

**Clinical application of immunocytochemical detection of ALK
rearrangement on cytology slides for detection or screening of lung
adenocarcinoma**

(ALK 転座を有する肺癌の検出, 擦過細胞診標本を用いた免疫細胞化学染色の有用性)

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Abstract

Immunohistochemical screening of Anaplastic lymphoma kinase (ALK) rearrangement has been regarded essential and routinely carried out to select treatment for lung adenocarcinoma. However, difficulty to approach a tumor by transbronchial lung biopsy (TBLB), it often fails to obtain tumor tissues whereas tumor cells are contained in cytology specimens simultaneously obtained when the bronchoscopy is done. Therefore we evaluated the expression of ALK protein by using immunohistochemistry (IHC) on TBLB specimens and immunocytochemistry (ICC) on brushing smear cytology slides in the same cases, and compared the concordance rate of IHC and ICC results. ICC was carried out on routine Papanicolaou-stained slides after cytology diagnosis and decolorization. Results: Eighteen patients with adenocarcinoma were extracted in the Hirosaki University Hospital and the Hirosaki National Hospital. IHC and ICC results showed a very high concordance rate: sensitivity of ICC in comparison with IHC was 85.7% (6/7), specificity was 100% (11/11),

positive predictive value was 100% (6/6), and negative predictive value was 91.6% (11/12). Detection of ALK rearrangement using ICC on routine Papanicolaou cytology slides is considered to be advantageous for lung cancer treatments.

Keywords: Adenocarcinoma, Anaplastic large cell lymphomakinase, Immunohistochemistry, Immunocytochemistry, FISH

1. INTRODUCTION

Lung cancer is the leading cause of cancer-related deaths in the world. Majority of lung tumor consists of non-small-cell lung cancer (NSCLC), which is often detected as advanced carcinoma associated with poor prognosis [1]. The treatment of NSCLC has been more personalized: rather targets specific oncogenic drivers than histology-driven empirical

drugs. This approach has led to the emergence of new molecularly targeted agents for specific cancer subtypes in NSCLC. Identification of activating mutations of the epidermal growth factor receptor (EGFR) is one of the most intriguing recent discoveries in the field of lung cancer research and treatment. Recent randomized phase 3 trials have found that, for patients with EGFR mutant tumors, first line EGFR tyrosine kinase inhibitor leads to a longer progression free survival compared with standard platinum based doublet chemotherapy [2-5]. More recently, anaplastic lymphoma kinase (ALK) gene rearrangement (ALK+) resulting in the echinoderm microtubule-associated protein-like 4 (EML4) - ALK fusion protein in NSCLC was first described in 2007 [6]. In recent clinical phase 1 trial, ALK inhibitor demonstrated a great effect in patients with ALK+ lung cancer [7, 8]. A small molecule inhibitor against ALK, crizotinib, had received accelerated approval by the Food and Drug Administration in 2011 to treat the patients with NSCLC harboring ALK+. In Japan, crizotinib was approved and has been used since 2012. Currently,

there are three methods for testing ALK+: fluorescent in situ hybridization (FISH), reverse transcription polymerase chain reaction (RT-PCR) and immunohistochemistry (IHC).

IHC is considered most useful for the screening of ALK+ because of the good correlation with the results by FISH and/or RT-PCR and its feasibility [9-11]. In the cases in which operative specimens are not obtained, pathological diagnosis of NSCLC is made on histological specimens by transbronchial lung biopsy (TBLB) or cytology specimens by brushing or aspiration simultaneously obtained by bronchoscopic examination. Since bronchoscopy usually cannot reach and disclose pulmonary lesions, a collection range is wider by brushing than TBLB, i.e. because of difficulty to approach a tumor by TBLB, TBLB often fails to obtain tumor tissues whereas tumor cells are contained in cytology specimens. Practically, since positive rate of tumor cells in cytology specimens has been shown to be higher than that in TBLB specimens [12, 13], a final diagnosis is often made by cytology specimens.

Immunocytochemistry (ICC) has been used for various types of samples such as pleural effusion and ascites. Evaluation of cytology specimens using ICC can facilitate determination of a tumor type and a site of origin [14]. In this study, we evaluated ICC for ALK+ in brushing smear cytology specimens simultaneously obtained with TBLB, and correlated the ICC results with the IHC results to determine whether ICC for ALK+ is a useful screening method or not.

2. MATERIALS AND METHODS

2. 1 Clinical samples

Medical records of patients who underwent bronchoscopy for lung cancer from 2009 to 2011 at the Hirosaki University Hospital and the

Hirosaki National Hospital were reviewed. Eighteen patients undergoing bronchoscopy which obtained both TBLB and brushing smear cytology specimens were extracted: TBLB specimens of 7 of the 18 patients had been shown the immunohistochemical positivity for ALK. They consisted of 11 men and 7 women and their median age with a median age of 66 years (range, 39 to 85 years). The number of never smokers and former/current smokers were 7 and 11, respectively. EGFR mutation was seen in 2 of the 18 patients. Characteristics of the patients are listed in Table 1.

2. 2 IHC

IHC was done using the commercially available antibody against ALK (dilution 1:100, clone 5A4; Novocastra, Newcastle, UK), and signals were detected using the EnVision FLEX Mini-kit (Dako, Tokyo, Japan).

Formalin-fixed and paraffin-embedded (FFPE) tissues in TBLB were sectioned at a thickness of 4 μm , and the tissue sections were deparaffinized and hydrated. Antigen retrieval was done for 40 min at 97°C in retrieval solution (pH 9.0, Dako). Peroxide blocking was carried out for 10 min with the blocking reagent (Dako). Then the slides were incubated with the primary antibody for 30 min at ambient temperature followed by incubation with the Mouse Linker (Dako) for 15 min at ambient temperature, and were incubated with the polymer reagent (Dako) for 30 min at ambient temperature. Staining was developed with diaminobenzidine (Dako). Then the slides were counterstained with hematoxylin, dehydrated and mounted.

2.3 ICC

Papanicolau-stained bronchoscopic brushing smear cytology slides containing cells cytologically diagnosed as adenocarcinoma were used.

After cytological diagnosis, the Papanicolau-stained cytology slides were decolorized in citric acid (pH 6.0) for 10 min at ambient temperature. Antigen retrieval for ICC was done for 10 min at 97°C in the same retrieval solution. Then ICC was carried out by using the same antibody and the detection kit as used in IHC.

2. 4 IHC and ICC Scoring

IHC and ICC expressions were quantified by three cytopathologists. Cytoplasmic ALK expressions were quantified using a three-value intensity score (0, 1+, 2+) and the intensity scores 1 and 2 are defined positive (Fig 1 and 2). To confirm ALK+, FISH or RT-PCR was performed by SRL Inc. (Tokyo, Japan).

3. Results

IHC and ICC results for detection of ALK+ are listed in Table 2. Of the 18 cases, the number of IHC-positive cases were 7: 6 with IHC score 2+ and 1 with score 1+. On the other hand, that of ICC-positive cases were 6 and all of them were ICC score 2+. The other 12 cases showed ICC-negative. The sensitivity, specificity, positive and negative predictive values of ICC in comparison with IHC were 85.7% (6/7), 100% (11/11), 100% (6/6), and 91.6% (11/12), respectively. The ALK IHC-positive 7 cases are listed in Table 3. Of the 7 cases, FISH could be conducted in 6 cases: 2 were evaluated as positive (case 2 and 5), 1 as negative (case 1) and 3 as indeterminate (case 4, 6 and 7). In the case 3, since tumor cells disappeared after deeper sectioning, FISH could not be done. RT-PCR was carried out only in the case 6 and ALK+ was detected. Only in the case 1, in which the FISH was negative, the result of IHC was different from that of ICC: IHC was positive whereas ICC was negative. Of the IHC-negative 11 cases, FISH were conducted in 4 cases and resulted in negative.

4. DISCUSSION

Lung adenocarcinoma with ALK+ has been found in approximately 5 to 9% of unselected patients with NSCLC [6, 9-11]. ALK+ NSCLC is more frequently observed in younger patients and in patients with lack of EGFR mutation. ALK+ patients are also generally never or light smokers, and predominantly have adenocarcinoma with an acinar or signet-ring pattern [15-18]. Although the selection of cases using these clinicopathological features increases the prevalence of ALK+, it's not able to identify all of cases with ALK+. High sensitive screening method is essential to detect patients with ALK+ for medical treatment. Although FISH has been regarded as a standard method to detect ALK+, it is not readily available as a routine method in practice [7]. FISH also has disadvantages such as relatively high cost and a long turnaround time. The evaluation of positive signals also requires considerable skill especially

when using biopsy specimens. In contrast, IHC has been widely involved in routine pathological diagnosis and easily feasible at most of pathology laboratories. Some studies have reported a difficulty in detecting ALK+ by IHC possibly due to a weak transcriptional activity of the promoter-enhancer region of EML4 that drives the expression of EML4-ALK. However, using high sensitive methods reported recently, the correlation of IHC-positive expression of ALK with ALK+ by FISH has been shown to be very high in NSCLC. Several reports described that both the sensitivity and the specificity of IHC were 90-100% in comparison with FISH [9, 11]. However depending on cases, we can often obtain only biopsy and cytology specimens because operation is not necessarily carried out in all cases with NSCLC. Moreover, because of difficulty to approach directly a tumor by bronchoscopy, TBLB often fails to obtain tumor tissues whereas tumor cells are contained in cytology specimens. That's why the evaluation of ALK+ using cytology specimens by ICC seems to considerably contribute to the treatment strategy in NSCLC

patients.

In the present study, we demonstrated that brushing smear cytology specimens were successfully stained using ALK antibody and EnVision FLEX+ method even after routine Papanicolau-staining. To our knowledge, this is the first report that brushing smear cytology specimens for routine Papanicolau evaluation are useful to detect ALK+ by ICC. Only in the case 1, the results of IHC and ICC were different from each other. Since ALK was negative by FISH in this case, the result of ICC seemed to be accurate. Some reports indicated that IHC weak positive case did not match the result of FISH [11, 19, 20]. In the present study, ICC and IHC score 2+ cases showed full match results. Since there were no cases we defined as staining score 1+ by ICC, the evaluation of ICC on cytology slides might provide clear results even on the Papanicolau-stained cytology slides. In 3 cases, FISH were indeterminate. This may be related to the fact that the biopsy specimens were fixed in formalin over 48 h in these 3 cases. The Japanese Lung Cancer Society

issues a guideline for ALK testing and recommends tissue fixation for 6 to 24 h. This recommendation is based on the American Society of Clinical Oncology/College of American Pathologists guideline recommendations for Human Epidermal Growth Factor Receptor 2 (HER2) FISH test in breast cancer [21].

The present study revealed a significant correlation between IHC and ICC. It should be noted, however, that a number of the cases with ALK+ and evaluated by FISH or RT-PCR for ALK+ was small. Therefore more larger-scale study with the molecular analysis might be required. Recently, Sakairi Y. et al reported that endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA) has been used to obtain mediastinal and hilar lymph nodes specimens in patients with lung cancer, and samples obtained with EBUS-TBNA are useful for detecting ALK+ by IHC and FISH or RT-PCR [22]. However, EBUS-TBNA cannot be applied to peripheral lesions of the lung. Our results are thought to be applied also to cytology specimens obtained by TBNA. Detection of

ALK+ using ICC on routine brushing smear cytology slides is considered to be useful for lung cancer treatments.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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Figure Legend

Fig 1. TBLB specimens of adenocarcinoma with hematoxylin and eosin staining and their corresponding IHC for ALK: one of IHC-negative (score 0) case (A and D), weak cytoplasmic staining (score 1+) in the case 1 (B and E), and strong cytoplasmic staining (score 2+) in the case 6 (C and F). (original magnification, $\times 200$).

Fig 2. Bronchial brushing cytology slides of adenocarcinoma with Papanicolau staining and their corresponding ICC for ALK: one of ICC-negative (score 0) case (A and C), strong cytoplasmic staining (score 2+) in the case 3 (B and D). ICC was done after decolorization of Papanicolau-staining on the same cluster of adenocarcinoma cells. (original magnification, $\times 400$).