

Original Article

Methionyl-tRNA synthetase and aminoacyl-tRNA synthetases interacting multi-functional protein-lacking exon 2 as potential diagnostic biomarkers for lung cancer

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Abstract: Cytological specimens from computed tomography (CT)-guided needle aspiration biopsy (CT-NAB) have relatively low sensitivity for lung cancer diagnosis. This study evaluated the usefulness of the dual immunofluorescence (IF) staining method using methionyl-tRNA synthetase (MARS), aminoacyl-tRNA synthetases interacting multi-functional protein-lacking exon 2 (AIMP2-DX2), and pan-cytokeratin (pan-CK) obtained from clinical specimens. One-hundred forty-five cytology specimens were prospectively collected from patients who underwent CT-NAB under the suspicion of lung cancer. The results of two combinations of MARS, AIMP2-DX2, and pan-CK dual IF staining were compared with those of conventional cytology by calculating the area under the curve (AUC). The results of combining dual IF with conventional cytology showed higher AUC than conventional cytology alone: cytology/MARS/AIMP2-DX2 (0.891 vs. 0.829, $P = 0.003$), cytology/MARS/pan-CK (0.916 vs. 0.829, $P < 0.001$), and cytology/AIMP2-DX2/pan-CK (0.877 vs. 0.829, $P = 0.005$). In specimens with non-diagnostic results in conventional cytology, MARS/AIMP2-DX2 dual IF staining showed sensitivity, specificity, and AUC of 60.0%, 86.4%, and 0.79, respectively. The dual IF staining method using two combinations of MARS, AIMP2-DX2, and pan-CK is an effective diagnostic tool that can improve the lung cancer diagnostic yield by complementing conventional cytology.

Keywords: MARS, AIMP2-DX2, pan-CK, biomarker, lung cancer

Introduction

CT-guided needle aspiration biopsy (CT-NAB) is a commonly used standard method for the pathologic diagnosis of pulmonary nodules. However, cytological specimens obtained by CT-NAB have a relatively low sensitivity of 56.3%-86.5% [1]. In a previous study, the non-diagnostic rate of CT-NAB was 27.6%, of which 40.4% of patients were finally diagnosed with lung cancer [2]. Factors associated with non-diagnostic results in CT-NAB include smaller lesion size (≤ 15 mm), needle penetrating the emphysema, and introducer needle located outside the lesion [3]. In this case, an insufficient volume of specimen is obtained, and thus vague expressions such as "suspicious for malignancy", "atypical cells", and "cellular paucity" are often used in conventional cytology results. When a non-diagnostic result is obtained, patients must undergo re-biopsy through

additional procedures or surgery, and treatment decisions are inevitably delayed.

Currently, immunohistochemical staining markers used to diagnose lung cancer include thyroid transcription factor-1 (TTF-1) and napsin A for adenocarcinoma, and p40, p63, and cytokeratin 5/6 for squamous cell carcinoma [4]. However, these markers are not specific for lung cancer; TTF-1 and napsin A are useful markers for determining cell origin, while p63 and cytokeratin 5 are markers for squamous cell carcinoma [5-9]. In addition, these markers are difficult to apply to NAB-derived samples. Because it is difficult to obtain sufficient specimens, non-specific staining patterns, such as an edge effect (adsorption), may be observed. To overcome these problems, we developed a new staining method and markers applicable to cytological specimens.

MARS and AIMP2-DX2 as potential diagnostic biomarkers for lung cancer

Aminoacyl-tRNA synthetases (ARSs) are a group of enzymes that play important roles in protein synthesis by charging tRNA with their cognate amino acids [10]. Methionyl-tRNA synthetase (MARS), one of the ARSs, plays an essential role in the initiation of translation by ligating methionine to tRNA [11]. In addition, MARS is overexpressed in lung cancer, and its overexpression is associated with poor clinical outcomes in lung cancer patients [12]. ARS exists in a complex-bound form called the multi-tRNA synthetase complex (MSC), which includes three ARS-interacting multifunctional proteins (AIMPs), named AIMP1, AIMP2, and AIMP3. AIMP2 not only functions as a simple scaffolding protein in MSCs, but also participates in lung epithelial cell differentiation [13]. An alternative splicing variant of AIMP2 lacking exon 2 (AIMP2-DX2) has been reported to be highly expressed in human lung cancer and is associated with poor prognosis in lung cancer patients [14, 15]. Pan-cytokeratin (pan-CK) is expressed in all epithelial cells and is a useful marker of epithelial origin cells [16, 17]. Pulmonary nodules consist of lung cancer cells, which originate from epithelial cells and fibroblasts, neovascular tissues and inflammatory cells, which originate from mesenchymal cells. Therefore, pan-CK can help distinguish mesenchymal origin cells from epithelial origin lung cancer cells.

The purpose of this study was to evaluate the diagnostic value of two combinations of MARS, AIMP2-DX2, and pan-CK in cytology specimens obtained by CT-NAB. We evaluated whether the immunofluorescence (IF) staining method of these candidates could be applied to rapid on-site examination (ROSE) for lung cancer diagnosis.

Materials and methods

Study design and subjects

To evaluate AIMP2-DX2 expression in mouse lung cancer, we performed immunoblot and immunohistochemistry (IHC) analyses using tissue lysates and paraffin-embedded tissue blocks from wild-type C57BL/6, LSL-Kras G12D, and LSL-Kras G12D: p53^{fl/fl} mice. To prepare mouse lung cancer tissue, LSL-Kras G12D and LSL-Kras G12D: p53^{fl/fl} mice inhaled AdCre particles at 8 weeks and were sacrificed at 24 weeks after inhalation (<http://mouse.ncicrf.gov/>). All animal studies were approved

by the Institutional Animal Care and Use Committee of Yonsei University (2014-0229-1) according to the guidelines of the American Association for the Assessment and Accreditation of Laboratory Animal Care. To evaluate AIMP2-DX2 expression in human lung cancer, specimens were prospectively collected from patients who (1) visited Gangnam Severance Hospital, affiliated with Yonsei University, (2) had a significant lung nodule (greater than 1.0 cm in diameter) on contrast-enhanced CT, and (3) underwent CT-NAB. Residual tissues and cytologic specimens were used after pathological examinations obtained from CT-NAB. A total of 175 formalin-fixed paraffin-embedded lung cancer tissue slides were analyzed by IHC, and 145 cytological specimens were analyzed by immunofluorescence staining. This study was approved by the Institutional Review Board of Gangnam Severance Hospital (approval no. 3-2014-0299 and 3-2018-0009) and was carried out in compliance with the Declaration of Helsinki, and all patients provided written informed consent.

Antibodies, cells, and other materials

Anti-MARS mouse antibodies (EPR9873(B)) from Oncotag® (#HFTAG201, Suwon, Kyungkido, Korea), AIMP2-DX2 rabbit antibodies from Biocon® (Suwon, Kyungkido, Korea), cyto-keratin AE1 + AE3 antibodies from LifeSpan BioSciences Inc. (#LS-C357999, WA, USA), and pan-CK (KRT777) rabbit antibodies from MyBioSource (#MSB4380427, CA, USA) were used in this study. Molt-4, Daudi, and H460 cells were obtained from the Korean Cell Line Bank (Seoul, Korea). ThinPrep PreservCyt® solution was obtained from Hologic Inc. (#70097-002, Marlborough, MA, USA) and the Envision Kit and DAB from Dako (#K3468, Carpinteria, CA, USA).

Immunoblotting

Mouse tissues were harvested using 2× LSB lysis buffer containing protease and phosphatase inhibitors (GenDepo, Korea) on ice. After homogenization and sonication, 30-50 mg of lysates was separated by gel electrophoresis on 7.5%-12% polyacrylamide gels and transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Inc., Richmond, CA, USA). The expression level of each protein was measured using ImageJ (<http://rsbweb.nih.gov/ij/>)

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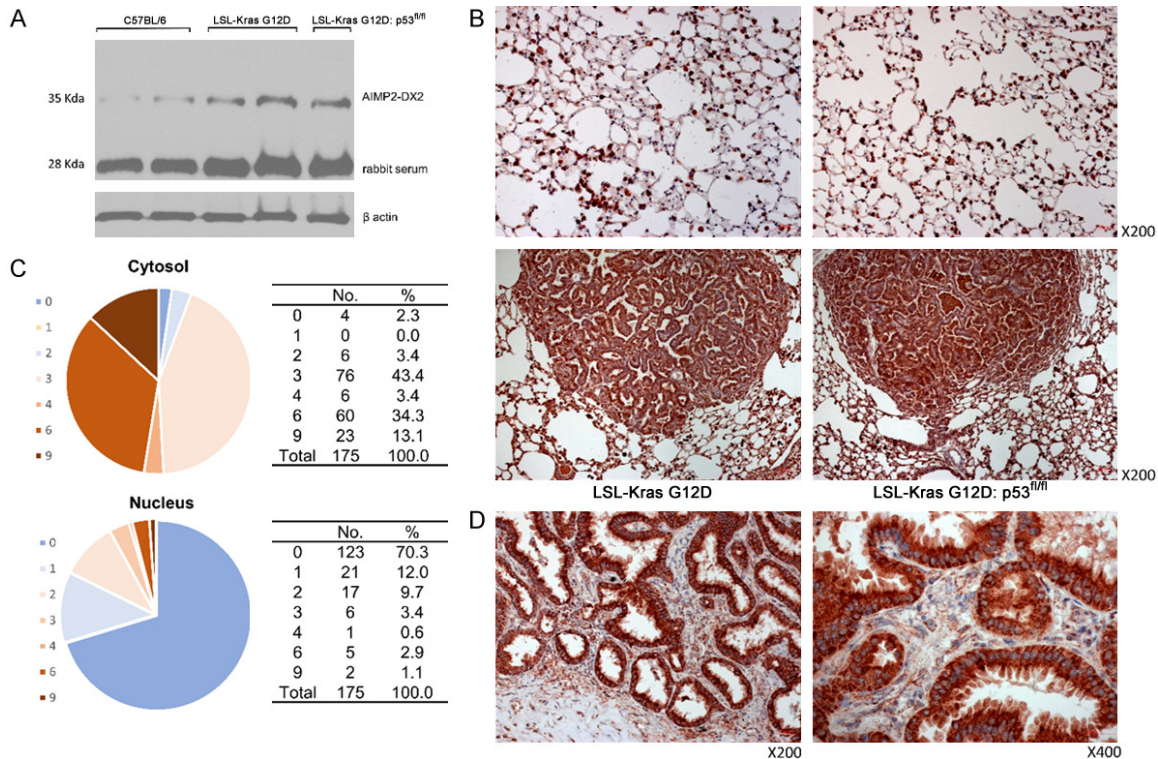


Figure 1. Expression of AIMP2-DX2 in mouse and human lung tissue by immunoblotting and immunohistochemistry. A. Immunoblot analysis of AIMP2-DX2 expression in wild type C57BL/6 mice, LSL-Kras G12D and LSL-Kras G12D: p53^{fl/fl} lung cancer model mice. B. Lung cancer progression and AIMP2-DX2 expression in LSL-Kras G12D mouse model over time. C. Normal-appearing mouse tissues adjacent to lung cancer tissues in LSL-Kras G12D and LSL-Kras G12D: p53^{fl/fl} lung cancer model mice. D. Distribution of cytoplasmic and nuclear expression of AIMP2-DX2 in 175 human lung cancer cases as assessed by immunohistochemistry.

and quantified relative to that of β -actin (**Figure 1D**).

Immunohistochemistry (IHC)

AIMP2-DX2 expression was analyzed by IHC using the LABS®2 System (Dako, Carpinteria, CA, USA) in mouse lung tissue and human lung cancer tissue. Specimen sections were deparaffinized, rehydrated, immersed in H₂O₂ methanol solution, and then incubated overnight with primary antibodies against AIMP2-DX2 obtained by rabbit immunization at a 1:2000 dilution. Sections were incubated for 10 min with a biotinylated linker and processed using avidin/biotin IHC techniques. 3,3-Diaminobenzidine (DAB) was used as a chromogen in conjunction with the Liquid DAB Substrate kit (Novacastra, UK).

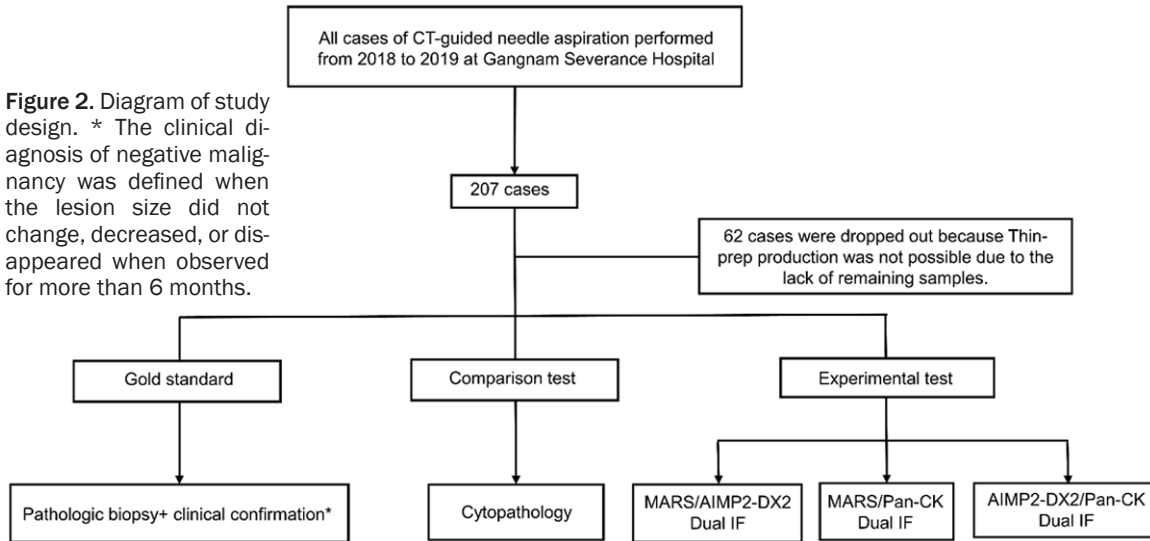
Immunofluorescence (IF) staining

Thinprep slides were immersed in 1X PBS for 5 min and then permeabilized with 0.2% PBS-T

for 30 min at room temperature. After washing with 1X PBS, the sections were blopan-cytokeratinized with 2% goat serum for 1 h and then incubated with primary antibody mixtures prepared in PBS for 90 min. Three combinations of primary antibody mixtures were as follows: (1) MARS diluted at a ratio of 1:250 and pan-CK diluted at a ratio of 1:200, (2) AIMP2-DX2 diluted at a ratio of 1:200, and pan-CK diluted at a ratio of 1:200; and (3) MARS diluted at a ratio of 1:250 and AIMP2-DX2 diluted at a ratio of 1:200. After washing, the sections were incubated with 1:1000 diluted secondary antibody mixture containing anti-rabbit-AF488 and anti-mouse-AF555 at room temperature for 1 h and then allowed to react with 4', 6-Diamidino-2'-phenylindole dihydrochloride (DAPI) for 1 min to counterstain the nuclei. The slides were mounted with coverslips using ProLong Gold Antifade Reagent® (P36930, Invitrogen, Carlsbad, CA, USA), and the slides were shaded and stored frozen at -20°C. Stained slides were observed using a Carl-Zeiss Imager M2 fluorescence

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Figure 2. Diagram of study design. * The clinical diagnosis of negative malignancy was defined when the lesion size did not change, decreased, or disappeared when observed for more than 6 months.



microscope (Imager M2, Carl Zeiss, Oberkochen, Germany), and images were analyzed using ZEN-lite software (Carl Zeiss).

Interpretation criteria and statistical analysis

The following four criteria were applied for the classification of cells as non-small cell lung cancer (NSCLC): (1) a nucleus diameter $\geq 10 \mu\text{m}$; (2) no identification in the nucleus; (3) prominent nucleoli; and (4) MARS positivity, AIMP2-DX2 positivity, and pan-CK positivity. The MARS, AIMP2-DX2, and pan-CK staining results of clinical specimens were interpreted by comparing the staining intensity of the reference sample. Two independent researchers (K-L and T-K) who were blinded to the pathological reports analyzed the slides; in case of discrepancy in the results between the two investigators, a third researcher (YS-C) also evaluated the slides. The area under the curve (AUC) was calculated and compared using the DeLong's method. The optimal cutoff point for separating the smaller nodule group from the larger nodule group was determined by the receiver operating characteristic (ROC) curve using the Youden index. Kappa (κ) statistics were used to evaluate the interobserver agreement among the pathologist and author's NSCLC diagnoses.

Results

AIMP2-DX2 were highly expressed in NSCLC tissue

To evaluate AIMP2-DX2 as a lung cancer-specific biomarker, its expression was estimated in

Kras-LSL G12D and Kras-LSL G12: p53^{fl/fl} lung cancer models and human lung cancer tissues. In mouse model, overexpression of AIMP2-DX2 showed in lung cancer than normal lung tissue by immunoblotting (**Figure 1A**). In addition, AIMP2-DX2 was highly expressed in lung cancer tissue than normal-appearing tissues adjacent to lung cancer (**Figure 1B**). To evaluate the AIMP2-DX2 expression in human lung cancer, we investigated 175 paraffin-embedded NSCLC tissue samples. AIMP2-DX2 expression was scored as follows: product of intensity and frequency, score 0-2: negative/trace AIMP2-DX2 expression; score more than 3: positive AIMP2-DX2 expression. One hundred sixty-five (94.3%) cases had positive AIMP2-DX2 cytoplasmic expression (**Figure 1C**) and showed its strong expression in NSCLC specimens (**Figure 1D**). Kim *et al.* reported that MARS was overexpressed in NSCLC tissue samples and was associated with shorter disease-free survival in patients with NSCLC [12]. Jung *et al.* documented that AIMP2-DX2 was detected in 58.3% of blood samples collected from lung cancer cases, which was related to poor clinical outcomes [18]. Taken these results together, it showed that both MARS and AIMP2-DX2 were specifically overexpressed in lung cancer.

Clinicopathological characteristics of study cases

Two hundred seven cytology specimens were prospectively collected during CT-NAB, and 145 specimens were prepared with Thinprep slides (**Figure 2**). The clinical characteristics of the

Table 1. Characteristics of patients from whom CT-guided needle aspiration cytology specimens were collected (n = 145)

Characteristics	Value
Mean age (years)	67.4 ± 11.0
Gender	
Male	94 (64.8)
Female	51 (35.2)
Mean diameter of primary lesion (cm)	3.69 ± 1.93
Final diagnosis	
Benign	34 (23.4)
Adenocarcinoma	79 (54.5)
Squamous cell carcinoma	18 (12.4)
Small cell lung cancer	3 (2.1)
Lung cancer, unspecified	3 (2.1)
Metastatic cancer from other organs	8 (5.5)

Values are n (%) unless otherwise defined.

enrolled patients are shown in **Table 1**. The mean age of the study population was 67.4 ± 11.0 years, and 94 (64.8%) were male. In 122 patients, the diagnosis was confirmed by pathological examination. For the remaining 23 patients, diagnosis was made based on findings from imaging studies performed during a clinical follow-up for at least 6 months. In the final diagnosis, 79 cases (54.5%) were adenocarcinoma and 18 cases (12.4%) were squamous cell carcinoma, whereas 34 cases (23.4%) were confirmed as benign pulmonary nodules.

Conventional cytology versus two combinations of MARS/AIMP2-DX2/pan-CK dual IF staining

In the reference sample, comprised of H460, Daudi, and Molt-4 cells at fixed ratio of 1:1:1, MARS/AIMP2-DX2 dual IF showed dual positive colocalization (orange) in most cells. In combinations of MARS/pan-CK and AIMP2-DX2/pan-CK, dual IF results showed dual positive colocalization (orange) according to the proportion of H460 cell lines (**Figure 3A**). However, colocalization of the two substances was not always present, and some cells were seen as red and green color separately.

In clinical specimens, NSCLC cells were defined by the presence of dual positive colocalization (orange color) signal in the cytosol (**Figure 3B**), and benign specimens presented no dual IF

staining (**Figure 3D**). **Figure 3C** and **3E** represent the results of conventional cytology in NSCLC case (same specimen provided in **Figure 1B**) and benign case (same specimen provided in **Figure 1D**), respectively. Of the collected 145 specimens, 47 were diagnosed with benign disease and 98 were diagnosed with NSCLC. Of the 98 cases diagnosed with NSCLC, the positive rate of the conventional cytology result was 75 cases (76.5%). The proportion of dual IF staining was 76 cases (77.6%) for MARS/AIMP2-DX2, MARS/pan-CK, and 78 cases (79.6%) for AIMP2-DX2/Pan-CK (**Table 2**). In all combinations of these three markers, the staining percentage of co-localization in the NSCLC specimen were reported higher than those of the benign specimens (14.9% vs 77.6% in MARS/AIMP2-DX2, 17.0% vs. 77.6% in MARS/pan-CK, and 25.5% vs. 78.6% in AIMP2-DX2/Pan-CK, **Figure 3F**).

In addition, we compared the diagnostic yield of conventional cytology with MARS/AIMP2-DX2, MARS/pan-CK, and AIMP2-DX2/pan-CK dual IF staining (**Table 3**). The sensitivity of conventional cytology using pap staining was 76.5%, specificity was 89.4%, and AUC was 0.829. On the other hand, those of dual IF of MARS/AIMP2-DX2 were 81.4%, 87.2%, and 0.863, respectively, which tended to increase sensitivity and AUC compared to those of conventional cytology. Those of MARS/pan-CK dual IF were 74.5%, 87.2%, and 0.829, respectively, and the AUC was equivalent to that of conventional cytology. Those of AIMP2-DX2/pan-CK were 82.7%, 74.5%, and 0.814, respectively, which tended to increase sensitivity compared to that of conventional cytology. However, when comparing the ROC curves of the cytology and dual IF staining results, the difference was not significant (**Figure 4A**).

Conventional cytology versus combinations of dual IF staining with cytology

In addition, we compared the diagnostic yield when combined with the results of cytology and dual IF staining. The sensitivity of cytology combined with MARS/AIMP2-DX2 was 86.7%, and specificity was 85.1%, and AUC was 0.891, which showed significantly increased sensitivity and AUC compared to those of conventional cytology (DeLong's method, cytology vs. cytology/MARS/AIMP2-DX2: $P = 0.003$). For the combination of cytology and MARS/pan-CK

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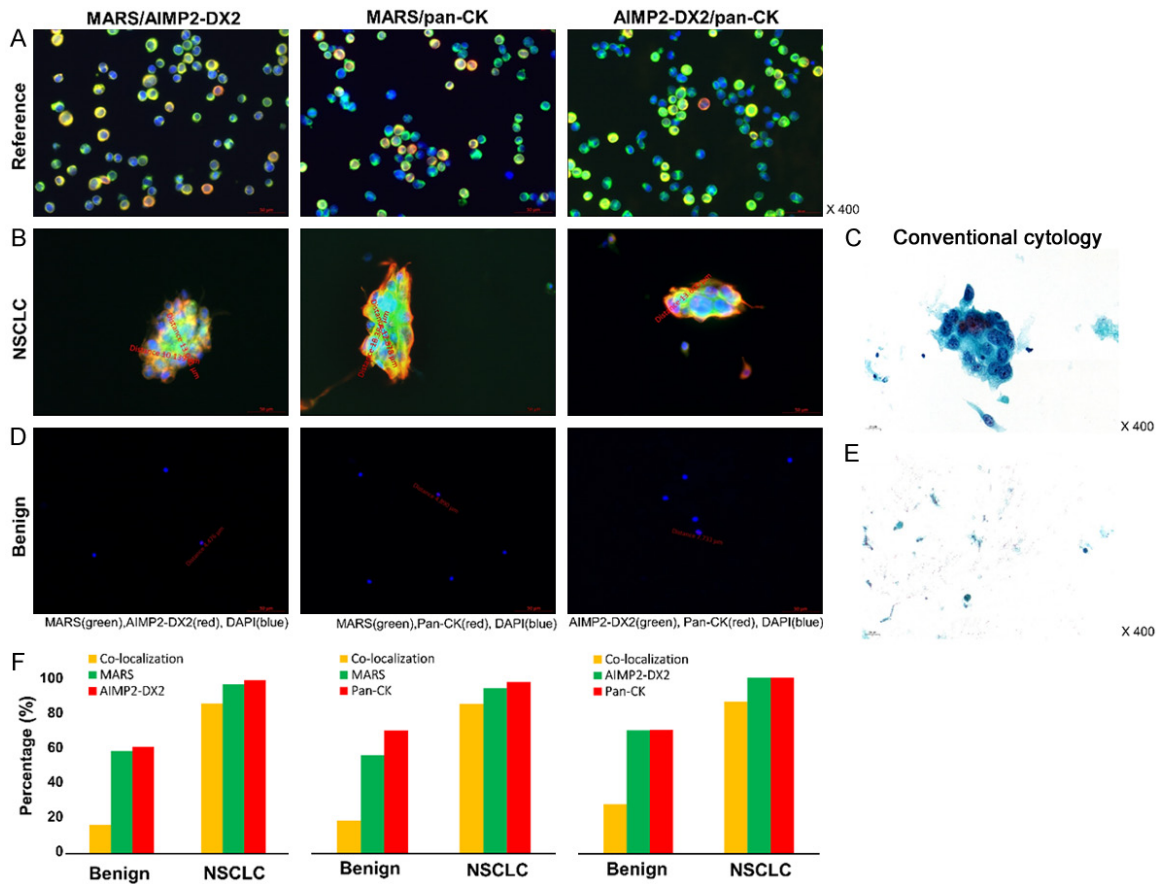


Figure 3. IF staining images of MARS, AIMP2-DX2, and pan-CK. (A) IF staining for MARS/AIMP2-DX2, MARS/pan-CK, and AIMP2-DX2/pan-CK in reference samples comprised of H460, Daudi, and Molt-4 cells at a fixed ratio of 1:1:1. (B) Dual IF staining images of MARS/AIMP2-DX2, MARS/pan-CK, AIMP2-DX2/pan-CK, and (C) image of conventional cytology in the CT-NAB-derived sample (final diagnosis: NSCLC). (D) Dual IF staining images of MARS/AIMP2-DX2, MARS/pan-CK, AIMP2-DX2/pan-CK, and (E) image of conventional cytology in the CT-NAB-derived sample (final diagnosis: benign disease). Images were taken using a Carl-Zeiss Imager M2 fluorescence microscope. (F) Staining percentages of MARS, AIMP2-DX2, pan-CK, and co-localization in the benign and NSCLC specimens.

Table 2. Comparison of the results in conventional cytology versus dual IF staining

Clinicopathologic diagnosis	Conventional cytology		MARS/AIMP2-DX2 dual IF		MARS/pan-CK dual IF		AIMP2-DX2/pan-CK dual IF	
	positive	negative	positive	negative	positive	negative	positive	negative
Malignancy	75	23	76	22	76	22	78	20
Benign	5	42	7	40	8	39	12	35

MARS, methionyl-tRNA synthetase; AIMP2, aminoacyl-tRNA synthetase interacting multifunctional protein 2; pan-CK, pan-cytokeratin; IF, immunofluorescence.

dual IF, sensitivity was 74.5%, specificity was 85.7%, and AUC was 0.916, showing a significantly higher specificity and AUC compared to those of other results (DeLong's method, cytology vs. cytology/MARS/pan-CK: $P < 0.001$). When combining the results of cytology and AIMP2-DX2/pan-CK dual IF, the sensitivity was 77.6%, specificity was 89.4%, and AUC was 0.877, which showed significantly increased sensitivity and AUC compared to those of con-

ventional cytology (DeLong's method, cytology vs. cytology/AIMP2-DX2/pan-CK: $P = 0.005$). The results of comparing ROC curves are presented in **Figure 4B**.

Comparison of the diagnostic yields from non-diagnostic result in conventional cytology

Among the 42 patients with specimens that reported atypical cells or suspicious findings for

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Table 3. Comparison of diagnostic yields in conventional cytology versus dual IF staining

	Sensitivity (%)	Specificity (%)	AUC	p-value
Conventional cytology	76.5	89.4	0.829	
MARS/AIMP2-DX2 dual IF	81.6	87.2	0.863	0.456*
MARS/pan-CK dual IF	74.5	87.2	0.829	0.984*
AIMP2-DX2/pan-CK dual IF	82.7	74.5	0.814	0.643*
Cytology + MARS/AIMP2-DX2 dual IF	86.7	85.1	0.891	0.003 [†]
Cytology + MARS/pan-CK dual IF	74.5	95.7	0.916	<0.001 [†]
Cytology + AIMP2-DX2/pan-CK dual IF	77.6	89.4	0.877	0.005 [†]

AUC, area under the curve; MARS, methionyl-tRNA synthetase; AIMP2, aminoacyl-tRNA synthetase interacting multifunctional protein 2; pan-CK, pan-cytokeratin; IF, immunofluorescence. *DeLong's method, cytology vs. dual IF results, [†]DeLong's method, cytology vs. cytology + dual IF results.

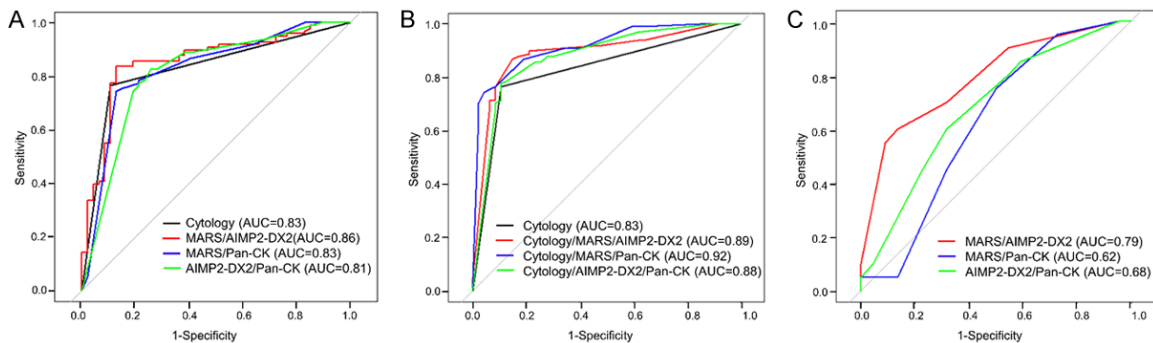


Figure 4. ROC curve analysis of the diagnostic value for lung cancer. (A) ROC curves of conventional cytology versus dual IF staining. (B) ROC curves of conventional cytology versus combination of cytology with dual IF staining. (C) ROC curves of IF staining in non-diagnostic cytology result (C).

Table 4. Comparison of results from non-diagnostic result in conventional cytology

Clinicopathologic diagnosis	MARS/AIMP2-DX2 dual IF		MARS/pan-CK dual IF		AIMP2-DX2/pan-CK dual IF	
	positive	negative	positive	negative	positive	negative
Malignancy	13	7	8	12	9	11
Benign	2	20	15	7	6	16

MARS, methionyl-tRNA synthetase; AIMP2, aminoacyl-tRNA synthetase interacting multifunctional protein 2; pan-CK, pan-cytokeratin; IF, immunofluorescence.

malignancy by conventional cytology, the final clinicopathological analysis revealed 20 malignancies and 22 benign lesions. Among the 20 malignancies, 13 were positive and 7 were negative according to the MARS/AIMP2-DX2 dual IF staining (Table 4). Cytologic diagnosis that was indeterminate by conventional cytology was markedly improved by MARS/AIMP2-DX2 dual IF staining. The sensitivity, specificity, and AUC were 60.0%, 86.4%, and 0.790, respectively (Table 5; Figure 4C).

Discussion

In this study, lung cancer diagnosis using dual IF staining combinations of MARS and AIMP2-

DX2 as lung cancer-specific markers, and pan-CK as an epithelial cell marker, showed good diagnostic performance. When our dual IF staining was combined with the conventional cytology results, all three combinations, MARS/AIMP2-DX2, MARS/pan-CK, and AIMP2-DX2/pan-CK, showed superior diagnostic yield than cytology alone. Moreover, the MARS/AIMP2-DX2 dual IF staining had good diagnostic power for specimens with non-diagnostic cytology results (sensitivity, 60.0%; specificity, 86.4%; AUC, 0.79). Therefore, the application of MARS/AIMP2-DX2 dual IF can improve the diagnostic yield of undiagnosed specimens using conventional cytology.

Table 5. Comparison of the diagnostic yields from non-diagnostic result in conventional cytology

	Sensitivity (%)	Specificity (%)	AUC
MARS/AIMP2-DX2 dual IF	60.0	86.4	0.790
MARS/pan-CK dual IF	75.0	50.0	0.622
AIMP2-DX2/pan-CK dual IF	60.0	68.2	0.680

AUC, area under the curve; MARS, methionyl-tRNA synthetase; AIMP2, aminoacyl-tRNA synthetase interacting multifunctional protein 2; pan-CK, pan-cytokeratin; IF, immunofluorescence.

In our study, there was no significant difference in the results when comparing the ROC curves of dual IF staining with those of conventional cytology for lung cancer diagnosis. Therefore, we additionally evaluated the agreement between conventional cytology and dual IF staining using Cohen's kappa. There was a substantial agreement between conventional cytology and dual IF staining ($\kappa = 0.71$ for cytology vs. MARS/AIMP2-DX2, $\kappa = 0.64$ for cytology vs. MARS/pan-CK, and $\kappa = 0.62$ for cytology vs. AIMP2-DX2/pan-CK). The reason for the disagreement between the two test results was that pathologists tended to judge conservatively, while dual IF staining was judged blinded to the patients' clinical information. Because the two test results did not match, the dual IF staining method could be used as a complementary diagnostic method.

ROSE provides real-time feedback on information about the quantity and quality of needle aspiration samples. Several studies reported that the application of ROSE increased the diagnostic yield, decreased the complication rate by reducing the number of needle penetrations, and reduced costs by avoiding additional diagnostic procedures [19-21]. However, ROSE is difficult to apply in clinical practice because it can be time-consuming and costly since it requires a cytopathologist to be present at the examination site [22]. Recently, telecytology has emerged as a cost-effective and time-effective method for ROSE. When rapid assessment was performed using telecytology on specimens from small biopsies, the diagnostic yield was increased and the proportion of non-diagnostic specimens decreased by 3% [23, 24]. In the present study, the lung cancer-specific MARS and AIMP2-DX2 dual IF staining method was able to derive diagnostic results simply and intuitively; hence, it could be candidate biomarkers for ROSE using telecytology.

There are some limitations to our study. First, we used residual specimens left after routine pathologic studies. Considering this fact, a better diagnostic yield might be expected if appropriate samples are used in the future study. Second, only 23% of the patients enrolled in our study had benign results, and the rest had malignant results. This is because our study was a prospective study, and invasive

CT-NAB was performed only when lung malignancy was suspected in the imaging study. Third, liquid-based cytology slides (Thinprep) were used in this study for MARS and AIMP2-DX2 IF staining, whereas direct smear staining was used in conventional cytology specimens. Further large-scale prospective studies are required to determine whether there are differences in dual IF staining between liquid-based cytology slides and direct smears.

In conclusion, the accuracy of the diagnosis of pulmonary nodules is critical for patient's treatment decisions. The results of combined cytology and two combinations of dual IF staining using MARS, AIMP2-DX2, and pan-CK showed improved diagnostic yield compared to conventional cytology alone. Moreover, the MARS/AIMP2-DX2 dual IF staining showed good diagnostic performance for specimens with non-diagnostic cytology results. Therefore, IF staining using ARSs could be a specific and complementary diagnostic method for lung cancer.

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Disclosure of conflict of interest

None.

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