ORIGINAL ARTICLE

High concordance between Immunohistochemistry and RT-PCR in diagnosing ALK rearrangement in lung adenocarcinoma cytologic samples

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ABSTRACT

Introduction: Lung cancer, predominantly lung adenocarcinoma, remains a major health challenge in Indonesia, with late-stage detection being common. This study explores the use of quantitative real-time polymerase chain reaction (qRT-PCR) for assessing ALK rearrangement from smear samples, a significant shift towards less invasive diagnostic methods, by assessing its concordance with immunohistochemistry (IHC) in detecting Anaplastic Lymphoma Kinase (ALK) rearrangements in lung adenocarcinoma patients.

Materials and Methods: This observational cross-sectional study analyzed 175 lung adenocarcinoma samples lacking EGFR mutations collected between 2018 and 2022. IHC was performed with the Ventana ALK D5F3 clone antibody on cell blocks or core needle biopsy specimens. The EML4-ALK fusion rearrangement status was determined using quantitative RNA qRT-PCR analysis on the smear specimen from transthoracic needle aspiration (TTNA) from the same sample. Only specimens with viable tumor cells were included, ensuring the exclusion of metastatic or necrotic samples.

Results: ALK rearrangements were identified in 16.2% (23/142) of samples via IHC and 14.8% (21/142) via qRT-PCR. Prevalence did not significantly differ by age and sex. The study found a 98.5% concordance rate between the two methods, with a κ coefficient of 0.95 (95% CI, 0.91-0.98), indicating almost perfect agreement.

Conclusion: The high concordance between IHC and qRT-PCR underscores their reliability in detecting ALK rearrangements, crucial for the precise diagnosis and treatment of lung adenocarcinoma in Indonesia. These findings support the use of either method, depending on available resources and expertise, to enhance lung cancer management.

KEYWORDS:
Lung cancer; Adenocarcinoma; Anaplastic Lymphoma Kinase (ALK); Immunohistochemistry (IHC); Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

INTRODUCTION

Lung cancer remains the leading cause of cancer-related morbidity and mortality worldwide. In 2020, Indonesia experienced 34,783 new lung cancer diagnoses, and 30,843 fatalities attributed to lung cancer, representing 13.2% of the nation's total cancer death toll.[1] These numbers underscore the urgent need for effective management strategies.

Lung cancer is primarily classified into two types: small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC), with NSCLC making up approximately 80–85% of all lung cancers.[2,3] NSCLC is further subdivided into squamous-cell carcinoma, large cell carcinoma, and adenocarcinoma. Often, adenocarcinoma is the most common and usually presents at an advanced stage with limited treatment modalities.[4] The National Comprehensive Cancer Network (NCCN) for NSCLC emphasizes the importance of molecular testing in advanced and metastatic NSCLC.[5] The emergence of targeted therapies using these molecular markers has transformed the field of precision medicine. The rapid advancement in molecular biology has

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led to the identification and confirmation of lung cancer driver genes, paving the way for the development of molecularly targeted medications and the onset of the era of targeted drug therapy.[5]

The Anaplastic Lymphoma Kinase (ALK) rearrangement has emerged as a distinct molecular subtype in NSCLC since 2007, with the prevalence approximately 5%.[6,7] Patients typically present with adenocarcinoma histology and are often light smokers or have never smoked.[8] Research has found that echinoderm microtubule-associated protein-like 4 (EML4)-ALK fusion gene is typically exclusive of other carcinogenic genes such as EGFR, ROS1, KRAS, and others.[9] EML4 represents the most frequent partner for ALK in lung cancer.[10,11] Thus, detection of the EML4-ALK fusion gene is crucial for administering targeted therapy.[9] The NCCN recommends testing the ALK rearrangements in patients with metastatic nonsquamous NSCLC. This recommendation is based on the efficacy of ALK inhibitors like alectinib (which is covered by national insurance of Indonesia), brigatinib, ceritinib, crizotinib, or lorlatinib. Moreover, since 2023, the detection of ALK rearrangements in early-stage NSCLC (stages IB-IIIA, and IIIB [only T3, N2]) may evaluate the possibility of adjuvant therapy with atezolizumab or pembrolizumab.[5]

Three primary techniques exist for ALK testing: immunohistochemistry (IHC), fluorescence in situ hybridization (FISH), and real-time polymerase chain reaction (RT-PCR).[10,12] The FISH method of testing was considered as the gold standard of fusion gene detection including ALK fusions, but this method is notably labor-intensive and its implementation remains limited, particularly in developing countries.[12,13] In regions such as Indonesia, IHC and RT-PCR are widely accessible and become feasible alternatives for biomolecular testing, especially ALK rearrangements. Previous studies have consistently demonstrated a substantial agreement between the results obtained through IHC or RT-PCR when compared with those derived from FISH.[10,12,14–17]

The increasing accessibility of molecular testing methods in Indonesia presents a unique opportunity to study the prevalence of oncogenic mutations in lung adenocarcinoma patients, especially important in advanced stages where extensive tissue resection is limited. The role of minimally invasive diagnostic methods like transthoracic needle aspiration (TTNA) becomes crucial, although they yield only smear samples. In Indonesia, biomarker testing has traditionally relied on protein-based techniques such as IHC, which necessitate tissue or cell block preparation.[18–20] This study aims to determine the feasibility of using qRT-PCR to accurately evaluate ALK rearrangement from cytology smear samples, marking a notable advancement in the validation of less invasive sample types for molecular diagnostics, while investigating the concordance between IHC and RT-PCR analyses within a patient group. To our knowledge, no study in Indonesia has compared RT-PCR with IHC for ALK testing. However, research from other countries has demonstrated a high concordance between RT-PCR and IHC in ALK testing.[21,22] Achieving this could facilitate more precise and early interventions, ultimately improving treatment outcomes by leveraging the adaptability of diagnostic tests to various sample types, thus enhancing the prospects for effective management of lung adenocarcinoma.

MATERIALS AND METHODS

Study Design

This study was an observational cross-sectional study employing consecutive sampling methods. The research was conducted using NSCLC samples collected between 2018 and 2022 from the Department of Anatomical Pathology, Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada and Cito Clinical Laboratory, Yogyakarta, Indonesia. Ethical approval for specimen collection was granted by the Medical and Health Research Ethics Committee (MHREC) of the Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada, Universitas Gadjah Mada - Dr. Sardjito General Hospital, Yogyakarta, Indonesia (Ref. No.: KE/FK/0532/EC/2020). The study adhered to the principles outlined in the Declaration of Helsinki. Given its retrospective and non-interventional design, the Ethics Committee granted a waiver for the implementation of a free informed consent.

Sample Collection, Preparation, and Selection

All specimens were primarily obtained via TTNA cytology. In cases where clinical conditions permitted, a core needle biopsy was also performed on the same patient, resulting in two types of samples (i.e., cytology and histology) from a single individual. For TTNA cytology samples, a portion of the aspirated material was prepared as a Diff-QuickTM stained smear, while the remaining material (residual clotting material from the syringe) was processed into a formalin-fixed, paraffin-embedded (FFPE) cell block, particularly when a core needle biopsy was not feasible. The cytology smear was reserved for RNA extraction and subsequent RT-PCR analysis, whereas the FFPE cell block and any available core needle biopsy specimens were utilized for IHC examination.

A total of 175 specimens of native Indonesian, that initially tested negative for EGFR mutations were evaluated for inclusion in this study. Only the specimens that were identified as lung adenocarcinoma were selected for additional analysis. Each sample was required to exhibit at least 30% tumor cell content (according to AmoyDx® EML4-ALK Fusion Gene Detection Kit [Amoy Diagnostics, Xiamen, China] and Lindeman et al., 2018),[23] following post-EGFR mutation testing to ensure sufficient cellular material for subsequent analyses. Furthermore, the availability of both a cytology smear (for qRT-PCR) and FFPE cell block or core needle biopsy (for IHC) sample from the same patient was necessary. Specimens indicated metastatic lung cancer originating from non-pulmonary primary sites, or predominantly displayed necrosis, which rendered adequate molecular and histological analysis unfeasible are excluded from the analyses.

Immunohistochemistry (IHC) Analysis

ALK rearrangement was evaluated using Ventana® ALK (D5F3 clone antibody) CDx assay (Roche Diagnostics, Indiana, USA) on FFPE cell block tissue sections or core needle biopsy tissue sections with a thickness of 4 µm, following the

manufacturer's standard protocols. This kit is already certified for in vitro diagnostics (IVD). The ALK status interpretation was determined based on specific criteria provided by the clone antibody manufacturer. A positive result required strong granular cytoplasmic staining in tumor cells, irrespective of the percentage of positive tumor cells. Certain staining elements were excluded, including light cytoplasmic stripping in alveolar macrophages, staining in neural cells (e.g., nerve and ganglion cells), glandular epithelial staining, scattered lymphoreticular cells within lymphocytic infiltrates, and background staining in normal mucosa or necrotic tumor areas. Absence of strong granular cytoplasmic staining in tumor cells indicating negative ALK status.

Two independent pathologists, blinded to each other's assessments and to the RT-PCR results, evaluated the stained slides according to the manufacturer's interpretation guide. Results were categorized as positive or negative. In instances of discordant interpretations, a third blinded pathologist reviewed the slides to reach a consensus.

RNA Extraction

RNA extraction was performed on scraped TTNA smear preparations using the RibospinTM II RNA Purification Kit (Cat. No. 314-150, GeneAll Biotechnology Co., Ltd., Seoul, Korea) according to the manufacturer's instructions. This manual process was conducted by trained personnel to maintain RNA integrity and quality. Samples intended for immediate analysis were stored at 4°C, while those designated for long-term storage were kept at -70°C to prevent degradation.

Reverse Transcription Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

The RNA samples in this study were analyzed using the AmoyDx® EML4-ALK Fusion Gene Detection Kit (Amoy Diagnostics, Xiamen, China), which is certified under CE-IVD (https://www.amoydiagnostics.com/products/amoydx-eml4-alk-fusion-gene-detection-kit) standard. This analysis was conducted in strict adherence to the manufacturer's guidelines. Quantitative real-time PCR (qRT-PCR) was performed on the Bioneer Exicycler96TM Real-Time Quantitative Thermal Block (ver. 4, Bioneer inc., California, USA) platform. The PCR conditions followed were as recommended by the manufacturer's protocol. The AmoyDx® EML4-ALK Fusion Gene Detection Kit (Amoy Diagnostics, Xiamen, China) validation process, conducted in accordance with vendor's recommendation, ensured the kit's reliability and the accuracy using Bioneer Exicycler96TM Real-Time Quantitative Thermal Block (ver. 4, Bioneer inc., California, USA) platform. Interpretation of the PCR results was based on the FAM fluorescence channel cycle threshold (Ct) values. Samples with a Ct value of less than 30 were classified as positive for EML4-ALK fusion genes. Conversely, samples with a Ct value of 30 or higher were deemed negative, suggesting no detectable EML4-ALK fusion or values below the kit's limit of detection (LOD).

Statistical Analysis

Statistical evaluations in this study were conducted using Microsoft Excel 2020 and IBM SPSS Statistics 27.0 software. The analysis categorized data into two types: categorical data, presented as frequencies and percentages, and quantitative data, expressed as mean values. For categorical data, the Chi-square test was used to assess associations, while for quantitative data, the independent t-test was employed to compare means between groups. For non-parametric data, the Mann-Whitney U test was used as an alternative to the independent t-test.

A univariate analysis was employed to gain insights into each variable. For binomial variables, the confidence interval was determined using the Clopper-Pearson method, specifically set at a 95% confidence interval (CI). The concordance rate was determined from the contingency table.[24,25] The 95% CI was estimated for κ. κ coefficient of ≤ 0 indicates no agreement, while coefficients between 0.01 and 0.20 reflect slight agreement. κ coefficient between 0.21 and 0.40 indicates fair agreement, and values between 0.41 and 0.60 are considered moderate. Substantial agreement is represented by coefficient between 0.61 and 0.80, and κ coefficient of more than 0.80 will correspond to an almost perfect agreement as per the scale proposed by Landis and Koc.[24]

RESULTS

After applying inclusion and exclusion criteria, 142 samples met all the necessary requirements and were included in the final analysis. The baseline characteristics of the samples is shown in Table I. The mean age of the patients was 60 years old, with 24 (16.9%) samples were 50 years of age or younger and 118 (83.1%) samples were older than 50. Of the 142 samples, a total of 79 samples (55.6%) were from males, and 63 samples (44.4%) were from females.

All the samples were successfully tested for ALK rearrangement status by IHC and qRT-PCR. The immunostaining pattern of ALK in lung adenocarcinoma was assessed using the Ventana anti-ALK (D5F3) antibody. The ALK IHC assay demonstrates significant cytoplasmic protein expression, characterized by a distinct granular pattern, in cases that produced positive results (Figure 1A&1B). For testing with IHC, 23/142 (16.2%) samples were positive for ALK rearrangement, while the remaining 119/142 (83.8%) samples had negative ALK rearrangement status (Table II). The result was similar with qRT-PCR testing. There were 21/142 (14.8%) samples with positive ALK rearrangement and 121/142 (85.3%) samples with negative ALK rearrangement (Table II).

The prevalence of individuals aged > 50 years old was greater than those ≤ 50 years old in both the IHC (8.3% versus 17.8%; p>0.05) and qRT-PCR (8.3% versus 16.1%; p>0.05) results, but these differences were not statistically significant. The prevalence of ALK rearrangement was also more common in males than females, as indicated by both IHC (20.3% versus 11.1%; p>0.05) and qRT-PCR (19.0% versus 9.5%; p>0.05) results. However, these differences also did not reach statistical significance.

In terms of the agreement between the IHC and qRT-PCR result, it was determined that cases could be classified as

Table I: Baseline characteristics of the sample

Characteristics	N (%)
Age	
≤ 50 years old	24 (16.9%)
> 50 years old	118 (83.1%)
Sex	
Male	79 (55.6%)
Female	63 (44.4%)

Table II: Characteristics of IHC for ALK rearrangement and qRT-PCR for EML4-ALK fusion

Characteristics	ALK rearrangement status by IHC		p-value	EML4-ALK fusion status by qRT-PCR		p-value
	ALK rearrangement (+) N (%)	ALK rearrangement (-) N (%)		ALK rearrangement (+) N (%)	ALK rearrangement (-) N (%)	
Prevalence	23 (16.2%)	119 (83.8%)		21 (14.8%)	121 (85.2%)	
Age			0.251			0.328
≤ 50 years old	2 (8.3%)	22 (91.7%)		2 (8.3%)	22 (91.7%)	
> 50 years old	21 (17.8%)	97 (82.2%)		19 (16.1%)	99 (83.9%)	
Sex		0.142			0.115	
Male	16 (20.3%)	63 (79.7%)		15 (19.0%)	64 (81.0%)	
Female	7 (11.1%)	56 (88.9%)		6 (9.5%)	57 (90.5%)	

Table III: Cross-tabulation of IHC for ALK rearrangement and qRT-PCR for EML4-ALK fusion

Examination Methods	EML4-ALK fusion status by RT-PCR		Total
	ALK rearrangement (+) N	ALK rearrangement (-) N	
ALK rearrangement status by IHC			
ALK rearrangement (+)	21	2	23
ALK rearrangement (-)	0	119	119
Total	21	121	142

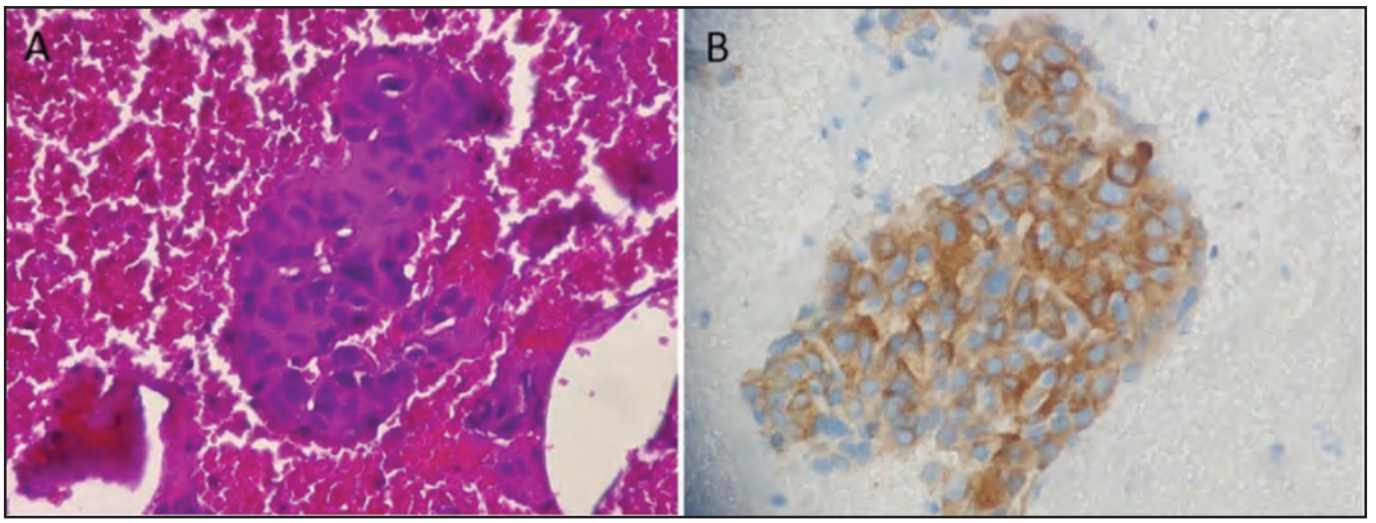

Fig. 1: A. Photomicrograph of the cell block from TTNA sample that revealed a lung adenocarcinoma (Cell block, HE, 400x); B. The immunostaining pattern of ALK in lung adenocarcinoma using the Ventana anti-ALK (D5F3) antibody. ALK IHC demonstrates a significant level of protein expression in the cytoplasm, characterized by a granular pattern, in the cases that tested positive (Cell block, Anti-ALK antibody, 400x)

either IHC negative/qRT-PCR negative or IHC positive/qRT-PCR positive. The cross-tabulation of ALK rearrangement status by IHC with D5F3 antibody in relation to ALK rearrangement status by qRT-PCR is shown (Table III). All 21 (100%) of positive ALK rearrangement status samples tested with qRT-PCR also showed positive results with IHC.

Only two (8.7%) samples that were tested positive with IHC showed negative results with qRT-PCR. Meanwhile, all the negative samples tested with IHC showed the same result with qRT-PCR. The concordance between the two tests was 98.5% with a corresponding κ coefficient of 0.95 (95%CI, 0.91–0.98). The κ coefficient is greater than 0.80, which corresponds to almost perfect agreement.

DISCUSSION

In this study, we reported a prevalence of ALK gene rearrangement between 14.8% (by qRT-PCR) and 16.2% (by IHC) in a sample of 142 Indonesian patients. These observations mark a higher prevalence rates of ALK gene rearrangement in NSCLC patients, which globally reported to be between 2-7% among individuals with adenocarcinoma histology subtype, predominantly comprising of light smokers or non-smokers patients; its presence is usually exclusive to other driver mutations.[5,7,8,13] Comparable neighboring countries, including Thailand, Malaysia, and Vietnam, have also exhibited a reduced incidence of ALK rearrangement ranging from 4.1% to 9.7%.[26–29] Our earlier study also reflected this trend, revealing that 20% samples (seven out of 35 cytology samples) of the Indonesian population exhibiting a positive EML4-ALK rearrangement.[30] Additionally, this finding gains further significance when compared to the more common and well-established data of EGFR mutation in the Indonesian lung cancer patients, where a larger study of 1,874 patients found a 44.5% positive rate of EGFR mutation.[31]

Considering the prevalent EGFR-targeted treatments in Indonesia, our research emphasizes the necessity of also focusing on ALK rearrangements. Although EML4-ALK fusion genes generally do not coexist with other oncogenic mutations, such as EGFR, ROS1, or KRAS, exceptions have been reported in a minority of patients.[32] These rare cases, where co-occurring actionable oncogenic drivers are present, highlight an area of clinical complexity that warrants further exploration.[32]

ALK rearrangements may contribute to osimertinib resistance in NSCLC patients with EGFR mutations, underscoring the importance of identifying and targeting ALK rearrangements even within this subset.[33] The exploration and consideration of various ALK inhibitors are crucial steps towards enhancing treatment outcomes for Indonesian patients. The NCCN guideline strongly recommends testing for both ALK and EGFR mutations, supported by substantial evidence to optimize therapeutic strategies.[5]

A systematic review and meta-analysis showed higher specificity for cell specimens than for tumor specimens for detecting ALK rearrangement with IHC compared to FISH in NSCLC patients.[34] In this study, we utilized the cell block, a smaller form of formalin-fixed paraffin-embedded, obtained from fine-needle aspirate or fluid sediment that can be utilized for IHC study. The retention of cytologic material in the cell block for methods designed for IHC and molecular studies enhances its diagnostic precision.[35]

The IHC has been demonstrated to be a cost-effective, fast, and accurate method for detecting ALK rearrangements in tissue or cell block samples, offering advantages over FISH due to lower requirements for specialized technical resources and expertise.[15,18] Both IHC and RT-PCR have shown high sensitivity and specificity, making them suitable for routine clinical practice.[15,18,36] Previous studies have shown a high concordance between IHC and FISH, some with rates of 98.4% for negative agreement and 98.5% for overall agreement.[14,18,36] This further validates the efficacy of IHC. Uruga et al.,[37] highlighted IHC's performance, with the D5F3 clone achieving 76-100% sensitivity and specificity compared to FISH in studies conducted between 2011-2018. Additionally, RT-PCR has demonstrated a 99.2% concordance with FISH in patients treated with crizotinib, affirming its reliability in detecting EML4-ALK fusion in NSCLC patients, with a specificity of 94% in cases without full-length ALK expression when compared to FISH and sequencing.[38,39] A comparison with NGS also confirmed RT-PCR's reliability.[40] With their validated sensitivity and specificity, both IHC, particularly using the D5F3 test, and RT-PCR are indispensable in the molecular diagnosis of NSCLC, offering efficient, reliable, and cost-effective alternatives for identifying ALK rearrangements and optimizing patient treatment plans.

We reported a strong concordance between the data obtained using IHC with Ventana D5F3 antibody and RT-PCR for EML4-ALK fusion. The observation that two samples were positive for the IHC D5F3 clone but negative according to the EML4-ALK RT-PCR test suggests the possibility of rare ALK fusion partners that are not typically identified by our RT-PCR kit. The observed discordance between testing methods can largely be attributed to the limited scope of EML4-ALK fusions detected by the RT-PCR assay and the dependency on the quality of RNA in the samples, as highlighted by numerous studies.[40–42] Both the methodology employed in this study, specifically IHC for D5F3 ALK rearrangement and RT-PCR for EML4-ALK fusion, have demonstrated a high level of sensitivity and high concordance with FISH in previous research.[15,38,39] While FISH is still included in some guidelines as a means to predict ALK inhibitor sensitivity, IHC and RT-PCR testing can be considered as reliable alternative methods for predicting the use of ALK inhibitors, particularly in Indonesia. Cytology specimens continue to be the most accessible type of sample.

These methods are especially useful for identifying patients who are eligible for treatment with tyrosine kinase inhibitors (alectinib, brigatinib, and crizotinib), particularly in instances where tissue samples are unavailable. Therefore, our findings can be utilized as a reliable reference to improve the ALK rearrangement assessment in Indonesia and another country where cytological samples or small biopsies are commonly used for diagnosing lung cancer. Both the IHC and RT-PCR methods are reliable for accurate detection of ALK rearrangements.

This analysis is particularly important in Indonesia and neighboring nations, where healthcare resources may be constrained, and effective diagnostic strategies are essential for enhancing patient outcomes while controlling expenses. The elevated concordance rates between IHC and RT-PCR indicate that both techniques are dependable for diagnosing ALK rearrangements, which are essential for targeted therapies in lung adenocarcinoma. The cost-effectiveness of these diagnostic methods can greatly impact clinical decision-making. Research indicates that molecular diagnostics, such as IHC and RT-PCR, facilitate earlier and more precise diagnoses, thereby potentially decreasing the overall treatment expenses linked to advanced lung cancer management.[43,44] This is especially crucial in areas with

limited healthcare budgets, as prompt and precise diagnostics may prevent the financial strain of treating advanced diseases.

Furthermore, the incorporation of economical diagnostic methods, such as the integration of IHC and RT-PCR, can enhance resource distribution within healthcare systems. The implementation of rapid on-site evaluation (ROSE) alongside endobronchial ultrasound transbronchial lung biopsy has demonstrated a reduction in diagnostic expenses while enhancing the efficiency of lung cancer diagnosis.[45] This corresponds with findings that highlight the necessity of employing less invasive and more economical diagnostic instruments to improve patient care while avoiding excessive expenses.[46]

In the future, we can improve the generalizability of our study by including samples from other types of NSCLC, such as squamous cell carcinoma. In our current study, we only focused on the samples of adenocarcinoma. In addition, this study does not employ FISH as a definitive benchmark due to limited sample availability and procurement method in this scope of the investigation. Hence, it is recommended as a potential subject for future investigation, to carry out concordance and diagnostic examinations employing other methodologies.

CONCLUSION

With high concordance, the status of ALK rearrangement in lung adenocarcinoma can be determined using either IHC or qRT-PCR. Depending on the availability of samples, the most suitable assay may be employed. In a country where cytological samples or small biopsies are commonly used for diagnosing lung cancer, the IHC or qRT-PCR methods can be relied upon for accurate testing of ALK rearrangements.

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CONFLICT OF INTEREST

The authors declared that they had no competing interests.

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