Guidance for ALK Gene Testing in Lung Cancer Patients

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Introduction

EML4-ALK gene translocation was initially reported in 2007 by Soda and Mano et al., Jichi Medical University (1). Translocation of the BCR-ABL gene causes chronic myelogenous leukemia, while an ABL tyrosine kinase inhibitor, imatinib, is known to have strong anti-tumor effects. Also, lung cancer with the EML4-ALK gene translocation was reported to respond well to the ALK inhibitor PF02341066 (crizotinib) (2). Crizotinib had already been developed when EML4-ALK translocation was discovered, thus clinical studies proceeded rapidly, and an application for its marketing approval was submitted to the Food and Drug Administration (FDA) and Pharmaceuticals and Medical Devices Agency (PMDA) in 2011, less than 4 years after discovery of the target. The FDA approved crizotinib on August 26, 2011 as a drug for treating non-small cell lung cancer accompanying ALK gene abnormality. In Japan, crizotinib is slated to be introduced for clinical care in early 2012. However, various precautions are necessary for its appropriate handling with EML4-ALK lung cancer. This article reviews recent findings, mainly about the precautions required for medical care of lung cancer with crizotinib, especially for diagnosis of ALK gene mutations.

1. ALK-translocated lung cancer

Soda, Noma et al. of Jichi Medical University identified the EML4-ALK fusion gene in 2009 by transfection of a cDNA expression library from a male lung cancer patient who had a history of light smoking into mouse 3T3 fibroblasts, and then collecting genes with transforming activity using the focus formation as an indicator. This was a modification of a method used for cloning RAS genes reported in the 1980s (1). Normally, the EML4 (echinoderm microtubule-associated protein-like 4) gene and ALK (anaplastic lymphoma kinase) genes are present on the short arm of chromosome 2, coded in opposite directions. Small inversions within these genes results in an EML4-ALK chimeric fusion gene (1) (Fig. 1).

ALK, a receptor-type tyrosine kinase, normally becomes activated via dimerization and subsequent ligand binding. However, EML4-ALK gene translocation is thought to result in constant ALK-dimerization, resulting in constitutive ALK activation (in the absence of ligand-binding), due to ALK-binding to the coiled-coil domain(3). Although oncogene activation via gene translocation is well known in hematologic malignancies, it has been thought to be rare in epithelial solid tumors. Thus, this finding is considered to be important. On the other hand, the Cell Signaling Technology group independently discovered ALK activation using systematic mass spectrometry analyses of phosphorylated tyrosine in lung cancer cells (4). Several hundred lung tumors were found to develop within several weeks after birth in EML4-ALK transgenic mice, while a rapid regression of those tumors was observed following ALK inhibitor administration (5).

2. Mechanism of ALK translocation

ALK gene translocation was originally reported in anaplastic lymphoma, followed by inflammatory myofibroblastic tumor (IMT).[1] In these reported cases, the partner of translocation was not EML4, but rather NPM, TPM3, TFG, ATIC, CLTC1, MSN, TPM4, ALO17, or MYH9 in cases of lymphoma, and TPM4, RANBP2, CARS, or SEC31L1 in IMT (6). ALK translocation often results in a fusion protein after exon 20 on the ALK side (upstream of the tyrosine kinase domain), with a fragile portion on exon 19 thought to be the likely site.

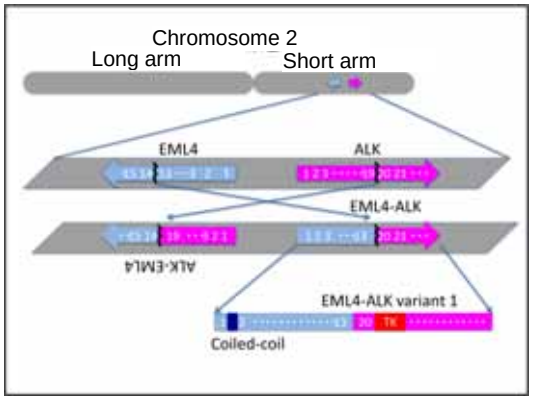

Fig. 1. Mechanism of EML4-ALK variant 1. The gene sequence is rotated in the opposite direction on the chromosomal short arm, then re-binds at breakpoints in the EML4 and ALK genes, forming EML4-ALK and ALK-EML4. Only EML4-ALK, which has both the coiled-coil domain (necessary for dimerization of EML4) and the tyrosine kinase domain of ALK, is believed to be active.

[1] IMT is a rare tumor consisting of spindle-shaped cells exhibiting the characteristics of myofibroblastic cells, and an accompanying infiltration of inflammatory cells (mainly lymphocytes and plasma cells). Primary lesions occur most commonly in the lungs, followed by various regions including mesenterium, intraperitoneal organs (i.e. liver, stomach, intestines, bladder, etc.), head, and limbs.

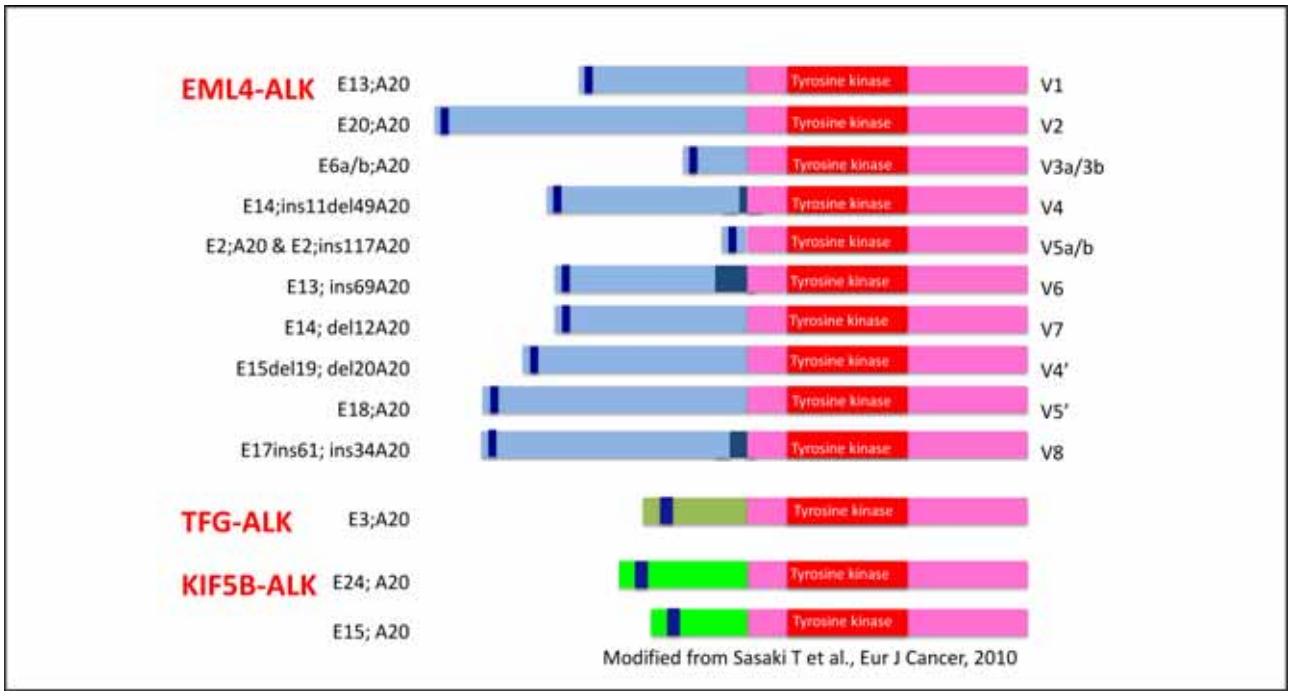

Figure 2. ALK translocations found in lung cancer (Horn L, Pao W. EML4-ALK: Honing in on a new target in non-small-cell lung cancer. J Clin Oncol 2009; 27: 4232-5.)

Conversely, translocation partners such as NPM, TPM3 and EML4 all have an oligomerized or coiled-coil domain. When these are fused with ALK, constant dimerization of ALK is achieved without ligand binding, resulting in activation and formation of a carcinogenic kinase. As another mechanism of ALK activation, point mutations in the kinase domain of the ALK gene (such as in EGFR gene alterations) have been reported in neuroblastomas.

EML4-ALK is specifically linked to lung cancer and has not been reported in other tumors, but more than 10 vairiants have been revealed to date (Fig. 2). The coiled-coil domain on the N-terminal side of EML4 and the kinase domain of ALK exon 20 are essential for transforming activity, and all known variants possess these domains. Some variants exhibit deletion or insertion of 10-70 base-pairs. Among them, two variants are common: the fusion of EML4 exon 13 and ALK exon 20 (variant 1), and the fusion of EML4 exon 6 and ALK exon 20 (variant 3a/b), (about 30% each; Fig. 3).

Takeuchi et al. detected a new ALK translocation in a lung cancer specimen found to be ALK-translocation positive through high-sensitivity immunostaining. In this case, exon 24 of the KIF5B gene was detected to be fused with exon 20 of ALK (7). Recently, fusion with exon 15 of KIF5B has also been reported (8). KIF5B is a protein involved in transportation of subcellular organelles that also possesses a dimerized domain. Therefore, in KIF5B-ALK, similar to EML4-ALK, ALK is believed to be constitutively activated as a result of dimerization.

Cell Signaling Technology comprehensively searched for the proteins subjected to tyrosine phosphorylation using a combination of immunoprecipitation and mass spectrometry in 41 lung cancer cell lines and more than 150 lung cancer specimens. An acceleration of ALK phosphorylation was detected in 1 specimen of the cell line H2228 and in 3 clinical specimens. EML4-ALK (E6;A20 and E13;A20) was identified in 3 specimens. Translocation with exon 3 of TFG gene (TRK fused gene) was detected in the remaining specimen,. This was the same type of fusion previously identified in lymphoma. TFG a coiled-coil domain as well.

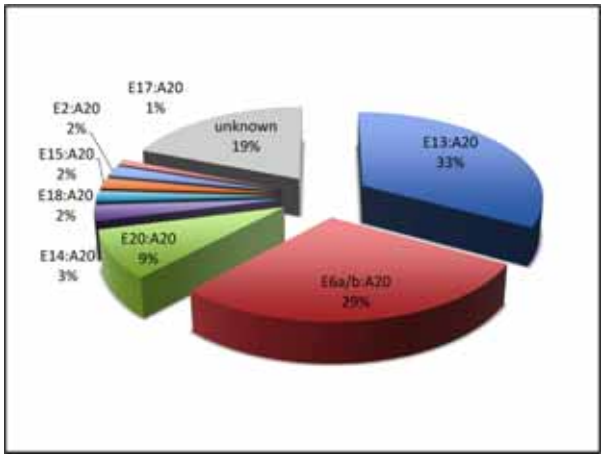

Figure 3. Frequency of EML4-ALK translocation in lung cancer (Sasaki T, Rodig SJ, Chirieac LR, Janne PA. The biology and treatment of EML4-ALK non-small cell lung cancer. Eur J Cancer 2010; 46: 1773-80)

3. Clinicopathological characteristics of ALK-translocated lung cancer

ALK-translocated lung cancer account for about 2 to 5% of all cases of non-small-cell lung cancer and its characteristics are shown in Table 1 Histologically, adenocarcinoma is predominant, where the frequency of ALK-translocation is approximately 4 to 5%. ALK-translocation is extremely rare in other histological types,. The first case of ALK-translocated lung cancer was reported in a smoker, but it is generally believed to be more common in non-smokers. There are no apparent racial differences, unlike in EGFR gene mutations (Table 1). ALK-positive lung cancer tends to be more common in younger generations, and the mean age for its onset is reported to be in the mid-50s. The median age of onset is approximately10 years earlier than for ALK-negative lung cancers. Sexual differences are not obvious, but it is slightly more common in women. This may be related to the sexual difference in smoking rates.

Nevertheless, ALK translocations have been detected in smoking and elderly lung cancer patients. Thus, it is impossible to definitely predict or rule out the presence of ALK solely by clinical background factors. In other words, it is critical to recognize that ALK status is unknown until specific testing is performed.

On the other hand, ALK translocation has been repeatedly shown to have a mutually exclusive relationship with other gene mutations seen in adenocarcinoma of the lung, such as EGFR, KRAS, and HER2. If any of these gene mutations have been detected, it is highly unlikely that ALK translocation will be present in that same patient. However, there has been one report of concomitant EGFR mutations, detected in 3 out of 50 untreated ALK-positive lung cancer patients (9).

Table1. The relationship between various clinicopathologic factors and ALK translocation

Reporter	Journal	Year	Overall		Histological type		Smoking history		Age		Sex	
			Number of cases	ALK+	Adenocarcinoma	Non-adenocarcinoma	Non-smoker	Smoker	ALK(+)	ALK(-)	Female	Male
Soda	Nature	2007	75	5(7%)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Rikova	Cell	2007	103	4(4%)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Shinmura	Lung Cancer	2008	77	2(3%)	2/50(4%)	0/27	0/22 (0%)	2/41(5%)	53	66	1/39 (2.5%)	1/38 (2.5%)
Perner	Neoplasia	2008	603	16(3%)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Koivunen	CCR	2008	306	6(2%)	8/208(4%)	0/97	4/69 (6%)	2/184 (1%)	55.9	61.9	5/124 (4%)	3/187 (2%)
Wong	Cancer	2009	266	13(5%)	11/209(5%)	2/12*	10/127	1/82	59(52-65)	64(55-71)	8/134	5/132
Boland	Hum Pathol	2009	335	6(2%)	N/A	N/A	N/A	N/A	69.8	69.6	N/A	N/A
Martelli	Am J pathol	2009	120	9(8%)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Rodig	CCR	2009	358	20(6%)	20/358(6%)	–	14/95(15%)	6/243(2%)	51(29-76)	66(29-90)	9/220(4%)	11/138(8%)
Shaw*	JCO	2009	141	19(13%)	18/130(14%)	1/11(9%)	19/85 (22%)	0/57 (0%)	52 (29-76)	65 (29-90)	8/85 (9%)	11/38 (29%)
Inamura	Mod Pathol	2009	363	11(3%)	11/253(4%)	0/110	6/105(6%)ADC	5/147(3%)ADC	56 ± 11	64 ± 9	5/134(4%)ADC	6/119(5%)ADC
Takahashi	Ann Surg	2010	211	5(2%)	5/211(2%)	0/102	4/92 (4%)	1/118 (1%)	70.0 ± 9.7	65.2 ± 10.1	4/100 (4%)	1/111 (1%)
Paik	JTO	2011	640	28(4%)	27/450(6%)	1/190(1%)	16/275(6%)	12/365(3%)	N/A	N/A	14/226(6%)	14/414(3%)
Yatabe	unpublished	2011	831	31(4%)	31/730(4%)	1/100(1%)	21/364 (6%)	6/379 (2%)	57 ± 9.9	65 ± 9.5	20/382 (5%)	11/447 (2%)
Total			**4429**	**175(4%)**	**133/2699(5%)**	**5/649(1%)**	**94/1234(8%)**	**34/1626(1%)**	**N/A**	**N/A**	**74/1444(5%)**	**63/1624(4%)**

*selected based on clinicopathologic factors

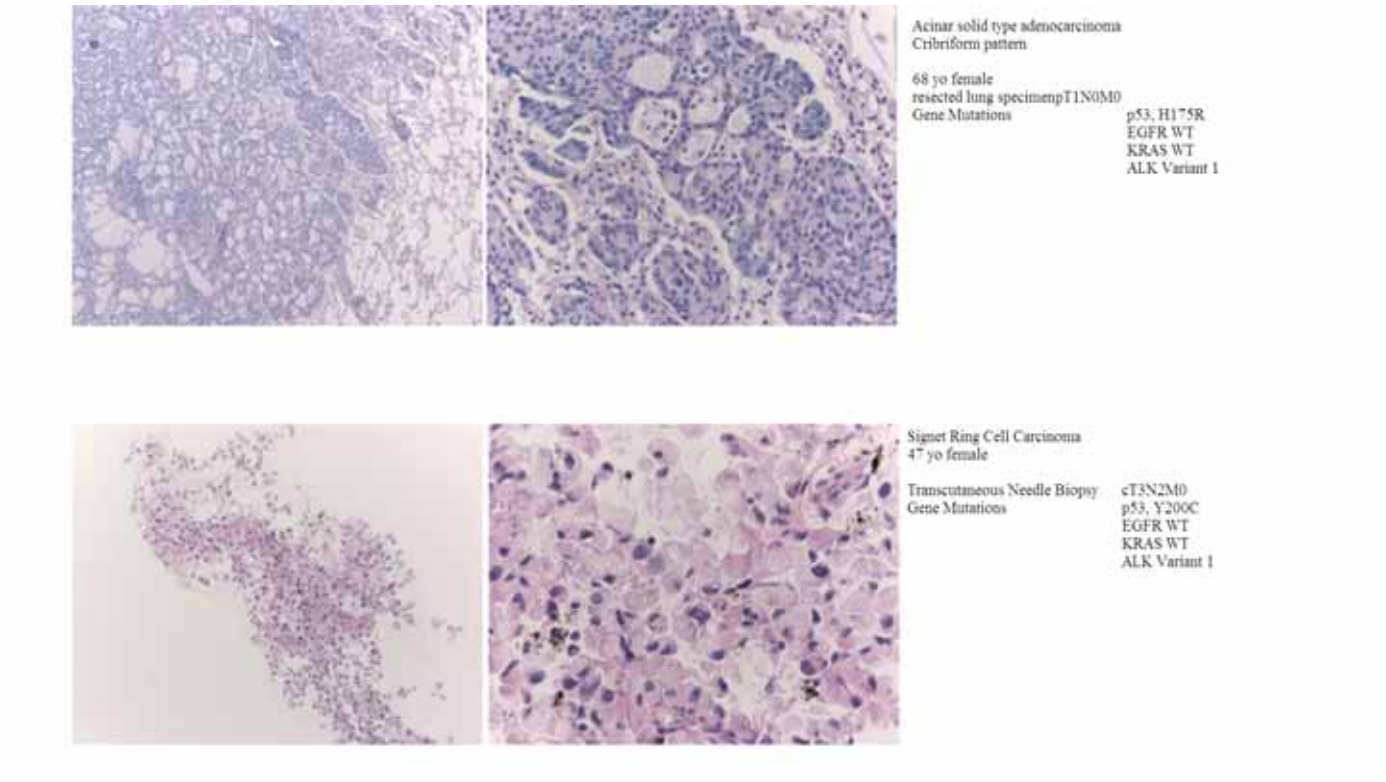

Fig. 4. Histological images of ALK lung cancer. In ALK-positive lung cancer, adenocarcinoma is said to commonly exhibit a characteristic cribriform pattern. These patterns are classified as the acinar adenocarcinoma or solid adenocarcinoma on the histological classification of adenocarcinoma. The ALK fusion gene is also commonly detected in adenocarcinoma with the morphology of signet ring cell carcinoma, but this component is usually only partially seen

Histopathological characteristics of ALK-positive lung cancer have also been described. Inamura et al. reported that the acinar type was predominant in 6 out of 11 cases of EML4-ALK lung cancer, while the papillary type was predominant in the remaining 5 cases (according to WHO classification, 4 cases were of the acinar type, 2 cases were of the papillary type, and 5 cases were of the mixed type)(10). All 11 cases tested positive for thyroid transcription factor 1 (TTF-1). The origin of the cells is believed to be from those derived from peripheral lung tissue, in which EGFR variants are prevalent. According to Rodig et al, ALK-positive lung cancer was present in 1 out of 22, 4 out of 124, 0 out of 46 and 11 out of 134 cases in which the predominant pattern was bronchiolo-alveolar carcinoma (BAC), acinar, papillary and solid, respectively, with the percentage being higher in the solid type (11). At the cellular level, the patients who had "signet ring cells," which were rich in mucin and had eccentric nuclei, accounted for 82% of the patients with ALK-positive lung cancer. In terms of data, the incidence of ALK translocation in the cases of adenocarcinoma in which the percentage of signet ring cells was 0%, 10% or less, and 10% or more was 3 out of 295 cases, 2 out of 21 cases and 12 out of 26 cases, respectively (11). Typical histological images of ALK-positive lung cancer are shown in Figure 4.

4. Clinical studies of ALK inhibitors

PF02341066 (crizotinib) was originally developed as an inhibitor of MET, but was later shown to also inhibit ALK. During the dose-escalating Part I of the phase I study, a significant effect was noted in ALK cases of lung cancer with ALK translocation, as well as in ALK-translocated cases of IMT. In Part II of the study, activation of ALK or MET was included in the eligibility criteria, to evaluate the antitumor effects of the recommended 250 mg twice daily dose. The results obtained in ALK-positive lung cancer testing were reported at the annual meeting of the American Society of Clinical Oncology (ASCO) in 2009, and in 2010, the results of the study equivalent to phase II data were reported in the New England Journal of Medicine (2). The updated results presented at ASCO 2011(12) are shown below.

1500 patients with lung cancer were screened by FISH, resulting inthe identification of 119 patients with ALK-positive lung cancer Most of these patients had received prior treatment. In Part 2, the patients with ALK lung cancer received treatment of a 28-day cycle at the same dose. Patients with ALK-positive lung

cancer tended to be young, non-smokers, and commonly showed adenocarcinoma. An anti-tumor effect could be evaluated in 116 cases, and 71 cases responded to treatment, including two cases with CR. The overall response rate was 61%, and the disease control rates at Week 8 and Week 16 after administration were 79% and 67%, respectively. Five cases had PD. The median progression-free survival (PFS) was 10.0 months (95% confidence interval, 8.2-14.7 months, N = 119). 114 out of 119 cases showed adverse events, and these were generally mild. Among them, 95 cases were of grade 1 or 2, and 19 cases were of grade 3 or 4. As a characteristic adverse event, visual disorder (after images, etc.) was reported in 62% of the cases, but was mostly transient, and ophthalmologic abnormalities were not observed.

Table 2. Merits and demerits of various methods for detecting ALK gene translocation

	IHC	FISH	RT-PCR
PROS	Capable of detecting unknown fusions.	Capable of detecting unknown fusions.	High sensitivity.
	Relatively easy to conduct.	Established as a method for diagnosing lymphoma.	High specificity.
	Capable of testing FFPE samples	Adopted as a screening method in clinical studies of crizotinib.	
	Short turnaround time.	Evaluation at the cellular level is possible.	
	Adopted at numerous facilities.	Capable of testing FFPE samples	
CONS	Fusion genes are not visually confirmed	Expensive.	RNA of good quality is required (sometimes difficult in FFPE)
	The results may vary significantly depending on the clone of the antibody and the detecting system	Relatively long turnaround time.	Multiplexing and multiple PCRs are required to cope with numerous patterns of translocation.
	False-positive and false-negative cases have been reported.	Technical experience is necessary	Incapable of detecting unknown fusions
		Evaluation of translocation on the short arm of chromosome 2 is sometimes difficult.	Incapable of confirming the presence of tumor cells

Considering these excellent results, the Second Committee on Drugs of the Ministry of Health, Labour and Welfare approved the designation of “orphan drug” on January 20, 2011. This allows for preferential consideration for promoting development and clinical applicationas well as with the J-NDA (Japan New Drug Application) process as a whole. Thus, an application for marketing approval has been submitted and the drug is expected to be approved in early 2012. In Japan, a phase III study comparing crizotinib with docetaxel or pemetrexed in the secondary treatment of ALK lung cancer (PROFILE 1007 study), and a phase III study comparing crizotinib with cisplatin + pemetrexed in the primary treatment (PROFILE 1014 study) are currently ongoing.

5. Crizotinib resistance

The PFS of crizotinib for ALK-positive lung cancer is 10.0 months, which is equivalent to the PFS of gefitinib and erlotinib for EGFR-mutated lung cancer. This shows that resistance develops, as it does against EGFR-TKIs even if the cancer is drug-sensitive at first.. Secondary mutation of ALK genes has been reported as the causative mechanism (13). Secondary mutations in two kinase domains, i.e., C1156Y and L1196M, have been reported, with in vitro resistance demonstrated in both cases. In particular, L1196M of ALK is homologous to T790M of the EGFR gene (involved in resistance of EGFR-TKIs), or T315I of ABL (involved in acquired CML resistance to imatinib), and is known as a gatekeeper mutation. New ALK inhibitors and HSP90 inhibitors currently under development have reported efficacy for such mutations, and in the future secondary treatment strategies will likely be selected based on resistance mechanisms. that A secondary L1152R mutation and resulting EGFR pathway activation were observed in DFCI076, a cell line derived from a crizotinib-resistant patient in other reports (9).

6. Diagnosis of ALK translocation

Currently available methods for detecting ALK abnormalities include immunohistochemistry (IHC), fluorescence in situ hybridization (FISH), and RT-PCR (including base sequence determination). The advantages and disadvantages of each method are summarized in Table 6, and are also reviewed below.

6.1. FISH

A DNA probe labeled with a fluorescent pigment is hybridized with the target gene in the sampleand the signal is examined by fluorescence microscopy. Since the eligibility criteria in the aforementioned crizotinib studies was positivity via FISH, this method can be regarded as the current standard. In the United States, an FDA-approved test must be used to appropriately select patients for crizotinib treatment, and presently only FISH is FDA-approved for ALK detection.

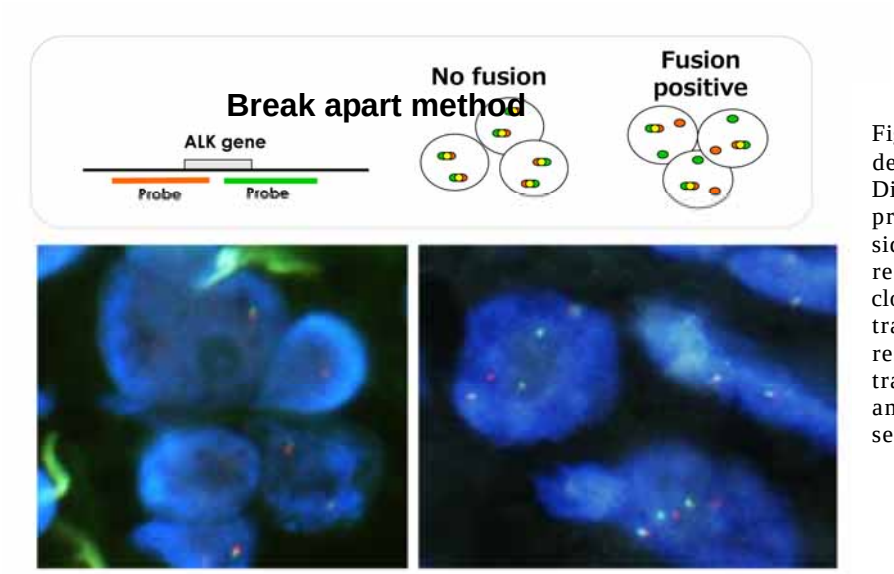

Figure 5. Break-apart FISH for detection of ALK translocation. Different-colored fluorescent probes are used to label the two sides of the gene breakpoint. The red and green probes are spatially close by, when there is no translocation, and an overlap results in a yellow signal. When a translocation is present, the red and green probes appear spatially separated

Two methods are available for FISH. In one method, probes are placed on the ALK gene and EML4 gene, respectively, and a fusion of these genes is detected (fusion assay). The other method involves placing two probes on the ALK gene with a breakpoint located in-between, and breakage of the gene and fusion with other genes are detected (break-apart assay; BAP)(Fig. 5). However, EML4 and ALK are originally present at relatively close proximity on the short arm of chromosome 2, and thus, the fusion signal is sometimes difficult to identify. Moreover, the fusion partner is not necessarily EML4. For these reasons, the latter method, i.e., break-apart assay, is currently used for most cases. In clinical studies of crizotinib, the cases were deemed positive when 15% or more of the tumor cells had an isolated red signal, or a signal of the isolated 3' end. A kit is available for purchase from Abbott Molecular (Vysis LSI ALK Dual Color, Break Apart Rearrangement Probe; Abbot Molecular, Abbott Park, IL), and many researchers, including those in the prior clinical study, have experience with this kit.

Chihara et al. examined the data from NEJM and pointed out that the response rate was 22/27 (81%) in the cases in which EML4-ALK translocation was confirmed by IHC or RT-PCR in addition to FISH, but was 25/52 (48%) in the cases in which translocation was confirmed by FISH only and other confirmation methods were not used. The difference was statistically significant (P = 0.007)(14). This calls attention to the difficulty of evaluation by FISH in individual cases, and highlights the need for establishment of a standard for evaluation, correlated with clinical response rate. (15).

6.1.1 Tissue Specimens for FISH

Regular formalin-fixed paraffin-embedded (FFPE) specimens are used in FISH. While relatively thick slices are often required for DNA extraction, slices of the same thickness as for IHC (3-4 μm thick) are required for FISH. Since stronger heat treatment and protease than ordinary IHC are needed, sliced tissues may easily

come off from the slides. It is essential to use slides coated with an anti-stripping agent. Typical coated slides include MAS , MAS-GP , and FRONTIER coated slides (Matsunami Glass).

Since the target of FISH detection is DNA, the test is highly affected by fragmentation of DNA in the specimens. DNA fragmentation may be triggered by over-fixation resulting from prolonged (more than 5 days) immersion in formalin and the decalcifying operation using formic acid.. Therefore, these procedures should be avoided, and the use of the tissue specimens exposed to such treatments as well. In particular, researchers should remember that specimens from bone metastases of lung cancer are routinely subjected to a decalcifying operation, and examination by FISH and immunostaining becomes impossible.

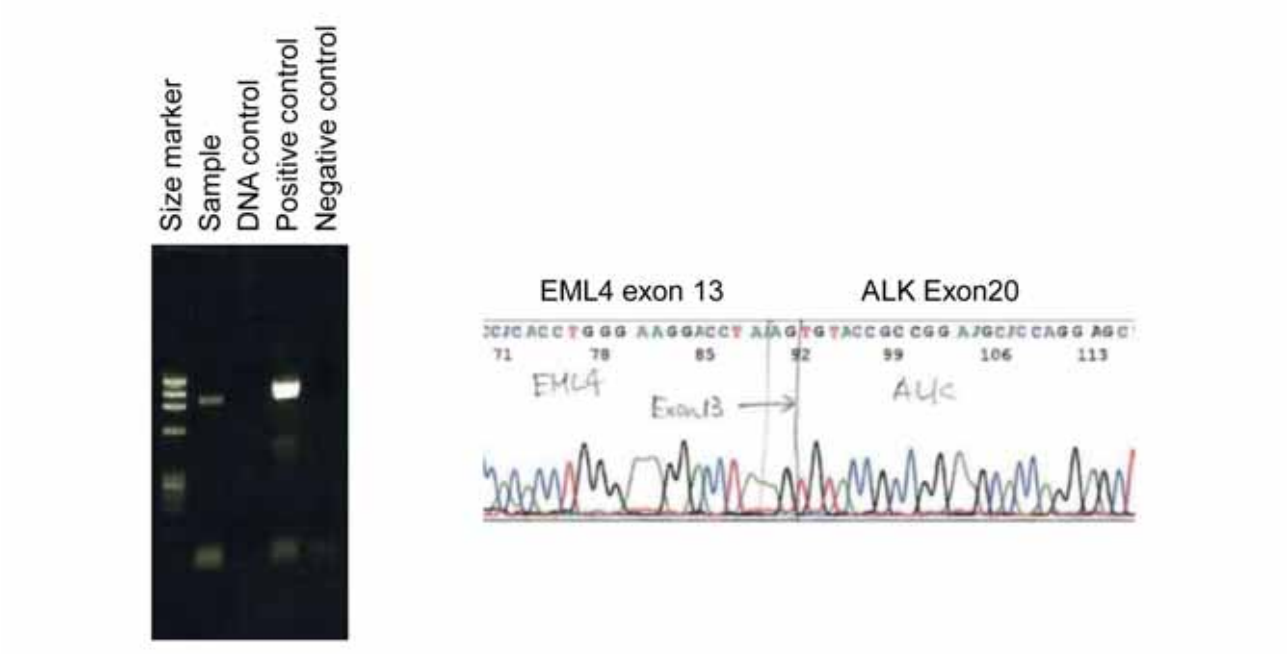

Figure 6. RT-PCR detection of EML4-ALK variant 1

In HER2 gene amplification detection of breast cancer specimens, guidelines have been published by ASCO / CAP (College of American Pathologists) in which the conditions for tissue fixation are described (http://www.cap.org/apps/docs/committees/immunohistochemistry/summary_of_recommendations.pdf). These guidelines state that fixation in neutral buffered formalin should be started within 1 hour after isolation of the specimen, that tumors should be sliced to the thickness of 5 to 6 mm, and that fixation should be completed between 6 and 24 hours. The guidelines also recommend that the time spent for these procedures (time until fixation, method of fixation, and time of fixation) should be recorded, and that the testing of non-stained specimens should be completed within 6 weeks after preparation. While these recommendations need to be followed as a general rule, fixative solutions other than formalin fixation are occasionally used in Europe and America, and neutral buffered formalin is recommended. However, even the use of ordinary newly-prepared formalin will not cause problems in the test. Similar consensus has been reached about the HER2 test of breast and gastric cancers in Japan.

While FISH enables morphological examination and identification of tumor cells, the observation is made in a dark field, and detailed observation such as that achieved by light microscopy is impossible. Therefore, specimens with small numbers of tumor cells should be avoided.

6.2 RT-PCR (reverse transcriptase[2]-PCR)

In EML4-ALK translocation, EML4 is fused in the opposite direction. Therefore, highly detailed detection of translocation can be expected if primers are set on the EML4 side and the ALK side, respectively, because PCR product is not produced in a normal state. Rather, it is produced only when translocation has occurred in the inverted position. Moreover, it is possible to determine base sequences in succession, if necessary, and

[2] Usually, genetic information flows from DNA to RNA and then to protein. However, retroviruses have an RNA-dependent DNA synthesizing enzyme. This enzyme is called reverse transcriptase.

it is also possible to verify the translocation mechanism at the nucleotide level. (Fig. 6).

However, as noted before, there are many variants of EML4-ALK, and this should be kept in mind during testing. Takeuchi et al. took this into consideration, and reported that many variants could be detected by multiplex PCR, in which two primers on the sense side are placed on exon 2 and exon 13 of EML4, and a primer on the anti-sense side is placed on exon 20 of ALK (16).

In this case, cDNA synthesized by reverse transcription of mRNA should be used as the specimen, because chromosomal DNA exceeds the size of usual amplifiable PCR products.[3] Identifying the variant by determining the size of the PCR product is possible, but there are associated difficulties. For example, in the case of variant 2 obtained as a result of fusion of exon 20 of EML4 with exon 20 of ALK, the PCR product becomes relatively large (1284 base pairs). To cope with this problem, high-quality RNA and advanced RT-PCR techniques are required, but extraction RNA of this length from ordinary FFPE specimens is difficult. Therefore, specimens need to be treated before fixation, but in the future this could be problematic when this technique is used in routine clinical tests. Moreover, since translocation of KIF5B-ALK and TFG-ALK cannot be detected using PCR primers designed to detect EML4-ALK,the inability to detect unknown fusion partners remains a problem.

6.2.1 Specimens of RT-PCR

Because the primers of the sequences which are not usually included in transcription products due to chromosomal inversion are used, increased sensitivity is expected. However, detection of long chimeric transcripts is necessary, and high-quality RNA is required. Moreover, since it is impossible to verify the presence of tumor cells after extraction, the verification of appropriate tumor tissue is vital. More specifically, it is necessary to collect tissue in a way that the specimen becomes paired with the tissues to be fixed in formalin after collection, and to treat the tissues immediately with RNA decomposition inhibitor (RNAlater/Qiagen, etc.). In the case of cytological specimens, the specimen must be thoroughly stirred in physiological saline or PBS to eliminate bias in the distribution of tumor cells. It should be taken into account that, since EGFR gene mutation is examined on the basis of DNA, the procedure differs from that using RNA specimens. A more commonly used method is to embed part of the tumor tissues in OCT compound, and to obtain DNA or RNA selectively from a region rich in tumor cells. The stamp method can be used for both biopsy tissues and isolated specimens. Since tumor cells selectively adhere to the slides (17), their alcohol fixed specimens become a good source of analysis. In the case of pleural effusion, etc., cell blocks should be prepared[4] because they can be applied to immunostaining and FISH.

6.3 Immunohistochemistry (IHC)

Immunostaining of ALK is useful for identifying lymphomas harboring ALK translocation, but EML4-ALK in lung cancer is often difficult to detect by ordinary immunohistochemical staining (18). Specifically, even if ALK translocation is present in lung cancer, the protein expression level is much lower than that seen in lymphoma. However, thanks to recent achievements, higher sensitivity and specificity for IHC detection of ALK have been reported for lung cancer.

6.3.1 Specimens

Non-stained sliced specimens are used for examination almost in the same way as the FISH method. Coated

[3] The DNA extracted from the nucleus of cells is genomic DNA (chromosomal DNA), which contain components that become blueprints for protein synthesis (exons) and parts which are not translated (introns). DNA is transcribed by messenger RNA (mRNA) first before protein synthesis, during which introns are omitted. This is called splicing. The process in which protein is synthesized from mRNA is called translation. DNA synthesized from RNA using the above-mentioned reverse transcriptase is called complementary DNA (cDNA), in which the introns are absent. In contrast, chromosomal DNA is sometimes known as gDNA.

[4] Several methods exist for preparation of cell blocks. While fibrin clotting method, glucomannan method, agarose method, etc. are known, the sodium alginate method is the easiest procedure that can be carried out by the following process.

Sodium alginate method (Junji Sano, et al., the Journal of the Japanese Society of Clinical Cytology 44: 291-7, 2005).

1. Centrifuge at 1500 rpm for 5 minutes.
2. Re-suspend in formalin solution and fix for 2 to 3 hours.
3. Centrifuge again. Discard the supernatant, and re-suspend the sediment in distilled water and wash.
4. Centrifuge again, and discard the supernatant. Add 0.5 mL of 1% sodium alginate and stir.
5. Add 1 or 2 drops of 1M calcium chloride solution to immediately gelatinize the solution.
6. Pick up the gel with tweezers, and embed in ordinary paraffin to prepare a specimen.

glass slides are needed to prevent detachment of sliced tissues resulting from the treatment with antigen activation It is possible to examine slides by immunostaining if at least one non-stained specimen is available, but it is recommended to prepare non-stained specimens for FISH as well. 3 or 4 non-stained specimens are usually needed, as well as spares. One of these specimens should be stained with HE to confirm the presence of tumor cells. In the case of TBLB (transbronchial lung biopsy) specimens in particular, precautions must be taken because tissues may be lost or tumor cells may disappear if the specimens are re-sliced after pathological diagnosis. As outlined in the FISH section, tissue fixation must be conducted according to the guidelines for HER2 test in invasive breast cancer, as delineated in ASCO/CAP guidelines.

6.3.2 Antigen activation

Methylene bridges, etc. are produced proteins as a result of formalin fixation, leading to steric hindrance and masking of antigenic determinants. The process of activation using heat or proteases is called antigen activation, and is an essential process in ALK staining, because the total amount of ALK expression is small. As shown in Table 3, microwaves, pressure pans, and autoclaves are used for heating, and various solutions for antigen activation are also used. Because the slices may be stripped off in this process, to prevent this detachment, glass slides coated with aminosilane or MAS should be used.

6.3.3 Primary antibody

Clones such as ALK1 (Dako), 5A4 (Novocastra, SantaCruz), SP8 (Lab Vision), ZAL4 (Zymed), P80 (Nichirei), and D5F3 (Cell Signaling technology) are available as anti-ALK antibodies. Table 6 shows the results of several examinations. According to Mino-Kenudson et al., D5F3 was significantly better than ALK1, but unfortunately, this antibody is not commercially available at present (18). In the study of three clones,(ALK1, 5A4 and SP8, by Takeuchi et al.) all three exhibited good sensitivity when the iAEP method (described below) was used for detection, but SP8 showed a high rate of false-positivity (7). On the other hand, several reports have shown that ALK1 exhibits poor sensitivity when ordinary detecting methods were used (Table 3).

6.3.4 Detecting method (signal amplification method)

In usual immunostaining, the most commonly used methods are the enzyme-antibody method, using alkaline phosphatase, and horse radish peroxidase (HRP) as labeling enzymes. The primary antibody treatment to counteract the target protein antigen, and HRP-bound secondary antibody treatment to counteract the primary antibody are conducted. After this, the HRP antibody may be visualized via reaction with diaminobenzidine (DAB).

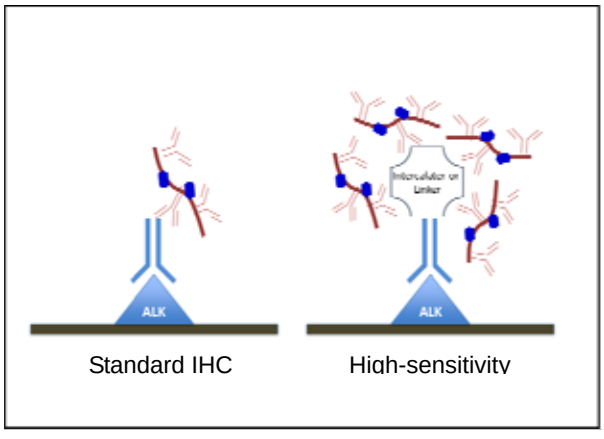

Fig. 7. High-sensitivity IHC. This figure illustrates the difference between standard IHC versus high-sensitivity IHC

A product of binding numerous HRP molecules using polymers on the secondary antibody application step has been marketed. In recent years, even more sensitive methods, such as EnvisonFlex+ (Dako) and iAEP (intercalated antibody-enhanced polymer) method (Nichirei), have become commercially available. In these methods, a step with a linker reaction is inserted after the reaction with the primary antibody to enable binding with more HRP-labeled polymers (Fig. 7). Takeuchi et al. found that only 3 out of 11 cases of ALK-

FISH-positive lung cancer tested positive by the standard IHC method using ALK-5A4 antibody, but all tested positive when the iAEP method was used(7). Additionally, Paik et al. reported 38 IHC-positive cases out of 640 cases without using any similar highly-sensitive method, by optimizing ALK-5A4 antibody (20). However, the concordance rate with FISH was low, and therefore this antibody optimization method is not ideal.

Yi et al. also reported Envision FLEX+[5] to have sensitivity of 90% and specificity of 97.8%, although they used the low-sensitive ALK1 antibody (21) (Table 3). Taking these reports into account it would be reasonable to anticipate a high rate of agreement with other methods of ALK detection by using ALK-5A4 antibody and a high-sensitivity detecting system (Fig. 8). Similarly, it is plausible to apply immunostaining methods to biopsy tissues and cytological cell block specimens (Fig. 9). Presently, however, examinations using surgical specimens are predominant, and reports on concordance rates amongst biopsy tissues are limited. Therefore, the method should be positioned as a procedure of analysis which can be conducted only at facilities where their consistency has been carefully validated.

Table 3. Summary of reports in literature regarding detection of ALK translocation by immunostaining and agreement with RT-PCR or FISH

Author			Tissue/section	Antibody	Supplyer	Dilution	Antigen retrieval	Buffer	Detection method	number of IHC-positive	Standard	number of Standard-positive	Concordance to Standard	Sensitivity & Specificity
Wong	Cancer	2009	Paraffin/not specified	Polyclonal	Invitrogen	1:1000	microwave	citrate (pH 6.0)	Dako HRP complex, not further specified	12 of 12	RT-PCR, direct sequencing	13 of 266	100% (tested in 12 of 13 ALK+ cases)	N.A.
Jokoji R	J Clin Pathol	2010	Paraffin/regular	5A4	Abcam	1:100	Microwave	Target Retrieval Solution (pH 9.0, Dako)	Envision Flex+	8 of 254	FISH	8 of 8	100%	
Martelli	Am J Pathol	2009	Paraffin/regular	ALK1, ALKc, 5A4	DAKO, Sigma, Santa Cruz	N/A	microwave	citrate (pH 6.0) or EDTA (pH 8.0)	REAL envision	0/662	N/A			
Shaw	J Clin Oncol	2009	Paraffin/not specified	ALK1	Dako	N/A	N/A	N/A	N/A	19/19	FISH (some RT-PCR)	19/141	100%	
Inamura	Mod Pathol	2009	Paraffin/not specified	ALK1	Dako	1:20	Heating	Target Retrieval Solution (pH 9.0, Dako)	EnVision +	11/253	RT-PCR (reported previously)	11/11	100%	
Takeuchi	Clin Cancer Res	2009	Paraffin/not specified	5A4	Abcam	1:50	97C, 40min	Target Retrieval Solution (pH 9.0, Dako)	iAEP method	11 of 21	RT-PCR	11 of 11	100%	100% and 100% respectively
				5A4	Abcam	1:50	97C, 40min	Target Retrieval Solution (pH 9.0, Dako)	EnVision+	2 of 21	RT-PCR	2 of 11	18% (2 of 11)	25% and 100% respectively
				ALK1	Dako	1:20	97C, 40min	Target Retrieval Solution (pH 9.0, Dako)	iAEP method	11 of 21	RT-PCR	11 of 11	100%	100% and 100% respectively
				ALK1	Dako	1:20	97C, 40min	Target Retrieval Solution (pH 9.0, Dako)	EnVision+	1 of 21	RT-PCR	1 of 11	9% (1 of 11)	9% and 100% respectively
				SP8	Abcam	1:100	97C, 40min	Target Retrieval Solution (pH 9.0, Dako)	iAEP method	19/21	RT-PCR	11 of 11	100% but 8 of 10 false positive	100% and 20%, respectively
				SP8	Abcam	1:100	97C, 40min	Target Retrieval Solution (pH 9.0, Dako)	EnVision+	2 of 21	RT-PCR	2 of 11	18% (2 of 11)	18% and 100%, respectively
Boland	Hum Pathol	2009	Paraffin/whole section	ALK1	Dako	1:100	Dako PT link	EDTA (pH 8.0)	ADVANCE (Envision Flex+)	6/335	FISH, and partly with RT-PCR	6 of 6	100%	
Rodig	Clin Cancer Res	2009	Paraffin/TMA	ALK1	Dako	1:2	pressure cooker(Decloaking Chamber BiocareMedical);	EDTA (pH 8.0)	EnVision+	4 of 239	FISH (applied to all cases examined)	10/243	40% (4 of 10)	40% and 100% respectively
				ALK1	Dako	1:2	pressure cooker(Decloaking Chamber BiocareMedical);	EDTA (pH 8.0)	tyramide amplification	8 of 239	FISH (applied to all cases examined)	8 of 10	80% (8 of 10)	80% and 100%, respectively
Mino-Kenudson	Clin Cacner Res	2010	Paraffin/mostly TMA (116 of 153 cases)	ALK1	Dako	1:2	pressure cooker (Decloaking Chamber [Biocare Medical])	EDTA (pH 8.0)	EnVision+	6 of 37	FISH	6 of 19	32% (6 of 19)	67% and 97%, respectively
				D5F3	Cell Signaling Technology	1:100	pressure cooker (Decloaking Chamber [Biocare Medical])	EDTA (pH 8.0)	EnVision+	21 of 153	FISH	21 of 22	95% (21 of 22)	100% and 97%, respectively
Yi	J Thorac Oncol	2011	Paraffin/whole section	ALK1	Dako	1:100	Dako PT link	EDTA (pH 8.0)	ADVANCE (Envision Flex+)	11 of 101	FISH	9 of 10	90% (9 of 10)	90% and 98%, respectively
Paik	J Thorac Oncol	2011	Paraffin/TMA	5A4	Novocatra	1:30	Heating	CC1 solution(Tris/borate/EDTA)	i-view detection kit (Ventana)	28 of 640	FISH	28 of 640	100% (28 of 28), but 10 of 612 false positive	100% and 98%, respectively
Yatabe	unpublished data		Paraffin/TMA	5A4	Santa Cruz		Dako PT link	Target Retrieval Solution (pH 9.0, Dako)	Envision Flex+	13 of 354	RT-PCR	12 of 354	100% , but 1 false positive	100% and 99%, respectively

[5] In the United States, this is sold as the “ADVANCE detection system,” but it is also known as the “EnvisionFlex+ System designed for use in large clinical laboratory centers.

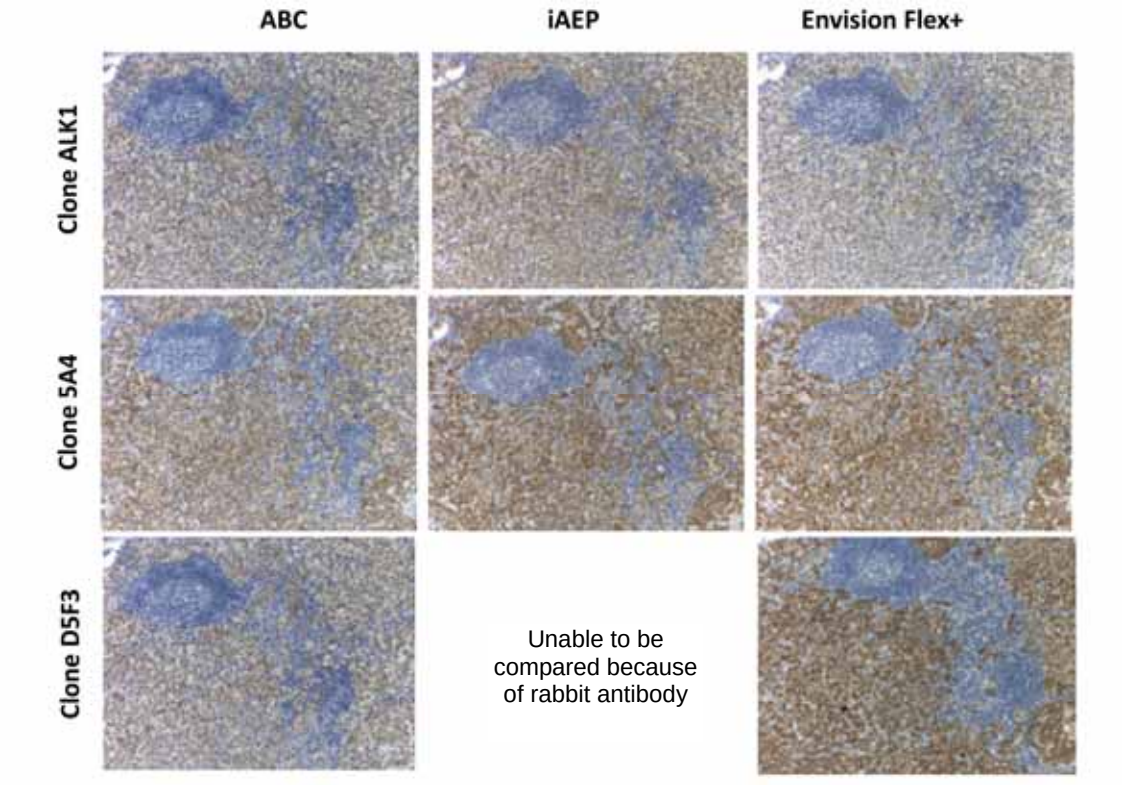

Figure 8-1 The differences in results listed by Antibody clone and detection system. In strongly positive cases, a positive signal can be achieved with any method.

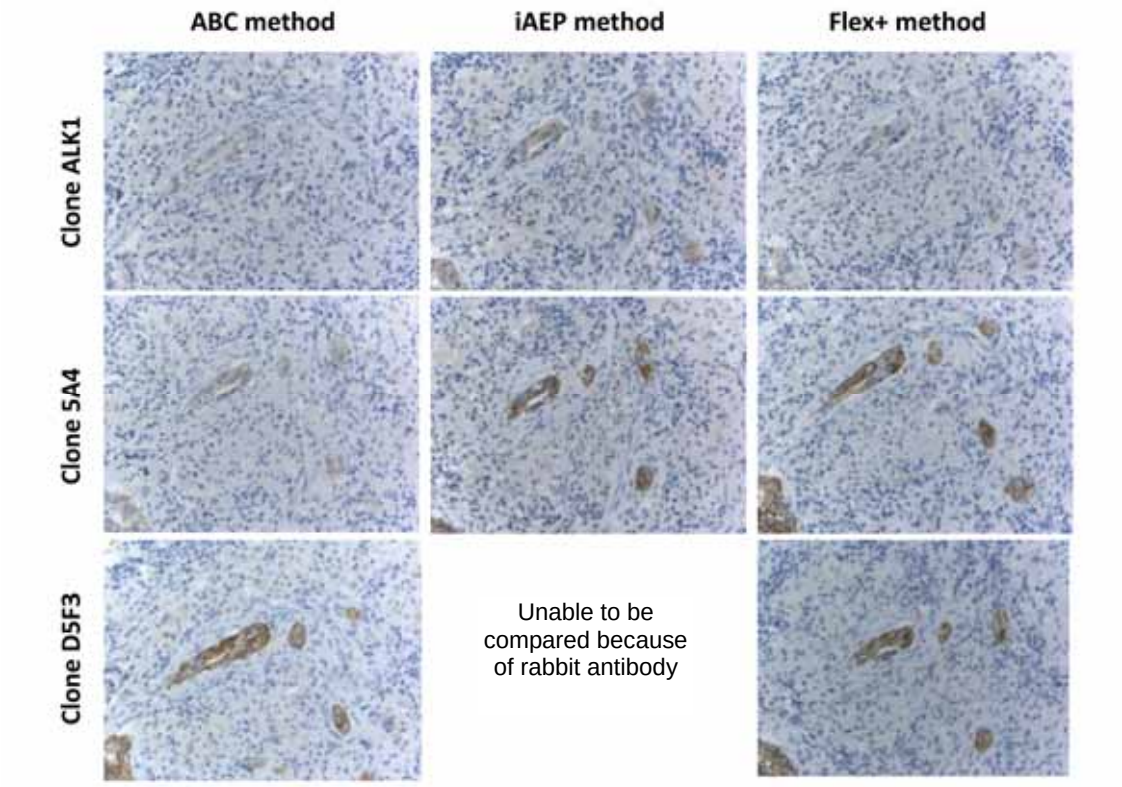

Figure 8-2 However, in some samples only using a high-sensitivity method is insufficient, and thus the selection of antibody clone becomes a critical issue.

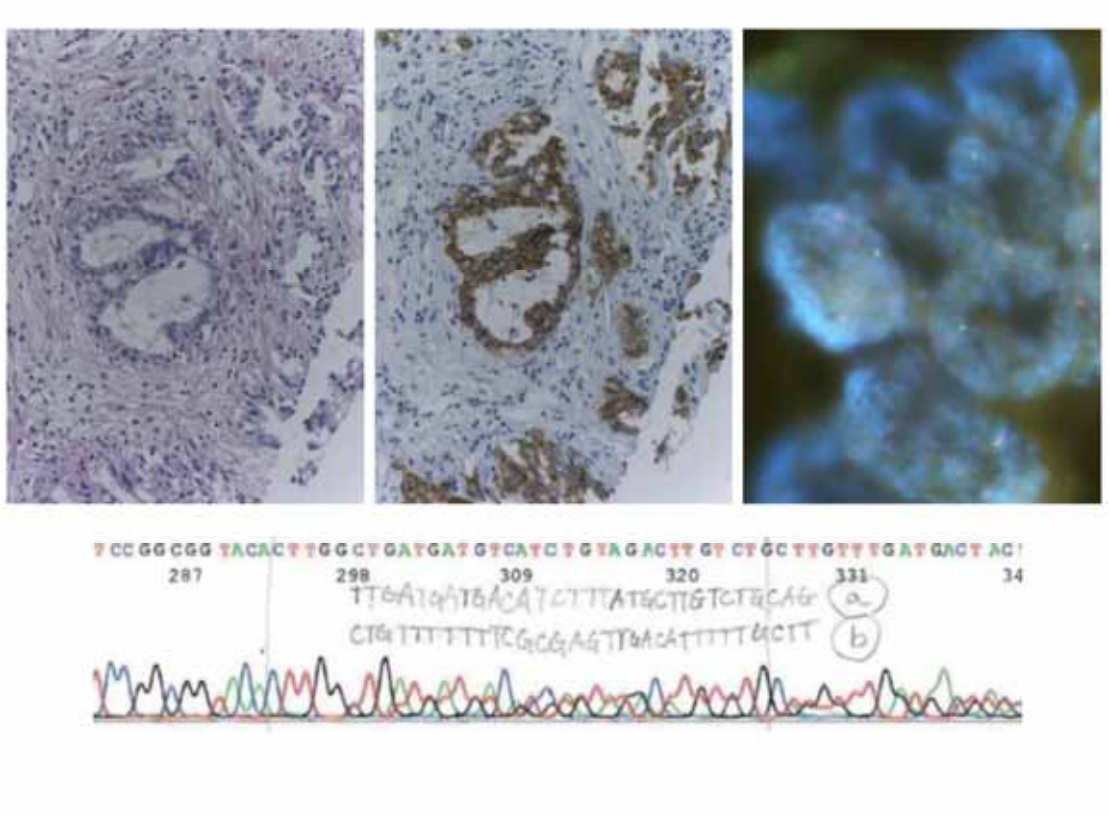

Figure 9-1 Example of detection in biopsied tissue

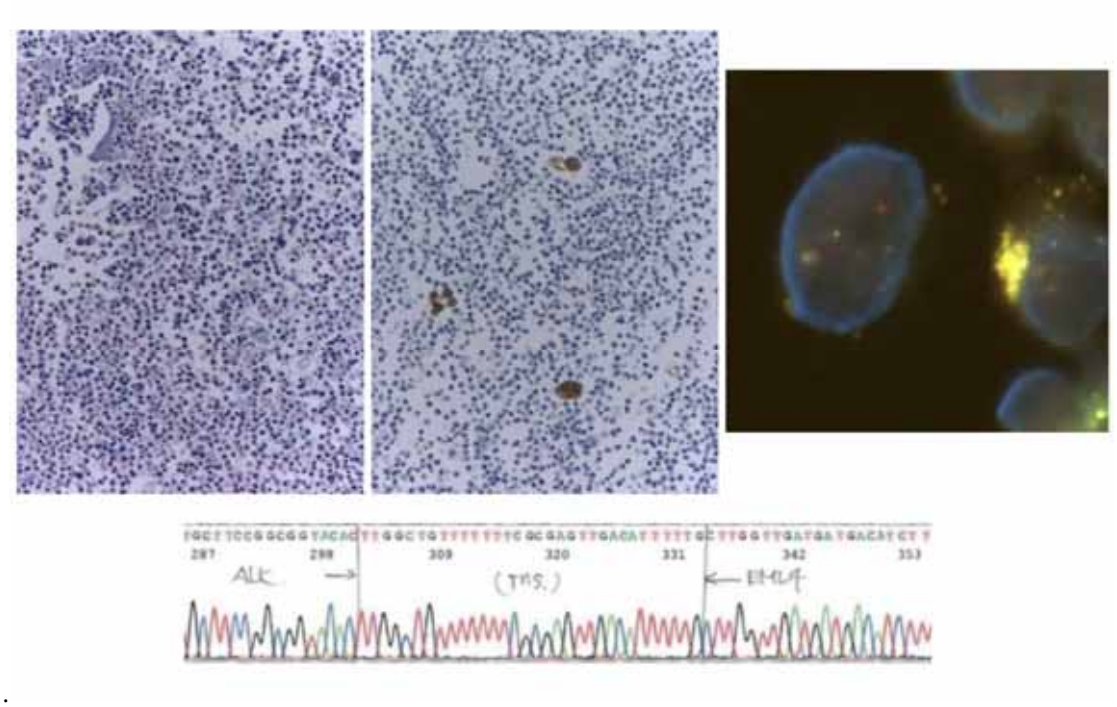

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Figure 9-2 Example of detection from a cytology cell block sample

7. Algorithm of ALK gene tests (Fig. 10)

It is important to consider the type of the patients to be tested and the timing of the test. What screening test will be efficient when crizotinib and other ALK inhibitors become available in actual clinical settings? While ALK has not been sufficiently examined at present, the most desirable algorithm, based on the findings obtained so far, is shown in Figure 10. The recommended approach would be to conduct screening by immunostaining, similar to that for HER2 tests in breast / gastric cancers (which is already in practice), and to focus on the most likely targets with FISH verification.

Even if the results of IHC screening are negative, verification by FISH may be considered. This is especially true in cases where ALK-positivity is strongly suspected based on clinical background (patients younger than 40 years, etc), and morphologic features (cribriform adenocarcinoma with mucus production, signet ring cell carcinoma, and TTF-1-positive mucous adenocarcinoma), in conjunction with EGFR/KRAS negative results. Although an IHC-positive case can still be FISH-negative, examination of such samples by RT-PCR is recommended when possible. Generally, these cases are rare, and would need examination by correlation to clinical response rates.

RT-PCR is expected to provide the most reliable verification of fusion if chimeric transcripts can be confirmed by direct base determination, etc. Therefore, verification of fusion genes by RT-PCR is strongly encouraged, especially when the only specimens available are derived from cytology samples. However, if the case is RT-PCR-negative, re-examination by immunostaining or FISH is recommended. On the contrary, it may be necessary for determining the eligibility for ALK inhibitor treatment.

Follow-up reports confirm the usefulness of IHC screening, but it is greatly affected by the method of antigen activation, anti-ALK antibody clones, and detecting methods. Although false-positivity is present in IHC, no reports focusing on this issue have been published thus far. In the case of FISH, experience is required when evaluating the method. In particular, the frequency of ALK-positive lung cancer is lower than the frequency of the gene mutations examined to date, and accumulation of experience and comparative examination present a challenge.

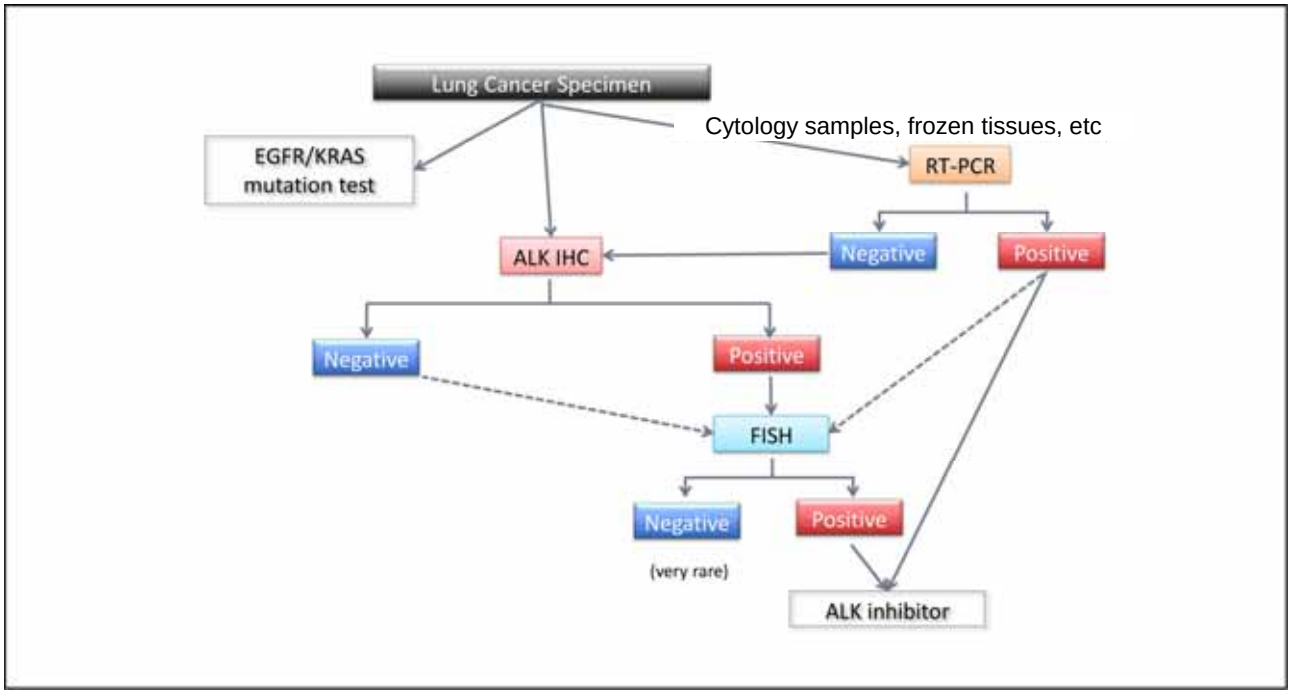

Fig. 10 Algorithm of ALK-translocated lung cancer diagnosis
Screening by IHC and verification by FISH are recommended for FFPE specimens in terms of convenience and cost. Even in ALK IHC-negative cases, verification by FISH is desirable if: (1) ALK-positive lung cancer is suspected clinically (patients younger than 40 years old) and (2) morphologically (signet ring cell carcinoma and TTF-1-positive mucus-producing adenocarcinoma showing cribriform growth pattern accompanying mucus production). Although the cases positive on ALK-IHC but negative on FISH are believed to be extremely rare, that possibility should considered in cases such as neuroendocrine tumors.
RT-PCR based on cytological specimens, such as pleural effusion and lavage fluid, can provide reliable verification of fusion if chimeric transcripts can be verified by direct base determination, and ALK inhibitors can be indicated on the

basis of this verification. However, verification by FISH is recommended even in positive cases. Even in negative cases, there is still a possibility of fusion with other gene partners, and examination by IHC will be necessary.
The advantages and disadvantages of IHC, FISH and RT-PCR need to be carefully considered. Even if the result of one test is positive, it is recommended to examine the presence of ALK-fusion by at least two methods. We recommend that these tests be conducted at testing centers in which testing accuracy has been validated and should be done for 2 years following marketing approval of the first ALK-inhibitor.

7.1 Proposal regarding ALK tests

As stated above, IHC could bean extremely useful screening procedure at facilities able to routinely conduct FISH, RT-PCR and IHC, and can repeatedly comparing the results of these three tests. However, high level controls are required to standardize staining conditions. Therefore, this guideline recommends that these tests be conducted at designated testing centers during a period of about 2 years, starting now. The EGFR gene mutation test has already become an essential test. The three companies reportedly possessingequivalent capabilities on the test, i.e., Mitsubishi Medience, SRL and BML, have been confirmed to have no problems in accuracy control of ALK tests as well. Accumulation of cases and testing experience at these examination centers is highly recommended. If another facility wishes to conduct ALK testing at its own laboratory, it needs to conduct comparative examination of the test results during this period, and to demonstrate sufficient concordance rates. Furthermore, each test has its own advantages and disadvantages (Table 2) so if possible, ALK-positivity should be confirmed by at least two tests.

Considerations regarding Health insurance coverage

Health insurance coverage is not provided at the time of producing this document for RT-PCR and FISH for rearrangement of ALK detection For N002 immunostaining, “5. Others, 400 points (per organ)” can be claimed. However, early coverage by health insurance allows for 690 points to be claimed in the same way as HER2 in mammary glands, and EGFR immunostaining in colorectal cancer. FISH has been approved as a drug for extracorporeal diagnosis, and is expected to be covered by health insurance in the near future.

Conclusion: Medical care in clinical settings and ALK

ALK-positive lung cancer accounts for only a small percentage of lung cancer. However, ALK inhibitors often exhibit marked efficacy in ALK-translocated lung cancer, and patients need to be chosen appropriately. However, diagnosis of ALK lung cancer is problematic in various aspects compared to other gene tests (e.g. EGFR in lung cancer, and HER2 in breast cancer and gastric cancer). It is recommended that The Japan Lung Cancer Society take initiative in accumulating clinical expertise to achieve success in individualized treatment of gene-mutated lung cancer, because despite its low incidence, a significant treatment effect can be expected. Our hope is that this guideline will help achieve that goal.

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