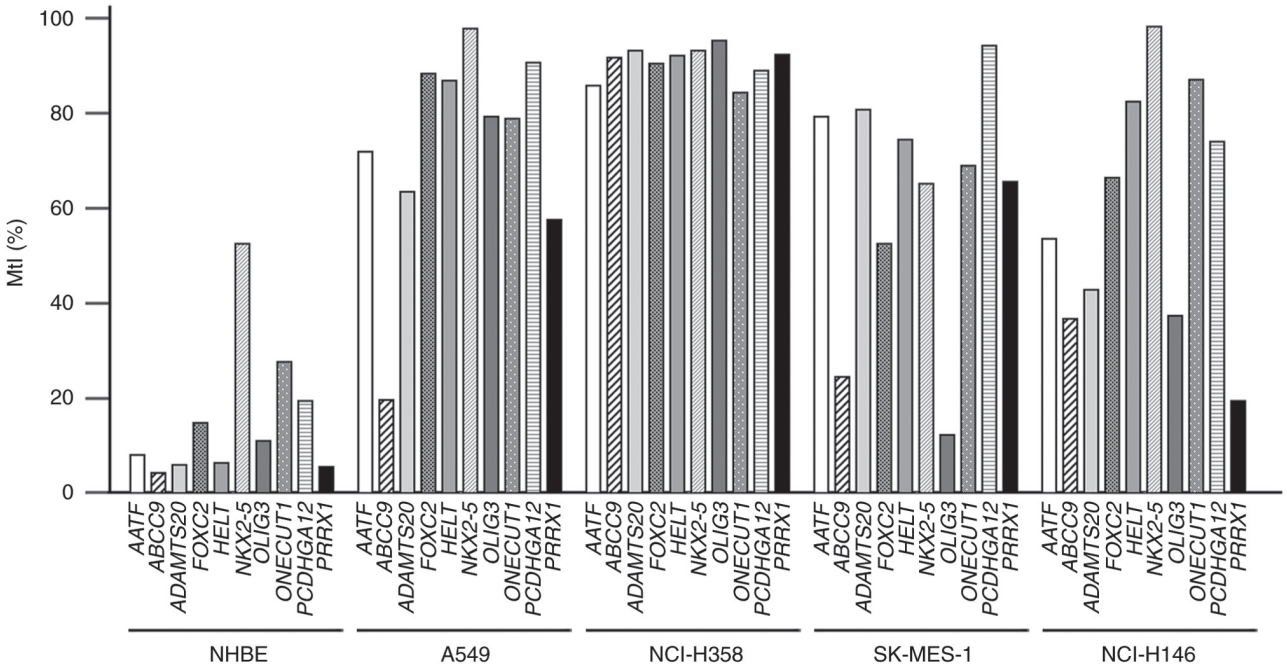

Figure 1. Bisulfite-pyrosequencing results of 10 candidate genes in cell lines. The methylation levels were calculated for all examined CpG dinucleotides in target regions. MtI values for each gene were plotted in four LC cell lines A549, NCI-H358, SK-MES-1 and NCI-H146 and in NHBE cells. MtI, methylation index; NHBE, normal human bronchial epithelial cell; *AATF*, apoptosis antagonizing transcription factor; *ABCC9*, ATP-binding cassette subfamily C member 9; *ADAMTS20*, ADAM metallopeptidase with thrombospondin type 1 motif 20; *FOXC2*, forkhead box C2 (mesenchyme forkhead 1); *HELT*, hey-like transcriptional repressor; *NKX2-5*, NK2 transcription factor-related, locus 5 (*Drosophila*); *OLIG3*, oligodendrocyte transcription factor 3; *ONECUT1*, one cut domain family member 1; *PCDHGA12*, protocadherin γ subfamily A, 12; *PRRX1*, paired-related homeobox 1.

Clinical validation of six methylated genes for detecting LC using BW specimens. A highly sensitive and accurate 3-plex LTE-qMSP in a single closed tube was developed to measure the methylation of target genes in BW samples. Methylation levels were determined using DNA from 68 patients with LC and 33 individuals with benign diseases. Results of the 3-plex LTE-qMSP showed that the methylation levels of all six genes were significantly higher in patients with LC than in individuals with benign diseases (Fig. 3). To determine sensitivity and specificity of individual genes for LC detection, ROC analysis was performed (Fig. 4). Given the optimal cut-off values, the sensitivity range of each gene was 52.9-80.9% and

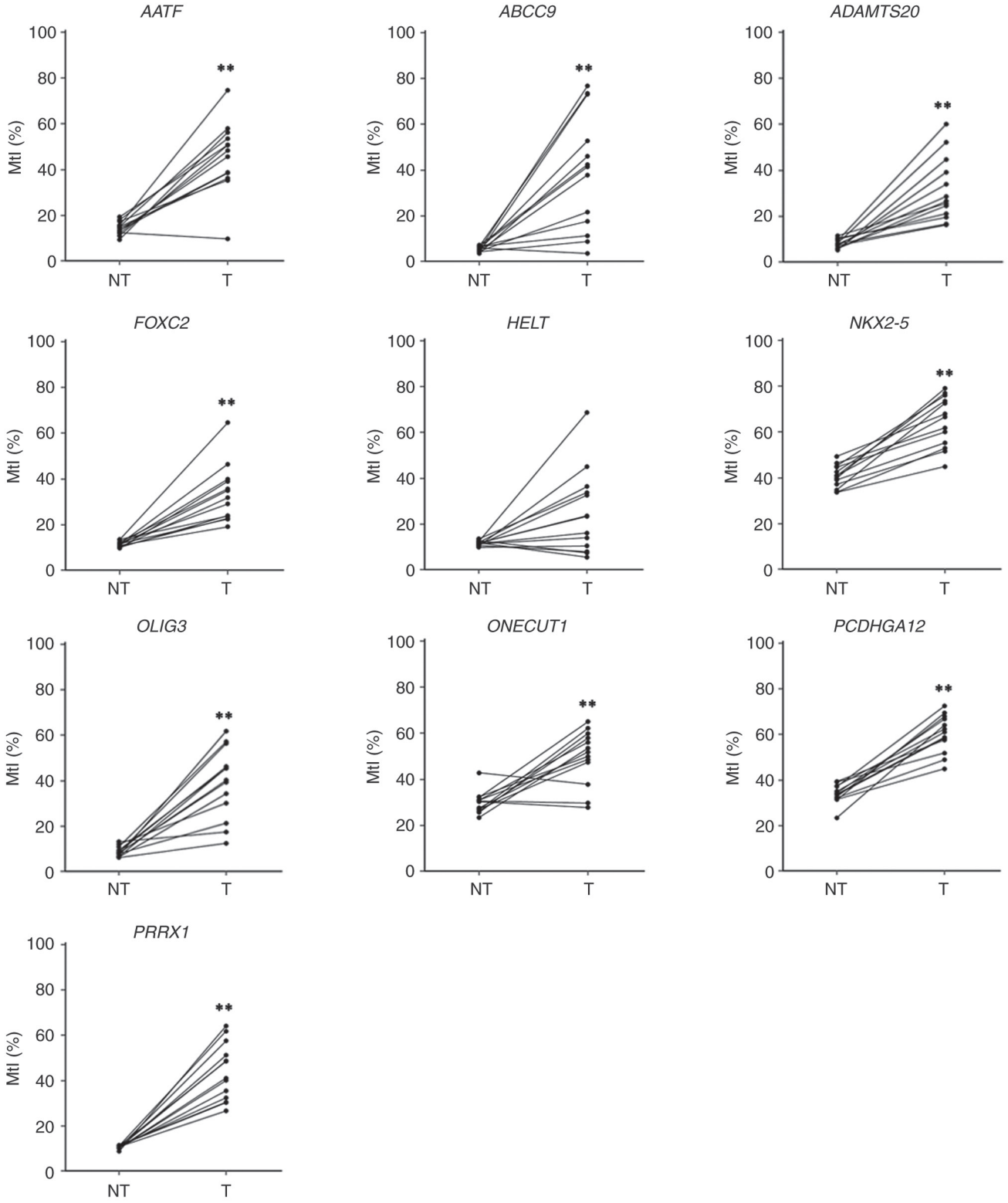

Figure 2. Assessment of methylation levels of 10 candidate genes in paired lung tissue by bisulfite-pyrosequencing. MtI for each gene was determined in primary lung tumor and paired adjacent NT tissue used for the CpG methylation microarray analysis. Samples from the same patients are linked with a straight line. **P<0.01 vs. NT. NT, non-tumor, T, tumor. MtI, methylation index; NHBE, normal human bronchial epithelial cell; *AATF*, apoptosis antagonizing transcription factor; *ABCC9*, ATP-binding cassette subfamily C member 9; *ADAMTS20*, ADAM metallopeptidase with thrombospondin type 1 motif 20; *FOXC2*, forkhead box C2 (MFH-1, mesenchyme forkhead 1); *HELT*, Hey-like transcriptional repressor; *NKX2-5*, NK2 transcription factor-related, locus 5 (*Drosophila*); *OLIG3*, oligodendrocyte transcription factor 3; *ONECUT1*, one cut domain family member 1; *PCDHGA12*, protocadherin γ subfamily A, 12; *PRRX1,* paired-related homeobox 1.

the specificity range was 81.8-97.0% for LC; AUC range was 0.696-0.859. The PPV range was 86.7-97.9% and NPV range was 48.2-69.8%. Cytology results of BW specimens showed a sensitivity of 50.0%, with a specificity of 100% (Table III).

Table III. Clinical performance of 6 genes and cytology in detecting lung cancer using bronchial washing samples.

Test	Cut-off (35-ΔC_T)	AUC (95% CI)	Sensitivity, % (95% CI)	Specificity, % (95% CI)	PPV, % (95% CI)	NPV, % (95% CI)
ADAMTS20	31.5	0.749 (0.653-0.830)	55.9 (43.3-67.9)	94.0 (79.8-99.3)	95.0 (83.0-98.7)	50.8 (43.8-57.8)
FOXC2	31.0	0.734 (0.637-0.817)	52.9 (40.4-65.2)	93.9 (79.8-99.3)	94.7 (82.2-98.6)	49.2 (42.6-55.8)
NKX2-5	31.0	0.696 (0.596-0.783)	57.4 (44.8-69.3)	81.8 (64.5-93.0)	86.7 (75.4-93.2)	48.2 (40.4-56.2)
OLIG3	28.5	0.793 (0.701-0.867)	67.6 (55.2-78.5)	90.9 (75.7-98.1)	93.9 (93.7-97.9)	57.7 (48.7-66.2)
PCDHGA12	30.5	0.859 (0.776-0.920)	80.9 (69.5-89.4)	90.9 (75.7-98.1)	94.8 (86.1-98.2)	69.8 (58.3-79.2)
PRRX1	25.0	0.830 (0.743-0.898)	69.1 (56.7-79.8)	97.0 (84.2-99.9)	97.9 (87.1-99.7)	60.4 (51.5-68.6)
Cytology	-	0.750 (0.654-0.831)	50.0 (37.6-62.4)	100.0 (89.4-100.0)	100.0	49.3 (43.4-55.2)
PCDHGA12 or *PRRX1*	-	0.891 (0.813-0.944)	82.4 (71.2-90.2)	87.9 (71.8-96.6)	93.3 (84.7-97.2)	70.7 (58.7-80.4)
PCDHGA12 or *PRRX1* or cytology	-	0.866 (0.784-0.926)	85.3 (74.6-92.7)	87.9 (71.8-96.6)	93.6 (85.2-97.3)	74.4 (61.7-83.9)

ADAMTS20, ADAM metallopeptidase with thrombospondin type 1 motif 20; *FOXC2*, forkhead box C2 (mesenchyme forkhead 1); *NKX2-5*, NK2 transcription factor-related, locus 5 (*Drosophila*); *OLIG3*, oligodendrocyte transcription factor 3; *PCDHGA12*, protocadherin γ subfamily A, 12; *PRRX1*, paired-related homeobox 1; AUC, area under ROC; PPV, positive predictive value; NPV, negative predictive value.

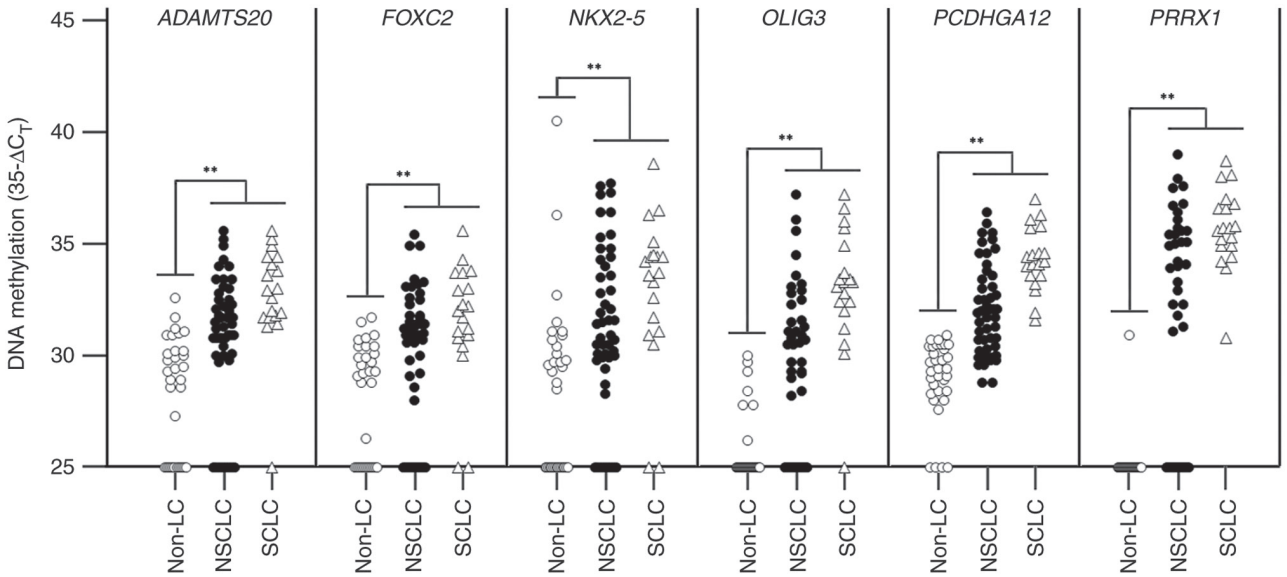

Figure 3. Clinical validation of six genes in bronchial washing samples using linear target enrichment-quantitative methylation-specific PCR. The distribution of methylation for every gene is depicted as scatter plots of 35-ΔC_T value. The differences in methylation levels between patients with lung cancer and individuals with benign diseases are statistically compared. **P<0.01. Non-LC, individuals with benign diseases, NSCLC, non-small cell lung cancer; *ADAMTS20*, ADAM metallopeptidase with thrombospondin type 1 motif 20; *FOXC2*, forkhead box C2 (mesenchyme forkhead 1); *NKX2-5*, NK2 transcription factor-related, locus 5 (*Drosophila*); *OLIG3*, oligodendrocyte transcription factor 3; *PCDHGA12*, protocadherin γ subfamily A, 12; *PRRX1*, paired-related homeobox 1.

In single marker analysis, *PCDHGA12* showed the highest sensitivity of 80.9% (55/68 patients with LC) and *PRRX1* achieved the highest specificity of 97.0% (32/33 individuals with benign diseases). *PCDHGA12* and *PRRX1* had relatively high diagnostic accuracies with AUCs of 0.859 and 0.830, respectively (Table III). A binary logistic regression analysis was conducted using all six markers to identify the best-performing biomarker combination in 36 splits (6 genes by 6 genes) of the dataset. *PCDHGA12* and *PRRX1* biomarkers were significantly associated with LC. These genes were highly co-methylated according to the correlation analysis (Spearman's correlation coefficient, 0.74). The overall sensitivity of the two-marker combination was 82.4% (56/68 patients with LC; 95% CI, 71.2-90.2%) with a specificity of 87.9% (29/33 individuals with benign diseases; 95% CI, 71.8-96.6%) and AUC of 0.891 (95% CI, 0.813-0.944; Table III). PPV was 93.3% (95% CI, 84.7-97.2%) and NPV was 70.7% (95% CI, 58.7-80.4%). The sensitivities for stage I, II, III and IV LC were 61.5% (8/13), 83.3% (5/6), 92.9% (13/14) and 85.7% (30/35), respectively (Table IV). For detecting LC, sensitivity of this

Table IV. Association between clinicopathological parameters, methylation of two-marker model, and cytology in bronchial washing specimens from 68 patients with LC.

		Methylation-positive (%)		
Parameter	Samples	Two-marker	Cytology	Combined
Sex				
Male	53	46 (86.8)	28 (52.8)	48 (90.6)
Female	15	10 (66.7)	6 (40.0)	10 (66.7)
P-value		0.119	0.560	0.035
Age, years				
<65	11	9 (81.8)	6 (54.5)	10 (90.9)
≥65	57	47 (82.5)	28 (49.1)	48 (84.8)
P-value		>0.999	>0.999	>0.999
Tumor location				
Central	34	33 (97.1)	21 (61.8)	33 (97.1)
Peripheral	34	23 (67.6)	13 (38.2)	25 (73.5)
P-value		0.003	0.089	0.013
Histology				
NSCLC	49	37 (75.5)	24 (49.0)	39 (79.6)
SCLC	19	19 (100.0)	10 (52.6)	19 (100.0)
P-value		0.015	0.795	0.052
Stage				
I	13	8 (61.5)	1 (7.7)	8 (61.5)
II	6	5 (83.3)	4 (66.7)	5 (83.3)
III	14	13 (92.9)	8 (57.1)	13 (92.9)
IV	35	30 (85.7)	21 (60.0)	32 (91.4)
P-value[a]		0.080	0.029	0.027

[a]Methylation positivity is compared between stages I-II and stages III-IV. P-value was calculated by Fisher's exact test. NSCLC, non-small cell lung cancer.

two-marker combination outperformed cytology (McNemar test). When combined with cytology results, its sensitivity was slightly increased to 85.3% (58/68 patients with LC; 95% CI, 74.6-92.7%), maintaining specificity, showing an AUC of 0.866 (95% CI, 0.784-0.926). PPV was 93.6% (95% CI, 85.2-97.3%) and the NPV was 74.4% (95% CI, 61.7-83.9%; Table III).

Subgroup analysis revealed that the methylation status of the two-marker combination was not associated with sex, age or stage (Fisher's exact test). However, it was associated with tumor location and histology (Fisher's exact test) (Table IV). Notably, the sensitivity for SCLC was 100% (19/19 patients with SCLC; Table IV). Specificity was not significantly affected by sex or age (Fisher's exact test).

Discussion

To the best of our knowledge, the present study is the first to demonstrate that the novel combination of methylation biomarkers *PCDHGA12* and *PRRX1* effectively detects early-stage LC. This test may identify patients with nodules who may not require invasive procedures. A large-scale prospective clinical study is warranted to validate its performance for clinical use.

For diagnosing LC, cytology examinations using BW specimens remain a preferred method due to their minimally invasive nature and safety profile during bronchoscopy, but often yield inconclusive results, even when performed by experienced professionals (11,14,15). Despite widespread use, the sensitivity of cytology remains relatively low, with a range of 23.5-32.1% (15). Specific biomarkers for LC are promising diagnostic adjuncts to confirm equivocal cytology findings. Development of molecular biomarker tests using BW specimens presents a promising strategy for enhancing diagnostic accuracy of bronchoscopy (16,17).

Aberrant DNA methylation is considered one of the most influential epigenetic biomarkers in various types of cancer, including LC (9,10,14). In the present study, a genome-wide search using CpG methylation microarray analysis was conducted to identify genes hypermethylated in LC. A total of 10 candidate genes consistently hypermethylated in primary lung tumors compared with paired adjacent non-tumor tissues were selected. A stepwise validation process using bisulfite-pyrosequencing assay identified six potential methylation biomarker candidates for diagnosing LC. Subsequent verification of those biomarkers using LTE-qMSP assay demonstrated that two methylation biomarkers, *PCDHGA12* and *PRRX1*,

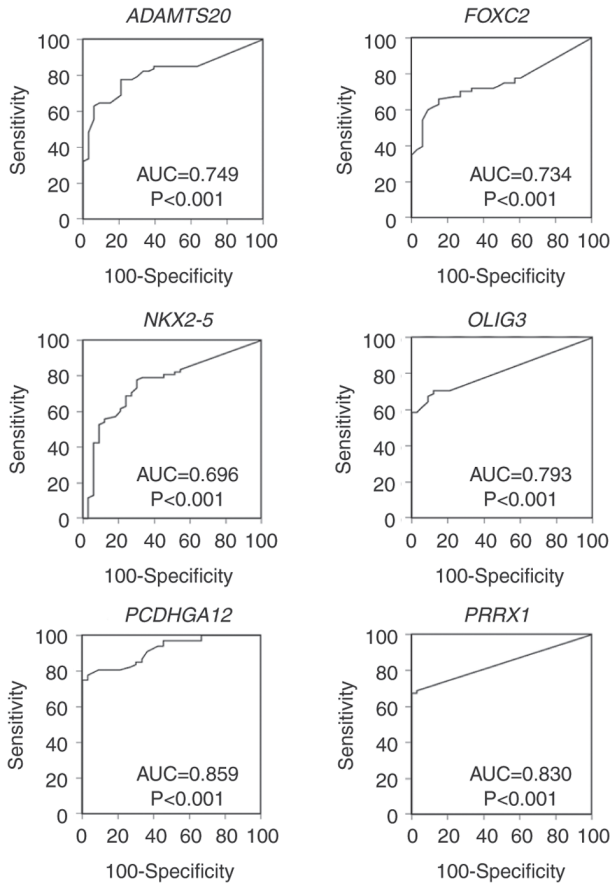

Figure 4. ROC analysis of six genes using BW samples. ROC curves for six genes were analyzed for differentiating LC from non-LC using BW samples. ROC, receiver operating characteristic; BW, bronchial washing; LC, lung cancer; AUC, area under ROC; *ADAMTS20*, ADAM metallopeptidase with thrombospondin type 1 motif 20; *FOXC2*, forkhead box C2 (mesenchyme forkhead 1); *NKX2-5*, NK2 transcription factor-related, locus 5 (*Drosophila*); *OLIG3*, oligodendrocyte transcription factor 3; *PCDHGA12*, protocadherin γ subfamily A, 12; *PRRX1*, paired-related homeobox 1.

had a high potential for detecting LC in BW specimens as a diagnostic adjunct to cytology.

Several studies have reported multiple methylation biomarkers for diagnosing LC using BW specimens (10,16,26-28). Combining cyclin-dependent kinase inhibitor 2A and retinoic acid receptor β2 methylation yields sensitivity of 69% and specificity of 87% (26). *SHOX2* had a sensitivity range of 68.0-75% and a specificity range of 94.0-95.0% (10,27). Roncarati *et al* (28) reported that a four-gene methylation panel [*RASSF1A*, cadherin 1, type 1, E-cadherin (epithelial), DLC1 ϱGTPase activating protein and peripherin] has high sensitivity of 97.0% and a moderate specificity of 74.0%. Recently, a prospective study was conducted using methylated homeobox A9 in bronchial lavage among patients with suspected LC; results of the study showed a sensitivity range of 73.1-80.0% and a specificity range of 85.3-75.6%, with a PPV range of 90.7-78.4% and an NPV range of 61.7-77.3% (16), which are comparable with those in the present study. Another study showed that the 23-gene expression classifier has the potential to detect LC in bronchial epithelial cells collected during bronchoscopy in two multicenter prospective studies with a high sensitivity range of 88.0-89.0% and a low specificity of 47.0% (29,30).

In the case of BW samples from patients with LC at an early stage, a few malignant cells may be present among the larger number of normal cells (10,28). A highly sensitive and accurate detection method should be used to measure neoplastic cell-specific biomarkers in BW specimens. In the present study, LTE-qMSP test was optimized for the 3-plex system in a closed single-tube to measure candidate methylation biomarkers. The clinical performance of six methylation biomarker candidates was evaluated using DNA from BW samples. Logistic regression analysis was used to build a two-biomarker combination model, comprising *PCDHGA12* and *PRRX1*, as the best performing biomarker for diagnosing LC. This two-biomarker combination model test achieved a high sensitivity of 82.4% and a specificity of 87.9%, with a PPV of 93.3% and an NPV of 70.7%.

The present study has several limitations including a small sample size that leads to insufficient statistical power, lack of information on smoking history, an imbalance of the male-to-female ratio of patients with LC (3.5:1.0), and the retrospective case-control study design. Additionally, the two-biomarker combination model test outperformed cytology in sensitivity. However, combining the test with cytology did not significantly improve diagnostic sensitivity. These data indicated that the two-biomarker combination model has a high potential to aid in diagnosing LC as an adjunct value to cytology using BW samples.

The two-biomarker model exhibited lower sensitivity for early-stage LC (I, II) than for late-stage LC (III, IV), which may be attributed to a smaller number of the neoplastic cells in BW samples (10). Notably, the results of the present study indicate markedly higher sensitivity of the two-biomarker model for patients with SCLC compared with that for patients with NSCLC, comparable with research by Jeong *et al* (31), which observed higher sensitivity of *PCDHGA12* for SCLC compared with that for NSCLC. The reasons for this difference warrant further investigation. In addition, the sensitivity for patients with squamous cell carcinoma reached 90.0% but decreased to 66.7% for patients with adenocarcinoma (ADC) in the present study. This decline may be attributed to decreased shedding of ADC cells into the airway (32).

The sensitivity of the two-biomarker model for peripheral LC was significantly lower than that for LC in the central region. At present, to overcome the limitations of standard flexible bronchoscopy in its ability to detect small lung nodules or peripheral lesions, radial-endobronchial ultrasound (R-EBUS) and electromagnetic navigation bronchoscopy (ENB) have been introduced. The American College of Chest Physicians guidelines for diagnosing and managing LC recommend ENB or R-EBUS to evaluate peripheral lung lesions that cannot be accessed with conventional flexible bronchoscopy (33). Therefore, it is worthwhile to investigate whether a two-biomarker test in BW specimens in conjunction with R-EBUS or ENB can improve diagnostic efficacy for patients with peripheral LC.

The present results underscore the potential use of two aberrantly methylated genes, *PCDHGA12* and *PRRX1*, as effective biomarkers for non-invasive diagnostic tests aimed at enhancing LC detection when used adjunctively with cytology in BW specimens. In clinical practice, inconclusive bronchoscopy results often require invasive procedures, potentially resulting

in benign diagnoses in a considerable number of cases, thus leading to cost inefficiency. The proposed application of the methylation biomarker test at an optimal cut-off value, coupled with cytology using BW samples, demonstrates promising sensitivity. This test facilitates identification of patients with a higher likelihood of harboring malignancies, thereby guiding selection of candidates for invasive bronchoscopy procedures. Moreover, the potential clinical benefits of a two-biomarker test are evident in cases classified as inconclusive, where a definitive cytological or histological diagnosis of malignancy is lacking. If the test using two methylation biomarkers is calibrated to optimal cut-off values, resulting in high NPV, it could provide evidence that patients with negative results may avoid unnecessary invasive procedures, thus conferring benefits to patients in a cost-effective manner. However, integrating the two-methylation biomarker model into routine clinical practice requires rigorous validation through large-scale prospective clinical trials.

Acknowledgements

The authors would like to thank Mrs Yangyei Seo and Mr. Jaejin Lee (Genomictree, Inc.), for their technical support with DNA extraction from BW samples.

Funding

The present study was supported by Genomictree, Inc. (Daejeon, South Korea; funding no. LC2-1-DEV).

Availability of data and materials

The data generated in the present study may be found in the Gene Expression Omnibus under accession number GSE246510 or at the following URL: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE246510.

Authors' contributions

SA conceived and designed the study. TJO, SHJ, SJK and MAW acquired, analyzed and interpreted the data. IBJ, MHL and JWS analyzed and interpreted data. TJO and SHJ confirm the authenticity of all the raw data. TJO wrote the manuscript. SA revised the manuscript for important intellectual content. SHJ, SJK and MAW provided administrative, technical or material support. SA supervised the study. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study adhered to local ethics guidelines and was approved by the Institutional Review Board of Chungnam National University Hospital (approval no. CNUH 2022-02-061-002) and the Konyang University Hospital (approval no. 2022-03-025). Written informed consent was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

Genomictree, Inc., provided funding for the study. TJO, SHJ, SJK, MAW and SA are employees of Genomictree, Inc. TJO and SA are shareholders of Genomictree, Inc. The other authors declare that they have no competing interests.

References

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soeromataram I, Jemal A and Bray F: Global caner statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 71: 209-249, 2021.
2. Tomasik B, Skrzypski M, Bieńkowski M, Dziadziuszko R and Jassem J: Current and future applications of liquid biopsy in non-small-cell lung cancer-a narrative review. Trans Lung Cancer Res 12: 594-614, 2023.
3. Blandin Knight S, Crosbie PA, Balata H, Chudziak J, Hussell T and Dive C: Progress and prospects of early detection in lung cancer. Open Biol 7: 170070, 2017.
4. Tsou JA, Galler JS, Siegmund KD, Laird PW, Turla S, Cozen W, Hagen JA, Koss MN and Laird-Offringa IA: Identification of a panel of sensitive and specific DNA methylation markers for lung adenocarcinoma. Mol Cancer 6: 70, 2007.
5. Oken MM, Hocking WG, Kvale PA, Andriole GL, Buys SS, Church TR, Crawford ED, Fouad MN, Isaacs C, Reding DJ, *et al*: Screening by chest radiograph and lung cancer mortality: The prostate, lung, colorectal, and ovarian (PLCO) randomized trial. JAMA 306: 1865-1873, 2011.
6. The National Lung Screening Trial Research Team; Aberle DR, Adams AM, Berg CD, Black WC, Clapp JD, Fagerstrom RM, Gareen IF, Gatsonis C, Marcus PM and Sicks JD: Reduced lung-cancer mortality with low-dose computed tomographic screening. New Eng J Med 365: 395-409, 2011.
7. Zhang C, Yu W, Wang L, Zhao M, Guo Q, Lv S, Hu X and Lou J: DNA methylation analysis of the SHOX2 and RASFF1A panel in bronchoavelolar lavage fluid for lung cancer diagnosis. J Cancer 8: 3585-3591, 2017.
8. Hu S, Tao J, Peng M, Ye Z, Chen Z, Chen H, Yu H, Wang B, Fan J and Ni B: Accurate detection of early-stage lung cancer using a panel of circulating cell-free DNA methylation biomarkers. Biomarker Res 11: 45, 2023.
9. Tsou JA, Hagen JA, Carpenter CL and Laird-Offringa IA: DNA methylation analysis: A powerful new tool for lung cancer diagnosis. Oncogene 21: 5450-5461, 2002.
10. Schmidt B, Liebenberg V, Dietrich D, Schlegel T, Kneip C, Seegebarth A, Flemming N, Seemann S, Distler J, Lewin J, *et al*: *SHOX2* DNA methylation is a biomarker for the diagnosis of lung cancer based on bronchial aspirates. BMC Cancer 10: 600, 2010.
11. Dietrich D, Kneip C, Raji O, Liloglou T, Seegebarth A, Schlegel T, Flemming N, Rausch S, Distler J, Fleischhacker M, *et al*: Performance evaluation of the DNA methylation biomarker *SHOX2* for the aid in diagnosis of lung cancer based on the analysis of bronchial aspirates. Int J Oncol 40: 825-832, 2012.
12. Wiener RS, Wiener DC and Gould MK: Risks of transthoracic needle biopsy: How high?. Clin Plum Med 20: 29-35, 2013.
13. Li P, Liu S, Du L, Mohseni G, Zhang Y and Wang C: Liquid biopsies based on DNA methylation as biomarkers for the detection and prognosis of lung cancer. Clin Epigenetics 14: 118, 2022.
14. Ma S, Yu X, Jin X, Qiu F, Chen X, Wang R and Cao C: The usefulness of liquid-based cytology of bronchoalveolar lavage fluids combined with bronchial brush specimens in lung cancer diagnosis. J Int Med Res 50: 3000605221132708, 2022.
15. Chrabańska M, Środa M, Kiczmer P and Droddzowska B: Lung cancer cytology: Can any of the cytological methods replace histopathology? J Cytol 37: 117-1121, 2020.
16. Wen SWC, Wen J, Hansen TF and Jakobsen A: Cell free methylated tumor DNA in bronchial lavage as an additional tool for diagnosing lung cancer-a systematic review. Cancers (Basel) 14: 2254, 2022.
17. Liang V, Li X, Li W, Zhu X and Li C: DNA methylation in lung cancer patients: Opening a 'window of life' under precision medicine. Biomed Pharmacother 144: 112202, 2021.
18. Esteller M: Epigenetics in cancer. N Eng J Med 358: 1148-1159, 2008.

19. Ibrahim J, Peeters M, Van Camp G and Op de Beeck K: Methylation biomarkers for early cancer detection and diagnosis: Current and future perspectives. Eur J Cancer 178: 91-113, 2023.
20. Anglim PP, Alonzo TA and Laird-Offringa IA: DNA methylation-based biomarkers for early detection of non-small cell lung cancer: An update. Mol Cancer 7: 81, 2008.
21. Wang Z, Xie K, Zhu G, Ma C, Cheng C, Li Y, Xiao X, Li C, Tang J, Wang H, *et al*: Early detection and stratification of lung cancer aided by a cost-effective assay targeting circulating tumor DNA (ctDNA) methylation. Respir Res 24: 163, 2023.
22. Rauch T, Wang Z, Zhang X, Zhong X, Wu X, Lau SK, Kernstine KH, Riggs AD and Pfeifer GP: Homeobox gene methylation in lung cancer studied by genome-wide analysis with a microarray-based methylated CpG island recovery assay. Proc Natl Acad Sci USA 104: 5527-5532, 2007.
23. Hong Y and Kim WJ: DNA methylation markers in lung cancer. Curr Genomics 22: 79-87, 2021.
24. Dejeux E, Audard V, Cavard C, Gut IG, Terris B and Tost J: Rapid identification of promoter hypermethylation in hepatocellular carcinoma by pyrosequencing of etiologically homogeneous sample pools. J Mol Diag 9: 510-520, 2007.
25. Chung W, Bondaruk J, Jelinek J, Lotan Y, Liang S, Czerniak B and Issa JJ: Detection of bladder cancer using novel DNA methylation biomarkers in urine sediments. Cancer Epidemiol Biomarkers Prev 20: 1483-1491, 2011.
26. Grote HJ, Schiemann V, Gedder H, Rohr UP, Kappes R, Gabbert HE and Böcking A: Aberrant promoter methylation of p16(INK4a), RARB2 and SEMA3B in bronchial aspirates from patients with suspected lung cancer. Int J Cancer 116: 720-725, 2005.
27. Ni S, Ye M and Huang T: Short stature homeobox 2 methylation as a potential noninvasive biomarker in bronchial aspirates for lung cancer diagnosis. Oncotarget 8: 61253-61263, 2017.
28. Roncarati R, Lupini L, Miotto E, Saccenti E, Mascetti S, Morandi L, Bassi C, Rasio D, Callegari E, Conti V, *et al*: Molecular testing on bronchial washings for the diagnosis and predictive assessment of lung cancer. Mol Oncol 14: 2163-2175, 2020.
29. Silvestri GA, Vachani A, Whitney D, Elashoff M, Porta Smith K, Ferguson JS, Parsons E, Mitra N, Brody J, Lenburg ME, *et al*: A bronchial genomic classifier for the diagnostic evaluation of lung cancer. N Eng J Med 373: 243-251, 2015.
30. Vachani A, Whitney DH, Parsons EC, Lenburg M, Ferguson JS, Silvestri GA and Spira A: Clinical utility of a bronchial genomic classifier in patients with suspected lung cancer. Chest 150: 210-218, 2016.
31. Jeong IB, Yoon YS, Park SY, Cha EJ, Na MJ, Kwon SJ, Kim JH, Oh TJ, An S, Park CR, *et al*: *PCDHGA12* methylation biomarkers in bronchial washing specimens as an adjunctive diagnostic tool to bronchoscopy in lung cancer. Oncol Lett 16: 1039-1045, 2018.
32. Ahrendt SA, Chow JT, Xu L, Yang SC, Eisenberger CF, Esteller M, Herman JG, Wu L, Decker PA, Jen J and Sidransky D: Molecular detection of tumor cells in bronchoalveolar lavage fluid from patients with early stage lung cancer. J Natl Cancer Inst 91: 332-339, 1999.
33. Liam CK, Lee P, Yu CJ, Bai C and Yasufuku K: The diagnosis of lung cancer in the era of interventional pulmonology. Int J Tuberc Lung Dis 25: 6-15, 2021.