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Combination Analysis of *PCDHGA12* and *CDO1* DNA Methylation in Bronchial Washing Fluid for Lung Cancer Diagnosis

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ABSTRACT

Background: When suspicious lesions are observed on computer-tomography (CT), invasive tests are needed to confirm lung cancer. Compared with other procedures, bronchoscopy has fewer complications. However, the sensitivity of peripheral lesion through bronchoscopy including washing cytology is low. A new test with higher sensitivity through bronchoscopy is needed. In our previous study, DNA methylation of *PCDHGA12* in bronchial washing cytology has a diagnostic value for lung cancer. In this study, combination of *PCDHGA12* and *CDO1* methylation obtained through bronchial washing cytology was evaluated as a diagnostic tool for lung cancer.
Methods: A total of 187 patients who had suspicious lesions in CT were enrolled. *PCDHGA12* methylation test, *CDO1* methylation test, and cytological examination were performed using 3-plex LTE-qMSP test.
Results: Sixty-two patients were diagnosed with benign diseases and 125 patients were diagnosed with lung cancer. The sensitivity of *PCDHGA12* was 74.4% and the specificity of *PCDHGA12* was 91.9% respectively. *CDO1* methylation test had a sensitivity of 57.6% and a specificity of 96.8%. The combination of both *PCDHGA12* methylation test and *CDO1* methylation test showed a sensitivity of 77.6% and a specificity of 90.3%. The sensitivity of lung cancer diagnosis was increased by combining both *PCDHGA12* and *CDO1* methylation tests.
Conclusion: Checking DNA methylation of both *PCDHGA12* and *CDO1* genes using bronchial washing fluid can reduce the invasive procedure to diagnose lung cancer.

Keywords: Lung Cancer; DNA Methylation; *PCDHGA12*; *CDO1*; Bronchial Washing Fluid

INTRODUCTION

In order to diagnose lung cancer, we relied on computer-tomography (CT) images. When suspicious lesions are observed on CT, lung cancer is diagnosed through biopsy. According to a previous study, the false positive rate of low dose CT for lung nodule was 94%.[1] Although low dose CT is used for lung cancer screening, CT has limitations because the rate of false

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Disclosure

The authors have no potential conflicts of interest do disclose.

Author Contributions

Conceptualization: Park SJ, Kang D, Lee M, Kwon SJ, Son JW, Jeong IB. Data curation: Park SJ, Kang D, Park YG, Hwang WJ, Son JW, Jeong IB. Formal analysis: Lee M, Lee SY, Oh T, Jang S, An S. Investigation: Kang D, Lee M, Lee SY, Park YG, Hwang WJ, Jeong IB. Funding acquisition: Son JW. Methodology: Park SJ, Lee M, Lee SY, Park YG, Oh T, Jang S, Hwang WJ, An S. Project administration: Son JW, Jeong IB. Resources: Oh T, Jang S, An S. Visualization: Oh T, Jang S, An S. Writing - original draft: Park SJ, Kang D. Writing - review & editing: Park SJ, Kang D, Kwon SJ, Son JW, Jeong IB.

positive is high. Therefore, biopsy is necessary for diagnosis of lung cancer. Compared with other procedures, bronchoscopy has lower risk for complications.[2] However, the sensitivity of peripheral lesion through bronchoscopy including washing cytology is low.[3] A new test with higher sensitivity through bronchoscopy is needed.

DNA methylation is an epigenetic mechanism that inactivates cancer suppressor genes and cancer related genes. DNA methylation has been studied as a cancer diagnostic biomarker because it was frequently found in cancer development.[4] Recently, DNA methylation is used as a diagnostic tool in various cancers.[5,6] In lung cancer, DNA methylation is used as a marker for diagnosis, treatment, and prediction of prognosis.[7-9] DNA methylation in tissue or body fluid is used recently for diagnosis of lung cancer.[9] Many studies have performed diagnosis of lung cancer through DNA methylation in bronchoalveolar lavage fluid (BALF).[10]

It is known that protocadherin affects tissue development and growth.[11] Genes that code for protocadherin can affect the development of various cancers.[11-13] Hypermethylation of protocadherin gamma subfamily A12 (*PCDHGA12*) affects various cancers including lung cancer.[14] We have found that DNA methylation of *PCDHGA12* in bronchial washing cytology has a diagnostic value for lung cancer in a previous study.[15] However, its specificity was low although its sensitivity was high.[15] Therefore, further study is needed to improve its low specificity.

We used a DNA methylation gene set to make up for the limitation of our previous study and decided to use cysteine dioxygenase type 1 (*CDO1*). *CDO1* is known to affect protein function and antioxidant defense mechanism.[16] *CDO1* promotor methylation is related to progression and malignancy of various tumors.[16-18] *CDO1* methylation can be used for diagnosis of cancers including lung cancer.[19-21]

We previously reported a highly sensitive and accurate two-step Linear Target Enrichment (LTE)-quantitative methylation specific real-time polymerase chain reaction (PCR) (qMSP) assay (LTE-qMSP) to detect *PCDHGA12* methylation using bronchial washing cytology.[15] However, this method had limitations, such as the risk of cross-contamination due to the two-step PCR procedure requiring multiple pipetting steps and open-up tubes. In this study, we applied the 3-plex one-step LTE-qMSP assay that uses a single closed-tube reaction to simultaneously detect *PCDHGA12* and *CDO1* methylation targets DNA. Both *PCDHGA12* methylation and *CDO1* methylation obtained through bronchial washing cytology were evaluated as a diagnostic tool for lung cancer. Additionally, we analyzed relationships of the combination of *PCDHGA12* and *CDO1* methylation with clinicopathological parameters including gender, age, cancer location, histology, and staging of lung cancer.

METHODS

Study design

A prospective study was designed to evaluate clinical performance of *PCDHGA12* and *CDO1* genes for detecting lung cancer using bronchial washing samples. Patients (n = 187) suspected with lung cancer scheduled for bronchoscopy at Konyang University Hospital (Daejeon, Korea) between May 2020 and November 2022 were enrolled. The indications for bronchoscopy of CT images suspected of lung cancer are shown in **Table 1**. Results of bronchoscopy or histopathology examination were not informed to the personnel involved in the laboratory work. Data analysis of LTE-qMSP results were independently performed to

Table 1. Indications for bronchoscopy of CT images suspected of lung cancer

CT findings
Mass (size > 30 mm)
GGO (size > 30 mm)
Cavitary lesion
Consolidation with central necrosis
Non-resolving pneumonia (consolidation, GGO, and mixed consolidation and GGO)
Single solid nodule
a. Size > 8 mm
b. 'Increase size of previously > 6 mm nodule at follow-up CT (until 2 yr)
Multiple solid nodule
a. Size > 8 mm
b. Increase in size of previously > 6 mm nodule at follow-up CT (until 2 yr)
Single subsolid nodule (GGO, partly solid)
a. Size > 6 mm
b. Increase in size of previous 6–30 mm GGO at follow-up CT (until 5 yr)
Multiple subsolid nodule (GGO, partly solid)
a. Size > 6 mm
b. Increase in size of previous 6–30 mm GGO at follow-up CT (until 5 yr)

CT = computer-tomography, GGO = ground-glass opacity.

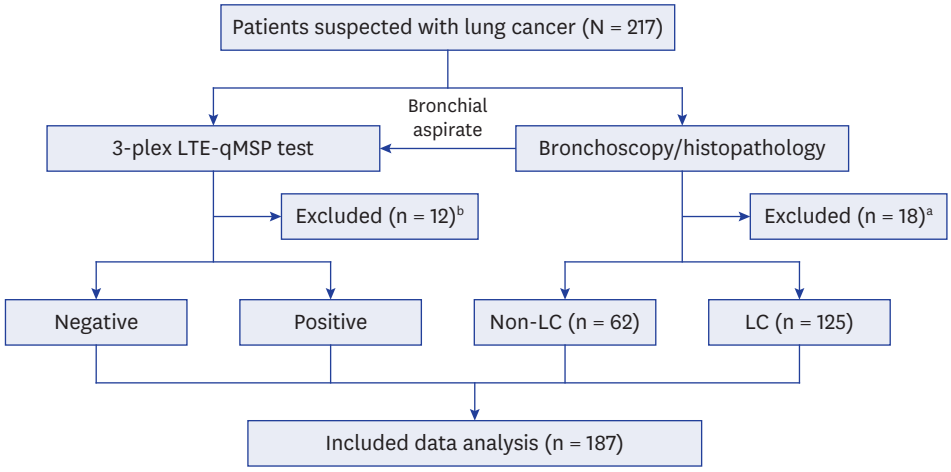

Fig. 1. Flowchart of the combined analysis of protocadherin gamma subfamily A12 and cysteine dioxygenase type 1 DNA methylation for lung cancer.
LTE-qMSP = Linear Target Enrichment-quantitative methylation specific real-time polymerase chain reaction, LC = lung cancer.
[a]Excluded due to insufficient specimen quantity; [b]excluded due to unclear diagnosis or metastatic cancer.

compare bronchoscopy findings and pathology outcomes as reference standards. The outline of this study is illustrated in **Fig. 1**. Among 217 patients, 12 patients were excluded due to metastatic cancer and 18 patients were excluded due to insufficient samples.

Clinicopathological and demographical characteristics of patients enrolled in this study are shown in **Table 2**.

Bronchial washing samples, DNA isolation, and bisulfite treatment

Fresh bronchial washing samples (10 mL each) were collected into preservative buffer (Genomictree, Inc., Daejeon, Korea) during bronchoscopy. Samples were kept at −20°C until DNA extraction.

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Table 2. Clinical and demographic characteristics of the patients test in this study

Characteristics	No. (%)
Non-lung cancer (benign)	62
Gender	
Male	30 (48.4)
Female	32 (51.6)
Mean age (range)	66.3 (29.0–88.0)
Lung cancer	125
Gender	
Male	100 (80.0)
Female	25 (20.0)
Mean age (range)	70.0 (31.0–93.0)
Pathological stage	
Stage I	29
Stage II	12
Stage III	22
Stage IV	62
Histologic type	
Squamous	42
Adenocarcinoma	53
NSCLC, other types[a]	4
Small-cell lung cancer	26
Location	
Central	58
Peripheral	67

NSCLC = non-small-cell lung cancer.
[a]Carcinoma, mucoepidermoid, neuroendocrine, pleomorphic carcinoma.

Genomic DNA was isolated using a solid phase magnetic bead-based GT NUCLEIC ACID PREP Kit (Genomictree, Inc.) according to the manufacturer's instructions. The 20 ng of the genomic DNA was then chemically modified with sodium bisulfite using an EZ DNA Methylation Gold kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. Bisulfite-converted DNA was purified and eluted with 18 μL of distilled water using a Zymo-Spin IC column (Zymo Research). Eluted DNA was immediately subjected to methylation analysis.[15]

Measurement of methylation for target genes in bronchial washing samples by 3-plex LTE-qMSP test

We developed a 3-plex LTE-qMSP test for measuring *PCDHGA12* and *CDO1* methylation targets and control gene in a single-tube. This test was performed by trained personnel who were unaware of bronchoscopy or histopathology results. A total of 20 ng of genomic DNA was used as an input. A 25 μL reaction mixture contained 5 μL of bisulfite-converted DNA, *PCDHGA12* and *CDO1* methylation-specific forward primer, *PCDHGA12* and *CDO1* methylation-specific reverse primer linked to a 5' universal tag sequence, *PCDHGA12* probe (5'-FAM), *CDO1* probe (5'-HEX), *COL2A1*-specific forward and reverse primers, *COL2A1* probe (5'-Cy5), universal tag sequence, and 5 μL of 5 x Fast qPCR PreMIX TaqMan Probe (Enzynomics, Inc., Daejeon, Korea). Real-time PCR was performed on an AB7500 FAST Real-Time PCR system (Thermo Fisher Scientific, Waltham, MA, USA). Thermal cycling conditions were as follows: 95°C for 5 minutes; 15 cycles of 95°C for 15 seconds and 70°C for 45 seconds; followed by 35 cycles of 95°C for 15 seconds and 60°C for 45 seconds. Heating and cooling rates were ≥ 4°C per second and ≥ 3.5°C per second, respectively. Primers and probes used for the 3-plex LTE-qMSP test are listed in **Table 3**.

We performed the 3-plex LTE-qMSP test once for each sample. The relative level of methylated gene in each sample was calculated as $35-{}^{\triangle}C_T$ [C_T of amplified target gene – C_T

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Table 3. Primer and probe sequences used in the 3-plex Linear Target Enrichment-quantitative methylation specific real-time polymerase chain reaction assay

Gene	Primers and probes	Sequences, 5′-3′[a]	Concentration, nmol/L
PCDHGA12	Sense	CGGTGCGTATAGGTATCGC	200
	Anti-sense	CTCGCGCGATACTCGAACTAAACAAACG	80
	Probe	FAM-CGCGTGATGGTTTTGGATGCGAACGAT-BQ1	200
CDO1	Sense	GAGAGATTGCGCGGAGTTTAC	200
	Anti-sense	CGAAAACGAAAAAACCCTACGAACACGACTC	40
	Probe	HEX-TTTTTGGGAAGGCGCGGAGTTCGGGGAAGT-BQ1	200
COL2A1	Sense	TAGGAGTATTAGTAATGTTAGGAGTA	100
	Anti-sense	CTACCCCAAAAAAACCCAATCC	100
	Probe	Cy5-AGAAGAAGGGAGGGGTGTTAGGAGAGG	100
Universal sequence tag		AAAGATTCGGCGACCACCGA	400

PCDHGA12 = protocadherin gamma subfamily A12, *CDO1* = cysteine dioxygenase type 1, *COL2A1* = collagen type II alpha 1 chain.
[a]Underlines indicated CpG dinucleotide sites.

of *COL2A1* (human reference gene)].[22] Higher values of 35-$^{\triangle}C_T$ indicated higher levels of methylation. If the C_T of target gene was undetectable, the value was set to 20, the value closest to the lowest 35-$^{\triangle}C_T$ for all test results.

Statistical analysis

We calculated receiver operating characteristic (ROC), area under ROC (AUC), and 95% confidence interval (CI) to determine the accuracy, sensitivity, and specificity of diagnosis using the MedCalc software, version 9.3.2.0 (MedCalc Software Ltd., Basel, Belgium). Statistical significance was considered when *P* value was less than 0.05. To calculate sensitivity and specificity, we categorized test results in a dichotomous manner: methylation-positive as '1' and methylation-negative as '0.' To describe demographic and other clinical characteristics, we used frequency and percentile (%). We also computed negative and positive predictive values.

Ethics statement

All participants in this study provided written informed consent. This study adhered to local ethics guidelines. The study plan was approved by the Institutional Review Board (IRB) of Konyang University Hospital in South Korea (IRB number 2022-03-025).

RESULTS

Clinical and demographic characteristics

A total of 187 patients were enrolled in this study, including 62 patients who were diagnosed with a benign disease and 125 patients who were diagnosed with lung cancer. Benign diseases were confirmed by histological examination through biopsy or improvement of CT lesion after 6 months follow-up. The mean age of patients with a benign disease was 66.3 years and the mean age of patients with lung cancer was 70.0 years. Among 125 lung cancer patients, there were 29 with stage I, 12 with stage II, 22 with stage III, and 62 with stage IV. In lung cancer patients, adenocarcinoma was the most common with 53 cases. Four patients were confirmed to have other types of NSCLC, including carcinoma, mucoepidermoid, neuroendocrine, and pleomorphic carcinoma. There were more peripheral cases than central cases (**Table 2**).

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Diagnostic performance of 3-plex LTE-qMSP test in detecting lung cancer using bronchial washing samples

The 3-plex LTE-qMSP test was performed using DNAs from 187 patients composed of 125 lung cancer patients at various stages (I to IV) and 62 non-lung cancer patients. Results revealed that significantly higher levels of *PCDHGA12* and *CDO1* methylation were present in DNAs from lung cancer patients than in DNAs from non-lung cancer patients ($P < 0.001$, Kruskal-Wallis test) (**Fig. 2**). Optimal cutoff values for *PCDHGA12* and *CDO1* were determined to be 30.5 and 28.5 of 35-$^{\triangle}C_T$ for detecting lung cancer, respectively (**Fig. 3**). For *PCDHGA12*, its sensitivity and specificity were 74.4% (93/125, 95% CI, 65.8–81.8%) and 91.9% (57/62, 95% CI, 82.2–97.3%), respectively, with an AUC of 0.832 (95% CI, 0.770–0.882, $P < 0.001$). For *CDO1*, its sensitivity and specificity were 57.6% (72/125, 95% CI, 48.4–66.4%) and 96.8% (60/62, 95% CI, 88.8–99.6%), respectively, with an AUC of 0.772 (95% CI, 0.705–0.830, $P < 0.001$). The combination of both genes revealed a sensitivity of 77.6% (97/125 95% CI, 69.3–84.6%) and a specificity of 90.3% (56/62, 95% CI, 80.1–96.4%), with an AUC of 0.840 (95% CI, 0.779–0.889) (**Table 4**). Cytology achieved a sensitivity of 39.2% (49/125 95% CI, 30.6–48.3%) and a specificity of 100% (62/62, 95% CI, 94.2–100%), with an AUC of 0.696 (95% CI, 0.625–0.761) (**Table 4**). Combining the two methylation genes and cytology for detecting lung cancer showed an overall sensitivity of 80.8% (101/125, 95% CI, 72.8–87.3%) and a specificity of 90.3% (56/62, 95% CI, 80.1–96.4%), with an AUC of 0.856 (95% CI, 0.797–0.903) (**Table 4**).

Subgroup analysis of methylation test and cytology for lung cancer

Results of subgroup analysis for lung cancer patients are shown in **Table 5**. Compared with *PCDHGA12* methylation test, combined test for methylation of both genes showed a higher sensitivity in males (81.0%), those with age ≥ 65 years (81.3%), those with a central location (94.7%), those with SCLC (90.9%), and those with stages III–IV (88.0%). Combining methylation of both genes and cytology had a significant diagnostic value for central lung cancer (sensitivity 94.7%, $P < 0.001$) and stage III–IV lung cancer (sensitivity 91.6%, $P < 0.001$). In all subgroup analyses, the sensitivity was higher when combining methylation tests for both genes than *PCDHGA12* or *CDO1* methylation test alone and cytology alone. **Table 6** shows results according to the location of lung suspicious lesion. There was no significant difference in DNA methylation according to the location of suspicious lesion. In this study, there were 10

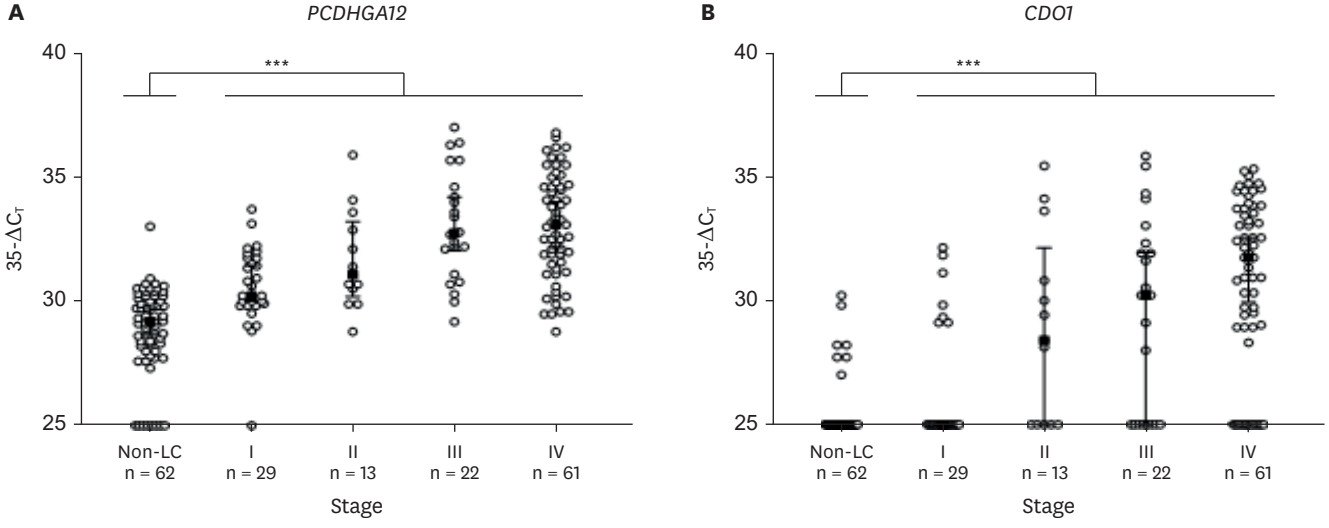

Fig. 2. The DNA methylation level of *PCDHGA12* gene (**A**) and *CDO1* (**B**) in lung cancer patients and non-LC patients.
PCDHGA12 = protocadherin gamma subfamily A12, *CDO1* = cysteine dioxygenase type 1, LC = lung cancer.

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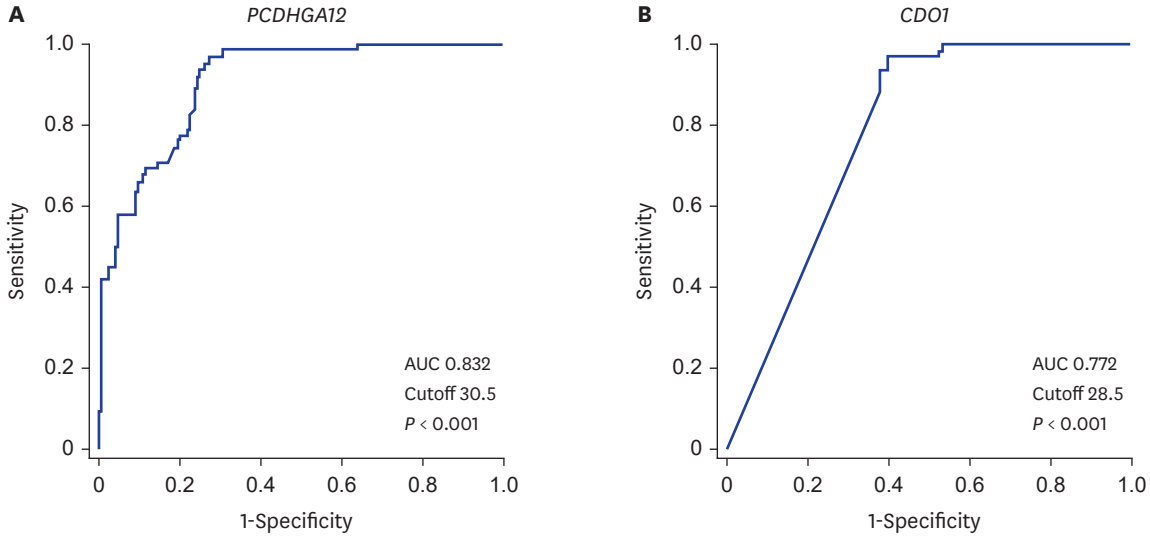

Fig. 3. Receiver operating characteristic curve for *PCDHGA12* gene (**A**) and *CDO1* (**B**) for diagnosing lung cancer in bronchial washing fluids.
PCDHGA12 = protocadherin gamma subfamily A12, *CDO1* = cysteine dioxygenase type 1, AUC = area under receiver operating characteristic.

Table 4. Clinical performance of the methylation test and cytology for detecting lung cancer

Test	Cut-off (35-ΔCT)	Sensitivity	Specificity	PPV	NPV
Methylation					
PCDHGA12	30.5	74.4 (65.8–81.8)	91.9 (82.2–97.3)	94.9 (88.9–97.7)	64.0 (56.7–70.8)
CDO1	28.5	57.6 (48.4–66.4)	96.8 (88.8–99.6)	97.3 (90.1–99.3)	53.1 (47.9–58.3)
Cytology		39.2 (30.6–48.3)	100.0 (94.2–100)	100.0	44.9 (41.5–48.4)
Combined					
PCDHGA12 or *CDO1*		77.6 (69.3–84.6)	90.3 (80.1–96.4)	94.2 (88.3–97.2)	66.7 (58.8–73.7)
PCDHGA12 or cytology		77.6 (69.3–84.6)	91.9 (82.2–97.3)	95.1 (89.3–97.8)	67.1 (59.3–74.0)
CDO1 or cytology		64.0 (54.9–72.4)	96.8 (88.8–99.6)	97.6 (91.0–99.4)	57.1 (51.2–62.9)
PCDHGA12 or *CDO1* or cytology		80.0 (71.9–86.6)	90.3 (80.1–96.4)	94.3 (88.6–97.3)	69.1 (61.0–76.3)

Values are presented as % (95% confidence interval).
PCDHGA12 = protocadherin gamma subfamily A12, *CDO1* = cysteine dioxygenase type 1, PPV = positive predictive value, NPV = negative predictive value.

patients who underwent surgery without biopsy for histological confirmation, of which 8 were diagnosed with cancer and the other 2 had a non-cancerous disease.

DISCUSSION

In our previous study, *PCDHGA12* methylation for diagnosing lung cancer had a sensitivity of 75.0%, a specificity of 78.9%, a positive predictive value of 84.9%, and a negative predictive value of 66.7%.[15] Compared with our previous study, the sensitivity and specificity were increased by combining methylation of both genes in the present study. The positive predictive value of combining methylation of both *PCDHGA12* and *CDO1* was also superior to that of the previous *PCDHGA12* methylation test, although the negative predictive value of combining methylation of both *PCDHGA12* and *CDO1* was not inferior to that of the *PCDHGA12* methylation test in the previous study. Additionally, when methylation of both genes was combined with cytology, sensitivity, specificity, positive predictive value, and negative predictive value all showed better results. According to previous studies on DNA methylation and lung cancer diagnosis, sensitivity was increased by adding different types of methylation markers compared to lung cancer diagnosis through a single DNA methylation marker.[10,23,24] In the present study, the sensitivity was increased by combining methylation of *PCDHGA12* and *CDO1* compared with our previous study.[15]

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Table 5. The relationship between clinicopathological parameters, methylation and cytology in bronchial aspirate samples from 125 lung cancer patients

Parameters	No. of samples tested	Sensitivity % (95% CI)					
		Methylation[a]	*P* value[b]	Cytology	*P* value[b]	Methylation or cytology	*P* value[b]
Gender			0.105		0.821		0.047
Male	100	81.0 (71.9–88.2)		40.0 (30.3–50.3)		84.0 (75.3–90.6)	
Female	25	64.0 (42.5–82.0)		36.0 (18.0–57.5)		64.0 (42.5–82.0)	
Age, yr			0.147		0.412		0.133
< 65	34	67.6 (49.4–82.6)		32.4 (17.4–50.6)		70.6 (52.5–84.9)	
≥ 65	91	81.3 (71.8–88.7)		41.8 (31.5–52.6)		83.5 (74.3–90.5)	
Cancer location			< 0.001		0.006		< 0.001
Central	57	94.7 (85.3–98.9)		52.6 (38.9–66.0)		94.7 (85.3–98.9)	
Peripheral	68	63.2 (50.6–74.6)		27.9 (17.7–40.1)		67.6 (55.2–78.5)	
Histology			0.157		1.000		0.241
SCLC	22	90.9 (70.8–98.9)		36.4 (17.2–59.4)		90.9 (70.8–98.9)	
NSCLC	103	74.8 (65.3–82.8)		35.9 (26.7–46.0)		77.7 (68.4–85.3)	
Stage			< 0.001		< 0.001		< 0.001
I–II	42	57.1 (40.9–72.2)		16.7 (7.0–31.4)		57.1 (40.9–72.2)	
III–IV	83	88.0 (79.0–94.1)		50.6 (39.4–61.8)		91.6 (83.4–96.6)	

CI = confidence interval, SCLC = small-cell lung cancer, NSCLC = non-small cell lung cancer.
[a]Protocadherin gamma subfamily A12 or cysteine dioxygenase type 1.
[b]*P* value was calculated by Fisher's exact test.

Table 6. The relationship between mass location, methylation and cytology in bronchial aspirate samples from 125 lung cancer patients

Parameters	No. of samples tested	Sensitivity % (95% CI)					
		Methylation[a]	*P* value[b]	Cytology	*P* value[b]	Methylation or cytology	*P* value[b]
Mass location			0.218		0.816		0.326
RUL	37	78.4 (61.8–90.2)		43.2 (27.1–60.5)		78.4 (61.8–90.2)	
RML	6	66.7 (22.3–95.7)		33.3 (4.3–77.7)		66.7 (22.3–95.7)	
RLL	37	64.9 (47.5–79.8)		40.5 (24.7–57.9)		70.3 (53.1–84.2)	
LUL	23	82.6 (61.2–95.0)		43.5 (23.2–65.5)		87.0 (66.5–97.2)	
LLL	19	94.7 (73.9–99.9)		31.6 (12.6–56.6)		94.7 (73.9–99.9)	
RML-RLL & LUL	1	100.0 (2.5–100.0)		0 (0–97.5)		100.0 (2.5–100.0)	
Unknown	2	100 (15.8–100.0)		0 (0–84.2)		100.0 (15.8–100.0)	

CI = confidence interval, RUL = right upper lobe, RML = right middle lobe, RLL = right lower lobe, LUL = left upper lobe, LLL = left lower lobe.
[a]Protocadherin gamma subfamily A12 or cysteine dioxygenase type 1.
[b]*P* value was calculated by Chi-squared test.

In this study, we improved the specificity of the methylation test as compared to our previous study. In the previous study, we used a two-step LTE-qMSP method to measure methylation in bronchial washing samples. However, this method involves open tubes and multiple pipetting steps, which increases the risk of cross-contamination and prolonged assay time. To overcome these limitations, we employed a one-step LTE-qMSP assay using a single closed-tube reaction to simultaneously detect multiple methylated targets of DNA.[25] This method has several advantages such as low false-positive and negative rates and minimal risk of PCR contamination.

Lung cancer is diagnosed through low dose CT and biopsy. As mentioned earlier, low dose CT has high false positive rate and biopsy has many complications.[1] Biopsy through bronchoscopy is difficult to find the exact bronchus that has lesion.[26] Recently, many studies are being conducted to diagnose lung cancer with non-invasive tests such as liquid biopsy to minimize complication through invasive test including bronchoscopy biopsy and surgical biopsy.[27,28] A liquid biopsy is a test for confirming substances present in blood or bodily secretion. Through liquid biopsy, lung cancer can be diagnosed by confirming circulating tumor cells, circulating tumor DNA, exosome, and microRNA in blood or bodily fluids including bronchial washing cytology.[27] However, bronchial washing cytology has limitation in screening or diagnosis for lung cancer due to its low sensitivity.[29] Therefore, finding a new tumor marker that can suggest lung cancer with a high sensitivity is needed to reduce invasive tests and supplement the false positive rate of a low-dose CT.

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Previous studies on bronchoscopy have used BALF.[10,30,31] BALF analysis is invasive. It can induce several complications to patients including respiratory failure, bronchospasm.[32] To diagnose lung cancer, percutaneous transthoracic needle aspiration biopsy can also induce complications including pneumothorax and air embolism.[33,34] In this study, we used simple bronchial washing fluid. It is less invasive than other procedures with complications. If data on DNA methylation tests through bronchial washing fluid are accumulated, invasive tests such as BALF analysis and percutaneous transthoracic needle aspiration biopsy for lung cancer diagnosis can be reduced. Through reducing the invasive tests, there are some advantages including lesser costs, shorter hospital days and lesser complications.[35]

Recent studies have tried to diagnose lung cancer through DNA methylation.[7-9] In our previous study, we found that methylation of *PCDHGA12* had a diagnostic value of lung cancer.[15] Compared with our previous study, the sensitivity was increased by combining methylation of *CDO1* and the specificity was increased by changing technological method. According to this study, it was found that diagnosis through bronchial washing cytology and both gene methylation can be helpful in increasing the diagnosis rate of lung cancer.

This study has several limitations. The small sample size was the first limitation. A total of 187 patients were enrolled. Statistical limitations could not be ruled out due to the small sample size. In subgroup analysis, some subgroups including male, central location lung cancer, and stage III–IV lung cancer has a significant diagnostic sensitivity. However, other groups dividing by age or histology did not show any significant difference in sensitivity. Further study is needed to overcome the limitation of subgroup analysis in this study. To increase the diagnostic value of lung cancer through DNA methylation, it is important to find optimal combination of DNA methylation. This study did not combine with methylation of other DNAs known to be associated with lung cancer including SOX17, TAC1, HOXA7, and RASSF1A.[10,36-38] Lastly, in this study, a statistically significant increase in sensitivity was shown as the stage of the patients in the subgroup progressed, with 57.1% in stages I and II compared to 88% in stage III and IV of lung cancer. The sensitivity of this study increases as the stage progresses, however patients with advanced lung cancer require biopsy for chemotherapy rather than surgery. Hence, the DNA methylation method will be more beneficial for screening rather than having a significant impact on diagnosis and treatment.

In conclusion, the sensitivity for lung cancer diagnosis was increased after combining results of both *PCDHGA12* and *CDO1* methylation tests. Therefore, checking DNA methylation of both genes using bronchial washing fluid can reduce invasive procedure for diagnosing lung cancer.

REFERENCES

1. National Lung Screening Trial Research Team, Aberle DR, Adams AM, Berg CD, Black WC, Clapp JD, et al. Reduced lung-cancer mortality with low-dose computed tomographic screening. *N Engl J Med* 2011;365(5):395-409.
PUBMED | CROSSREF
2. Tukey MH, Wiener RS. Population-based estimates of transbronchial lung biopsy utilization and complications. *Respir Med* 2012;106(11):1559-65.
PUBMED | CROSSREF
3. Detterbeck FC, Lewis SZ, Diekemper R, Addrizzo-Harris D, Alberts WM. Executive summary: diagnosis and management of lung cancer, 3rd ed: American College of Chest Physicians evidence-based clinical practice guidelines. *Chest* 2013;143(5 Suppl):7S-37S.
PUBMED | CROSSREF

4. Kulis M, Esteller M. DNA methylation and cancer. *Adv Genet* 2010;70:27-56.
PUBMED | CROSSREF
5. Kandimalla R, van Tilborg AA, Zwarthoff EC. DNA methylation-based biomarkers in bladder cancer. *Nat Rev Urol* 2013;10(6):327-35.
PUBMED | CROSSREF
6. Wang Y, Zhang M, Hu X, Qin W, Wu H, Wei M. Colon cancer-specific diagnostic and prognostic biomarkers based on genome-wide abnormal DNA methylation. *Aging (Albany NY)* 2020;12(22):22626-55.
PUBMED | CROSSREF
7. Darılmaz Yüce G, Ortaç Ersoy E. Lung cancer and epigenetic modifications. *Tuberk Toraks* 2016;64(2):163-70.
PUBMED | CROSSREF
8. Langevin SM, Kratzke RA, Kelsey KT. Epigenetics of lung cancer. *Transl Res* 2015;165(1):74-90.
PUBMED | CROSSREF
9. Mehta A, Dobersch S, Romero-Olmedo AJ, Barreto G. Epigenetics in lung cancer diagnosis and therapy. *Cancer Metastasis Rev* 2015;34(2):229-41.
PUBMED | CROSSREF
10. Zhang C, Yu W, Wang L, Zhao M, Guo Q, Lv S, et al. DNA methylation analysis of the SHOX2 and RASSF1A panel in bronchoalveolar lavage fluid for lung cancer diagnosis. *J Cancer* 2017;8(17):3585-91.
PUBMED | CROSSREF
11. Dang Z, Shangguan J, Zhang C, Hu P, Ren Y, Lv Z, et al. Loss of protocadherin-17 (PCDH-17) promotes metastasis and invasion through hyperactivation of EGFR/MEK/ERK signaling pathway in hepatocellular carcinoma. *Tumour Biol* 2016;37(2):2527-35.
PUBMED | CROSSREF
12. Chen T, Long B, Ren G, Xiang T, Li L, Wang Z, et al. Protocadherin20 acts as a tumor suppressor gene: epigenetic inactivation in nasopharyngeal carcinoma. *J Cell Biochem* 2015;116(8):1766-75.
PUBMED | CROSSREF
13. Taylor KH, Pena-Hernandez KE, Davis JW, Arthur GL, Duff DJ, Shi H, et al. Large-scale CpG methylation analysis identifies novel candidate genes and reveals methylation hotspots in acute lymphoblastic leukemia. *Cancer Res* 2007;67(6):2617-25.
PUBMED | CROSSREF
14. Lu Y, Lemon W, Liu PY, Yi Y, Morrison C, Yang P, et al. A gene expression signature predicts survival of patients with stage I non-small cell lung cancer. *PLoS Med* 2006;3(12):e467.
PUBMED | CROSSREF
15. Jeong IB, Yoon YS, Park SY, Cha EJ, Na MJ, Kwon SJ, et al. *PCDHGA12* methylation biomarker in bronchial washing specimens as an adjunctive diagnostic tool to bronchoscopy in lung cancer. *Oncol Lett* 2018;16(1):1039-45.
PUBMED | CROSSREF
16. Brait M, Ling S, Nagpal JK, Chang X, Park HL, Lee J, et al. Cysteine dioxygenase 1 is a tumor suppressor gene silenced by promoter methylation in multiple human cancers. *PLoS One* 2012;7(9):e44951.
PUBMED | CROSSREF
17. Harada H, Hosoda K, Moriya H, Mieno H, Ema A, Ushiku H, et al. Cancer-specific promoter DNA methylation of cysteine dioxygenase type 1 (CDO1) gene as an important prognostic biomarker of gastric cancer. *PLoS One* 2019;14(4):e0214872.
PUBMED | CROSSREF
18. Meller S, Zipfel L, Gevensleben H, Dietrich J, Ellinger J, Majores M, et al. CDO1 promoter methylation is associated with gene silencing and is a prognostic biomarker for biochemical recurrence-free survival in prostate cancer patients. *Epigenetics* 2016;11(12):871-80.
PUBMED | CROSSREF
19. Nishizawa N, Harada H, Kumamoto Y, Kaizu T, Katoh H, Tajima H, et al. Diagnostic potential of hypermethylation of the cysteine dioxygenase 1 gene (CDO1) promoter DNA in pancreatic cancer. *Cancer Sci* 2019;110(9):2846-55.
PUBMED | CROSSREF
20. Ushiku H, Yamashita K, Ema A, Minatani N, Kikuchi M, Kojo K, et al. DNA diagnosis of peritoneal fluid cytology test by CDO1 promoter DNA hypermethylation in gastric cancer. *Gastric Cancer* 2017;20(5):784-92.
PUBMED | CROSSREF
21. Wang P, Zhao H, Shi R, Liu X, Liu J, Ren F, et al. The role of plasma CDO1 methylation in the early diagnosis of lung cancer. *Zhongguo Fei Ai Za Zhi* 2020;23(5):314-20.
PUBMED | CROSSREF
22. Chung W, Bondaruk J, Jelinek J, Lotan Y, Liang S, Czerniak B, et al. Detection of bladder cancer using novel DNA methylation biomarkers in urine sediments. *Cancer Epidemiol Biomarkers Prev* 2011;20(7):1483-91.
PUBMED | CROSSREF

23. Kneip C, Schmidt B, Seegebarth A, Weickmann S, Fleischhacker M, Liebenberg V, et al. SHOX2 DNA methylation is a biomarker for the diagnosis of lung cancer in plasma. *J Thorac Oncol* 2011;6(10):1632-8.
PUBMED | CROSSREF

24. Zhai X, Li SJ. Methylation of RASSF1A and CDH13 genes in individualized chemotherapy for patients with non-small cell lung cancer. *Asian Pac J Cancer Prev* 2014;15(12):4925-8.
PUBMED | CROSSREF

25. An S, Oh T. Method for multiplex detection of methylated DNA. United States patent US11186866B2. https://patents.google.com/patent/US11186866B2/en. Updated 2021. Accessed November 30, 2021.

26. Funahashi A, Browne TK, Houser WC, Hranicka LJ. Diagnostic value of bronchial aspirate and postbronchoscopic sputum in fiberoptic bronchoscopy. *Chest* 1979;76(5):514-7.
PUBMED | CROSSREF

27. Li W, Liu JB, Hou LK, Yu F, Zhang J, Wu W, et al. Liquid biopsy in lung cancer: significance in diagnostics, prediction, and treatment monitoring. *Mol Cancer* 2022;21(1):25.
PUBMED | CROSSREF

28. Rolfo C, Mack P, Scagliotti GV, Aggarwal C, Arcila ME, Barlesi F, et al. Liquid biopsy for advanced NSCLC: a consensus statement from the International Association for the Study of Lung Cancer. *J Thorac Oncol* 2021;16(10):1647-62.
PUBMED | CROSSREF

29. Girard P, Caliandro R, Seguin-Givelet A, Lenoir S, Gossot D, Validire P, et al. Sensitivity of cytology specimens from bronchial aspirate or washing during bronchoscopy in the diagnosis of lung malignancies: an update. *Clin Lung Cancer* 2017;18(5):512-8.
PUBMED | CROSSREF

30. Villalba M, Exposito F, Pajares MJ, Sainz C, Redrado M, Remirez A, et al. TMPRSS4: a novel tumor prognostic indicator for the stratification of stage IA tumors and a liquid biopsy biomarker for NSCLC patients. *J Clin Med* 2019;8(12):2134.
PUBMED | CROSSREF

31. Topaloglu O, Hoque MO, Tokumaru Y, Lee J, Ratovitski E, Sidransky D, et al. Detection of promoter hypermethylation of multiple genes in the tumor and bronchoalveolar lavage of patients with lung cancer. *Clin Cancer Res* 2004;10(7):2284-8.
PUBMED | CROSSREF

32. Schnabel RM, van der Velden K, Osinski A, Rohde G, Roekaerts PM, Bergmans DC. Clinical course and complications following diagnostic bronchoalveolar lavage in critically ill mechanically ventilated patients. *BMC Pulm Med* 2015;15(1):107.
PUBMED | CROSSREF

33. Zhang H, Wang S, Zhong F, Liao M. Risk factors for air embolism following computed tomography-guided percutaneous transthoracic needle biopsy: a systematic review and meta-analysis. *Diagn Interv Radiol* 2023;29(3):478-91.
PUBMED | CROSSREF

34. Polat G, Özdemir Ö, Serçe Unat D, Karadeniz G, Ayrancı A, Unat OS, et al. Pneumothoraxes after CT-guided percutaneous transthoracic needle aspiration biopsy of the lung: a single-center experience with 3426 patients. *Tuberk Toraks* 2023;71(1):67-74.
PUBMED | CROSSREF

35. Na KJ, Park IK, Park S, Kang CH, Kim YT. Efficacy and cost-effectiveness of surgical biopsy for histologic diagnosis of indeterminate nodules suspected for early stage lung cancer: comparison with percutaneous needle biopsy. *J Korean Med Sci* 2020;35(28):e261.
PUBMED | CROSSREF

36. Ji XY, Li H, Chen HH, Lin J. Diagnostic performance of RASSF1A and SHOX2 methylation combined with EGFR mutations for differentiation between small pulmonary nodules. *J Cancer Res Clin Oncol* 2023;149(11):8557-71.
PUBMED | CROSSREF

37. Hulbert A, Jusue-Torres I, Stark A, Chen C, Rodgers K, Lee B, et al. Early detection of lung cancer using DNA promoter hypermethylation in plasma and sputum. *Clin Cancer Res* 2017;23(8):1998-2005.
PUBMED | CROSSREF

38. Marmor HN, Zorn JT, Deppen SA, Massion PP, Grogan EL. Biomarkers in lung cancer screening: a narrative review. *Curr Chall Thorac Surg* 2023;5:5.
PUBMED | CROSSREF