A screening method for the ALK fusion gene in NSCLC

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INTRODUCTION

Lung cancer research has recently made significant progress in understanding the molecular pathogenesis of lung cancer and in developing treatments for it. Such achievements are directly utilized in clinical practice. Indeed, the echinoderm microtubule-associated protein-like 4–anaplastic lymphoma kinase (ALK) fusion gene was first described in non-small cell lung cancer in 2007, and a molecularly targeted drug against the fusion was approved in 2011. However, lung cancer with the ALK fusion constitutes only a small fraction of lung cancers; therefore, efficient patient selection is crucial for successful treatment using the ALK inhibitor. Currently, RT-PCR, fluorescent in situ hybridization (FISH), and immunohistochemistry are commonly used to detect the ALK fusion. Although FISH is currently the gold standard technique, there are no perfect methods for detecting these genetic alterations. In this article, we discuss the advantages and disadvantages of each method and the possible criteria for selecting patients who are more likely to have the ALK fusion. If we can successfully screen patients, then ALK inhibitor treatment will be the best example of personalized therapy in terms of selecting patients with an uncommon genotype from a larger group with the same tumor phenotype. In other words, the personalized therapy may offer a new challenge for current clinical oncology.

Keywords: anaplastic large cell lymphoma kinase, non-small cell lung cancer, immunohistochemistry, RT-PCR, FISH, screening, molecular targeted drugs

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Table 1 | Clinicopathological characteristics of ALK-positive lung cancers.

| Characteristics | All patients (n = 811) | ALK (n = 31) | EGFR (n = 324) | KRAS (n = 62) | Null (n = 331) |
|-----------------|------------------------|-------------|---------------|--------------|--------------|
| **AGE, YEAR**   |                        |             |               |              |              |
| Median          | 63                     | 56\(^1\)    | 65            | 64           | 65           |
| Range           | 22–89                  | 35–76       | 31–89         | 41–80        | 22–83        |
| **SEX**         |                        |             |               |              |              |
| Female          | 362                    | 21\(^2\)    | 198           | 15           | 111          |
| Male            | 449                    | 10          | 126           | 47           | 210          |
| **SMOKING STATUS** |                    |             |               |              |              |
| Never smoked    | 359                    | 21\(^3\)    | 211           | 14           | 99           |
| Smoker          | 452                    | 10          | 112           | 48           | 222          |
| **HISTOLOGY**   |                        |             |               |              |              |
| Adenocarcinoma  | 713                    | 30          | 322           | 56           | 231          |
| Squamous cell   | 52                     | 1           | 0             | 2            | 48           |
| Adenosquamous   | 8                      | 0           | 1             | 1            | 7            |
| Other           | 38                     | 0           | 0             | 3            | 35           |
| **PATHOLOGICAL STAGE** |             |             |               |              |              |
| I               | 492                    | 18          | 225           | 40           | 168          |
| II              | 137                    | 4           | 34            | 10           | 72           |
| III             | 150                    | 8           | 54            | 9            | 70           |
| IV              | 32                     | 1           | 10            | 3            | 11           |

\(^1\)Statistically significant by the independent sample two-tailed t-test (p < 0.002).
\(^2\)Statistically significant difference in sex between the ALK-positive and negative tumors (p = 0.001).
\(^3\)Statistically significant difference in smoking status between the ALK-positive and negative tumors (p = 0.001).

CONCENTRATION OF THE PATIENTS WITH ALK-POSITIVE LUNG CANCER

CLINICOPATHOLOGICAL FEATURES

Because ALK-positive lung cancer constitutes less than 5% of all lung cancers (Sasaki et al., 2010), it is critically important to select those patients who are more likely to have the ALK mutation. We initially attempted to select the patient population based on their clinicopathological features. As shown in Table 1, the patients with ALK-positive cancers were characteristically younger (a median age of 56 vs. 63), more frequently female, and more frequently non-smokers. In addition, the ALK fusion has a mutually exclusive relationship with the EGFR, KRAS, and HER2 mutations. Therefore, we attempted to select the ALK-positive lung cancer patients based on these clinicopathological features. The prevalence of the ALK translocation in the female patients younger than 63 without the EGFR, KRAS, HER2, or p53 mutations was 25% (Figure 1). However, four patients were not included in this subset (Mitsudomi et al., 2010). The selection of patients using clinicopathological features alone was able to increase the prevalence of the ALK mutation in our sample but was not able to identify all of the patients with it.

 MORPHOLOGICAL FEATURES

An alternative method of selection uses the morphological features of the tumors. Table 2 contains several morphological features known to be characteristic of ALK-positive adenocarcinomas (Figure 2). These characteristics have been identified in studies comparing ALK-positive and ALK-negative lung cancers (Inamura et al., 2008; Rodig et al., 2009; Takeuchi et al., 2009; Yoshida et al., 2011), mostly using surgical specimens. Among these features, the presence of signet ring cells has the highest predictive value for ALK-positive lung cancer. According to Rodig et al. (2009) the ALK fusion was detected in 12 of the 26 tumors that they identified as having greater than 10% signet cells by area, although such tumors constituted only 8% of total lung adenocarcinomas. By contrast, the other morphological characteristics were more frequently seen in ALK-negative tumors. The solid-subtype was significantly more frequent in the ALK-positive cancers; however, the ALK-positive rate was 8% among the solid-subtype adenocarcinomas.

In practice, candidate patients for ALK inhibitor treatments typically have advanced cancer, and their biopsy specimens and/or cytology are the major source of samples. Therefore, predicting...
FIGURE 1 | Concentration of EML4–ALK translocation using clinicopathologic features (n = 345). Numbers in black squares indicate number of patients with ALK translocation. Incidence of ALK translocation became 25% when patients were confined to those without EGFR, KRAS, HER2, p53 mutations and to female patients younger than 63. However, four patients were not included in this subset (Mitsudomi et al., 2010).

FIGURE 2 | Morphological characteristics of ALK-positive lung cancer. The cribriform pattern (A) is composed of fused acini or glands. The solid-growth pattern consists of tumor cell nests without structural architecture (B). Solid adenocarcinoma cells with the ALK fusion often have vesicular nuclei with convoluted contours and deep grooves. This subtype may be misdiagnosed as squamous cell carcinoma or mucoepidermoid carcinoma in some cases. Signet ring cell carcinoma (C) is also characteristic of ALK-positive lung cancer. Rodig et al. (2009) found that 71% of the adenocarcinomas that consisted of more than 10% signet ring cells harbored the ALK fusion. One cellular characteristic of ALK-positive lung cancer is mucin production. In extreme cases, the alveolar space is filled with mucin (D), although the lumens of the neoplastic glands seldom open to the alveolar spaces. Although the majority of mucinous tumors are negative for TTF-1, the ALK-positive tumors (i.e., the signet ring cell carcinomas) are an exception.

THREE KEY METHODS OF DETECTING ALK FUSION

Currently, the methods of RT-PCR, FISH, and immunohistochemistry have been used to detect the ALK fusion gene. Because each method inevitably has both advantages and disadvantages (Table 3), we should be aware of their characteristics before applying them to clinical samples.

RT-PCR

RT-PCR is technically easy and rapid. The chromosomal inversion that characterizes the ALK fusion makes the sequence unique, and the PCR primer only hybridizes with the fusion chimeric transcript. This unique primer is responsible for the high sensitivity of this method. Soda et al. (2007) detected fusion mRNA in sputum that contained as few as 10 EML4–ALK-expressing BA/F3 cells. This method is used only for known fusion partners, however, and all 11 of the reported variants require skillful application...
of the technique because of the long PCR products. In addition, high-quality RNA is difficult to obtain in clinical practice. We found that 4 of the 361 tumors we screened had inconsistent RT-PCR and immunohistochemical results. Three of the tumors revealed both new and known variants of the EML4–ALK fusion transcript with 5′-RACE. Therefore, this method is difficult to implement in a routine clinical diagnostic laboratory. However, RT-PCR is one of the few methods that provide direct evidence of the chromosomal translocation. A demonstration of chimeric transcription is the best direct evidence of the translocation; if the

| Table 3 | Advantages and disadvantages of ALK detection methods. |
|---------|---------------------------------------------------------|
| **Pros** | **Cons** |
| RT-PCR  | A potentially rapid diagnostic method                  |
|         | Very sensitive                                          |
|         | More accurate                                           |
|         | Difficult to obtain high-quality of RNA                  |
|         | Not applicable for unknown partners                      |
|         | Difficult to confirm the presence of tumor cells         |
|         | Difficult to apply to archival tissues                  |
| FISH    | Applicable for any partners                             |
|         | Screening method in clinical trials                      |
|         | Established in many labs                                 |
|         | Applicable to archival tissues                           |
|         | Expensive                                                |
|         | Relative long turnaround time                            |
|         | Less sensitive                                           |
| IHC     | Applicable for any partners                             |
|         | Rapid turnaround time                                    |
|         | Established in many labs                                 |
|         | Applicable to archival tissues                           |
|         | Indirect demonstration of the fusion gene                |
|         | Occasional false negative results                        |
|         | High dependence on antibody clones and detection methods |

**FIGURE 3** | Fluorescent in situ hybridization method for ALK fusion detection. The probes, which are labeled with different fluoroses, are designed at telomeric and centromeric sides between break point (A). Representative FISH images of ALK-negative (B) and positive tumors (C) are displayed. When ALK is translocated, the signals are changed from merged yellow to single red and green. The current criteria of the gene rearrangement and positive ALK FISH are as follows. Cells are considered rearrangement positive when: (i) At least one set of orange and green signals are two or more signal diameters apart. (ii) There is a single orange signal without a corresponding green signal in addition to fused and/or broken apart signals. The tumor is considered positive ALK test when: (i) A sample is considered negative if <5 cells out of 50 (<5/50 or <10%) are positive. (ii) A sample is considered positive if >25 cells out of 50 (>25/50 or >50%) are positive. (iii) A sample is considered equivocal, if 5–25 cells (10–50%) are positive. If the sample is equivocal, a second reader should evaluate the slide.
results are negative, however, it is clinically difficult to determine whether the tumor is truly negative for the ALK fusion or whether it was not detected due to impaired RNA integrity or technical errors.

**FLUORESCENT IN SITU HYBRIDIZATION**

Fluorescent in situ hybridization is currently the gold standard method used in clinical trials to detect the ALK fusion gene, and it was the first FDA-approved method for detecting the ALK fusion (Kwak et al., 2010). The FISH technique has been established in several labs to perform the HER2 test for breast cancer. FISH has the significant advantage of allowing archival material to be used. FISH also has disadvantages, however, including a relatively high cost and a long turnaround time. The evaluation of positive signals also requires considerable skill, especially when using biopsy samples. A break-apart FISH probe has been used to detect the ALK fusion (Figure 3), and the probes are designed for the telomeric and centromeric sides of the break points. Therefore, this design is applicable in detecting any kind of fusion partner, and any type of ALK gene rearrangement could theoretically be detected using this technique. However, it is known that some tumors with RT-PCR proven and ALK immunohistochemistry (IHC)-positive ALK fusions show non-split signals under the current criteria (Figure 4).

**IMMUNOHISTOCHEMISTRY**

In contrast, immunohistochemical analysis is technically easy because it is integrated into routine pathological diagnosis. This assay has the advantages of rapidity, allowing the use of archival tissues, the ability to detect any partner genes, and simple comparisons of morphology. Although the immunohistochemical technique does not detect the ALK fusion gene itself, ALK is not detectable in any normal tissues other than the brain (Iwahara et al., 1997). Therefore, an ALK-positive reaction is associated with dysregulated expression of the gene, due to the altered promoter activity that is highly characteristic of an ALK inversion.

The early studies of ALK fusion immunohistochemistry reported that not all fusion-positive tumors yielded a positive immunohistochemistry result (Martelli et al., 2009). In contrast to the ALK fusion in anaplastic large cell lymphoma, the mRNA expression is lower in lung cancer; therefore, the false negative results appear to be caused by lower sensitivity. In general, the detection threshold is determined by the affinity of the primary antibody and the signal amplification system. In terms of the antibody affinity, Mino-Kenudson et al. (2010) compared two clones of the anti-ALK antibody and found large differences in their affinities for lung cancer cells. Nevertheless, it has been reported that highly sensitive immunohistochemistry is quite effective at detecting the ALK fusion gene. Takeuchi et al. (2009) compared the immunohistochemical results of combining three anti-ALK antibodies and two detection systems and found that all of the ALK-positive tumors could be detected by their highly sensitive immunohistochemical system, regardless of the differences between the clones. These two key factors, the antibody clones and the detection system, are important for detecting the ALK fusion by immunohistochemistry (Mitsudomi et al., 2011). Indeed, when

**FIGURE 4** | A case with EML–ALK-positive adenosquamous cell carcinoma, showing negative FISH. The tumor was a well-circumscribed nodule (A), in which both components of adenocarcinoma (B) and squamous cell carcinoma (C) were histologically identified. The chimeric transcript was detected with RT-PCR products (D), in addition to positive ALK-IHC (E). However, FISH results did not fulfill the positive criteria (F).
we compared the results obtained using the ALK1 antibody with a conventional detection system to those obtained using the (high-affinity) 5A4 antibody with a highly sensitive detection system (Envision FLEX+ system), we found that 3 of 12 ALK-positive tumors went undetected by the former method (Table 4; Figure 5). 5A4 and D5F3 are known to be high-affinity antibody clones.

Table 4 | Comparison between conventional (ALK1 and standard ABC methods) and optimized IHCs (5A4 and Flex+ system) using tissue microarray and biopsy specimens.

| TISSUE MICROARRAY (n = 361) | 5A4 positive | 5A4 negative |
|-----------------------------|--------------|--------------|
| ALK1 positive               | 10           | 0            |
| ALK1 negative               | 3            | 348          |

| BIOPSY (n = 43) | 5A4 positive | 5A4 negative |
|-----------------|--------------|--------------|
| ALK1 positive   | 3            | 0            |
| ALK1 negative   | 3            | 37           |

FIGURE 5 | Discordant results according to antibody clones and detection system. A biopsy specimen only contains a small number of tumor cells [H&E staining, (A)]. When ALK1 antibody and conventional detection system was used, IHC showed negative results for tumor cells [(B), arrowheads]. However, proper clone (clone 5A4) and highly sensitive detection method (Envision FLEX+) made tumor cells show clearly positive reactions (C). Gene rearrangement and chimeric transcript of EML4–ALK fusion had been shown with FISH and RT-PCR in this specimen.

Table 5 | Comparison of IHC results according to the difference of antibody clones (5A4 and D5F3).

|                  | D5F3          |
|------------------|---------------|
| 5A4              |               |
| Positive         | Negative      | Membranous only |
| Positive         | 12            | 0              | 94          |
| Negative         | 1             | 343            | 264         |

1 Chimeric transcripts of EML4–ALK were detected in all cases.
2 The tumor was negative for chimeric transcript of EML4–ALK and FISH.
3 The tumors were negative for chimeric transcript of EML4–ALK.
FIGURE 6 | A case of ALK-IHC positive small cell lung cancer. A lung nodule was biopsied from the 83-year-old female smoker. H&E section (A) shows typical morphology of small cell lung cancer and CD56/NCAM and synaptophysin were positive. Although it is little reports of SCLC with ALK fusion, the tumor cells were positive for ALK immunohistochemistry using clone 5A4 and Envision FLEX+ (B). No gene rearrangement and chimeric transcript of EML4–ALK have been detected, respectively.

When a highly sensitive detection system was used, either clone was able to detect all of the ALK-positive cancers, although D5F3 caused membranous staining in some ALK-negative tumors (Table 5). Because IHC does not directly demonstrate ALK fusion, there are certain pitfalls. Certain small cell lung cancers have positive reactions (Figure 6) but do not have the ALK translocation. This positive reaction did not appear to be associated with gene amplification, and the reason for the positive reaction was unknown. The ALK protein may be expressed in association with the neuroectodermal differentiation of small cell lung cancer, as it is expressed in the normal brain (Iwahara et al., 1997). Another pitfall is a certain rate of false negative reactions in signet ring cells, even when using a high-affinity antibody with a highly sensitive detection method. A large amount of cytoplasmic mucin can often push the cytoplasm into the rim, and the thinned cytoplasm weakly demonstrates a positive reaction in certain cases (Figure 7). Fortunately, pure signet ring cell carcinoma is extremely uncommon in lung cancer, and the other tumor components can show positive reactions, or a FISH analysis can be used instead.

ALK TESTING GUIDELINES BY THE JAPANESE LUNG CANCER SOCIETY

The ALK inhibitor crizotinib is expected to be introduced in Japan, and we face the practical application of the methods described above because this agent will be approved for patients with ALK fusion detected using any methods. This is in contrast with US policy, where crizotinib has been approved for ALK-positive patients only with an FDA-approved test. As noted, different precautions are necessary for individual methods as well as the appropriate handling of the tissues. Under the circumstances, the Japanese Lung Cancer Society issued a guideline for ALK testing (Figure 8). The guidance mentioned detailed the pros and cons of these methods, precautions for tissue handling, tissue application (biopsy,
cytology, and surgical specimens) and comparisons of the three methods, including the different results of the various antibody clones against ALK for immunohistochemistry. Although validation of this algorithm has recently begun in a large prospective cohort, it is stressed that concurrent multiple methods should be used to select ALK-positive patients.

CONCLUSION
The lung cancer with ALK fusion constitutes only a few percentages in overall lung cancer, thus the target proportion is very limited in comparison with the other molecular targeted treatments. However, if we succeed to achieve successful screening of the patients, the treatment with ALK inhibitor will be the most representative personalized therapy in terms of selecting the patients from those harboring the same category of tumors according to the infrequent gene alteration. In other words, the achievement may be a new challenge for the current clinical oncology.

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