Recommendations for the Analysis of *ALK* Gene Rearrangements in Non–Small-Cell Lung Cancer

A Consensus of the Italian Association of Medical Oncology and the Italian Society of Pathology and Cytopathology

Antonio Marchetti, MD, PhD, Andrea Ardizzoni, MD,† Mauro Papotti, MD,‡ Lucio Crinò, MD,§ Giulio Rossi, MD,‖ Cesare Gridelli, MD,¶ Massimo Barberis, MD,# Eugenio Maiorano, MD,** Nicola Normanno, MD,†† Gian Luigi Taddei, MD,‡‡ Giorgio Scagliotti, MD,‡ Claudio Clemente, MD,§§ and Carmine Pinto, MD‖‖*

Introduction: Recent clinical trials led to the approval of crizotinib (PF-02341066; Pfizer) by the U.S. Food and Drug Administration for the treatment of locally advanced or metastatic non–small-cell lung cancer (NSCLC) patients whose tumors are positive for anaplastic lymphoma kinase (*ALK*) alterations. The European Medicines Agency accepted the regulatory submission of crizotinib for the treatment of these patients. Therefore, *ALK* gene testing has become mandatory to choose the most appropriate therapy.

Methods: To help physicians, involved in the management of NSCLC patients to be treated with ALK inhibitors in Italy, the Italian Association of Medical Oncology and the Italian Society of Pathology and Cytopathology identified a large panel of Italian medical oncologists and pathologists that outlined recommendations for *ALK* testing in NSCLC patients.

Results: The guidelines produced include specific information on the target patient population, the biological material for molecular analysis, a section dedicated to the histocytopathologic diagnosis of NSCLC, and the methods for the assessment of *ALK* alterations that are summarized in this article.

Conclusions: Clinicopathologic recommendations were produced to guide the management of NSCLC patients who need to be tested for *ALK* rearrangements before treatment with ALK inhibitors.

Key Words: Anaplastic lymphoma kinase gene rearrangements, Guidelines, Non–small-cell lung cancer, Targeted therapy.

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Genetic lesions that drive the proliferation of cancer cells, known as driver mutations, can make specific tumors sensitive to therapeutic inhibitors targeting the mutated pathways. Several target-based therapies are now available for which treatment optimization is based on tumor testing for specific mutations and direct therapies against the mutant target.

In patients with non–small-cell lung cancer (NSCLC), inhibitors of the epidermal growth factor receptor (EGFR), such as gefitinib or erlotinib, have produced consistent responses in a subset of cases carrying activating *EGFR* mutations.[1–3] More recently, similarly positive outcomes have been reported for crizotinib in NSCLCs with rearrangement of the anaplastic lymphoma kinase (*ALK*) gene.

In 2007, *ALK* gene rearrangements were described for the first time as small inversions on chromosome 2p inducing a fusion of parts of the echinoderm microtubule-associated protein-like 4 (*EML4*) gene with parts of the *ALK* gene in NSCLC. The resulting fusion protein (EML4-ALK), with kinase activity, conferred a strong proliferative stimulus on the cells.[4,5]

Multiple distinct EML4-ALK chimeric variants have been identified, representing breakpoints within various *EML4* exons, all of which are transforming in vitro.[6,7] In addition to *EML4*, other fusion partners with the *ALK* gene (e.g., *KIF5B* and *TFG*) have been reported in NSCLC.[8] Clinical data regarding the fusion of ALK with other proteins are as yet scarce.

*Center of Predictive Molecular Medicine, Center of Excellence on Aging, University-Foundation, Chieti, Italy; †Medical Oncology Unit, University Hospital, Parma, Italy; ‡Division of Anatomic Pathology, San Luigi Hospital and University of Turin, Orbassano, Italy; §Department of Medical Oncology, Perugia Hospital, S. Andrea delle Fratte, Perugia, Italy; ‖Section of Pathologic Anatomy, Azienda Policlinico, Modena, Italy; ¶Division of Medical Oncology, S.G. Moscati Hospital, Avellino, Italy; #Division of Pathology and Laboratory Medicine, European Institute of Oncology, Milan, Italy; **Department of Pathological Anatomy, University of Bari "Aldo Moro", Bari, Italy; ††Cell Biology and Biotherapy Unit, INT-Fondazione Pascale, Naples, Italy; ‡‡Department of Human Pathology and Oncology, University of Florence, Florence, Italy; §§Pathology Department, Sant'Ambrogio Clinical Institute, Milan, Italy; and ‖‖Medical Oncology, S. Orsola-Malpighi Hospital, Bologna, Italy.

Antonio Marchetti and Carmine Pinto are co-corresponding authors.

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Address for correspondence: Antonio Marchetti, MD, PhD, Center of Predictive Molecular Medicine, Center of Excellence on Aging, University-Foundation, Via Colle dell'Ara, 66100 Chieti, Italy. E-mail: amarchetti@unich.it

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ALK rearrangement is present in 2% to 7% of NSCLC cases, and it is associated with distinct clinicopathologic features, including onset at a young age, absent or minimal smoking history, and adenocarcinoma histology.[4,7,9–15] The prognostic role of ALK gene rearrangement is still unclear, with most studies reporting an absence of correlation between ALK gene rearrangement and prognosis.[13,16]

ALK rearrangements are in general mutually exclusive with *EGFR* and *KRAS* mutations,[17] consistent with the notion that *ALK* rearrangement defines a unique molecular subset of NSCLC. Preclinical and clinical studies have shown that cancer cells harboring *EML4-ALK* and other *ALK* abnormalities are exquisitely sensitive to ALK inhibitors.[9,18]

Crizotinib (PF-02341066; Pfizer Labs, Division of Pfizer Inc, New York, NY) is a selective oral tyrosine kinase inhibitor of ALK and mesenchymal–epithelial transition growth factor (MET), which inhibits the tyrosine phosphorylation of ALK.[19,20] In a phase I clinical trial, the toxicity profile and the efficacy of crizotinib were evaluated in a cohort of patients with NSCLC and *ALK* rearrangement.[21]

The study data were recently updated. In the 119 patients treated, of whom 29% of cases had received one previous line of chemotherapy and 59% two lines or more, objective responses were observed in 61% of patients and disease stability in 27% of cases, with disease control in 88% of patients. Progression-free survival was 10 months. The principal toxicity grade 3 to 4 resulted in a reversible increase, after treatment interruption, of glutamic-pyruvate transaminase in 4% of the patients. The median overall survival of the study was not reached, with overall survival at 1 year of 74% and at 2 years of 54%.[22,23]

These data led to the accelerated approval of crizotinib in the United States, by the U.S. Food and Drug Administration (FDA) on August 26, 2011, for the treatment of patients with locally advanced or metastatic NSCLC positive for *ALK* gene rearrangement. The use of crizotinib has been restricted to patients whose tumors result positive to *ALK* alteration from a test approved by the FDA (currently the Abbott Vysis *ALK* Break Apart FISH Probe Kit, Abbott Molecular Inc., Abbott Park, IL).[24]

On July 19, 2012, the European Medicines Agency in Europe accepted the regulatory submission of crizotinib for the treatment of patients with advanced stage, ALK-positive, pretreated NSCLC.[25] The results of a phase I expanded cohort study were confirmed in a phase II completed study in which 136 patients, all pretreated from the second line onward, obtained a disease control of 90%, with an overall response of 50% and a progression-free survival not yet reached at a median follow-up of over 10 months.[26] This trial, PROFILE 1005, involved the *ALK* rearrangement centralized fluorescence in situ hybridization (FISH) test.

Crizotinib is undergoing evaluation in two randomized phase III trials with ALK-positive patients, PROFILE 1007 and PROFILE 1014, which compare the efficacy and the toxicity of crizotinib with standard chemotherapy as second-line and first-line treatments, respectively. Although a number of guidelines are available to help physicians involved in the management of patients with NSCLC carrying *EGFR* mutations, recommendations for NSCLC patients to be treated with inhibitors of the *ALK* gene are still lacking.

PATIENTS AND METHODS

The Italian Association of Medical Oncology (AIOM) and the Italian Society of Anatomic Pathology and Diagnostic Cytopathology (SIAPEC-IAP) identified a large panel of Italian medical oncologists, pathologists, and molecular biologists that met for the first time in April 2011. After a series of additional meetings, guidelines for *ALK* testing were written by a restricted steering committee and submitted to the panel of experts for their comments. The guidelines were published on the Web sites of both AIOM and SIAPEC-IAP in June 2012 (www.aiom.it, 2012; www.siapec.it, 2012).

RESULTS AND DISCUSSION

The main points of the recommendations for the analysis of *ALK* gene rearrangements in NSCLC, prepared by AIOM and SIAPEC-IAP, are summarized in the following paragraphs.

Clinical Indications for ALK Rearrangement Analysis

The analysis of molecular changes of *ALK* is necessary to choose the best therapeutic strategy in selected NSCLC patients in stages IIIB and IV which, in the presence of gene rearrangements, may benefit from treatment with ALK inhibitors. The *ALK* test is indicated in NSCLC patients with histotypes of adenocarcinoma, large-cell carcinoma, mixed tumors with adenocarcinoma, or not otherwise specified NSCLC, which present the highest probability of gene rearrangements.[27]

The detection of *ALK* alterations may be conducted on a surgical specimen, or biopsy or cytological samples of the primary tumor or of metastases.[16,28] In patients with the highest probability of *ALK* alterations, i.e., nonsmokers, light smokers (<15 packs/year or ≤5 cigarettes/day), and ex-smokers (≥15 years) with the abovementioned histotypes, for which adequate material is not available, a further biopsy may be required to permit subsequent molecular determination when clinically indicated.[12]

The integration of *ALK* analysis into a diagnostic algorithm for NSCLC is still a matter of debate within the international scientific community. For cases with wide tissue availability, the immunohistochemical test could be potentially useful for an initial selection of cases to be investigated in-depth through FISH.[29–32] According to another school of thought, with the current lack of a validated diagnostic kit for *ALK* gene expression in lung cancer, immunohistochemical analysis should be used for study or research and not for diagnostic purposes.[33] Given that *EGFR* and *KRAS* mutations and *ALK* rearrangement are in general mutually exclusive, information on the mutational status of *EGFR* and *KRAS*, wherever available, is another factor useful in the selection of patients to undergo *ALK* analysis.[17] Possible algorithms for *ALK* gene testing are reported in Figure 1. For cases of limited tissue availability, it is not possible to define an algorithm in that the choice of analyses to be conducted should be made on a case-by-case basis. The factors to consider are the line of treatment, and therefore, the availability or otherwise of a molecular drug for the relevant line of treatment and the clinicopathologic characteristics. For

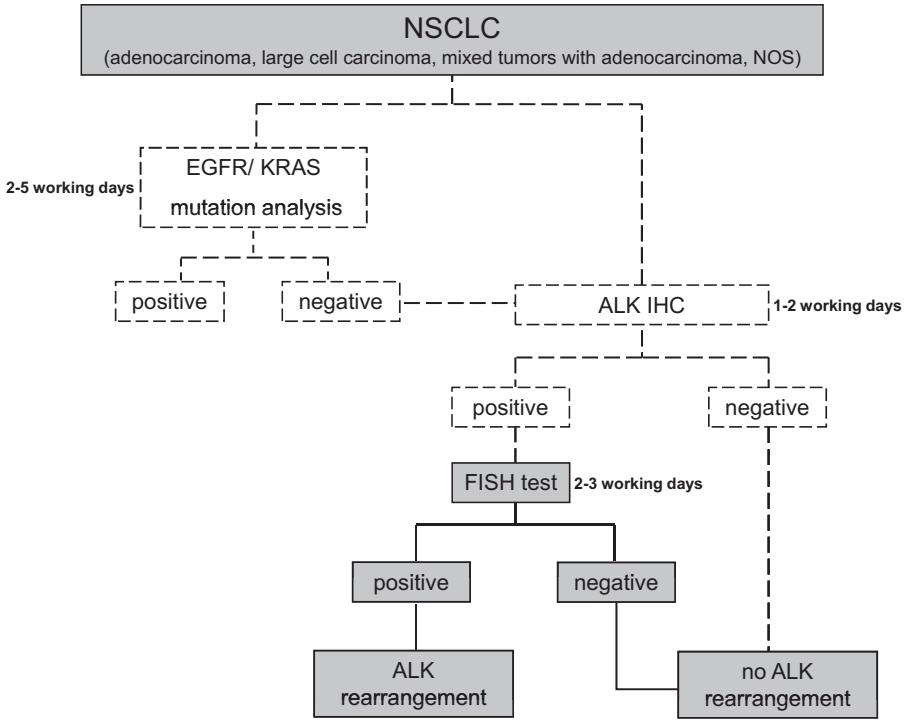

FIGURE 1. Possible ALK testing algorithms in NSCLC. Dot boxes and dot lines correspond to hypothetical ways to select patients for FISH analysis (grey boxes) which represent the elective method to assess *ALK* gene rearrangements. The time length of the main steps is reported. ALK, anaplastic lymphoma kinase; NSCLC, non–small-cell lung cancer; FISH, fluorescence in situ hybridization.

example, *ALK* mutations are more frequent in tumors rich in intracellular mucin or with a signet-cell appearance, which however are usually negative for *EGFR* mutations.[13,34–36] Nonetheless, in the light of the most recent discoveries, which could soon make new drugs available for patients with specific molecular alterations, the cases with limited tissue availability should represent an exception, at least for some of the above-listed patient categories.[37–40]

Diagnostic, Histological and Cytological Workup

As with *EGFR* gene mutations, rearrangements of the *ALK* gene can also be observed with absolute prevalence in certain particular histotypes of NSCLC.[27] Therefore, a detailed histopathologic workup is today an essential part of the diagnostic and therapeutic procedures for lung cancer patients.

NSCLC today represents a challenging diagnostic area for the surgical pathologist, for several reasons: (1) defining the histotypes has become an important aspect in the therapeutic approach to NSCLC patients; (2) the incidence of adenocarcinoma increased from the beginning of the 1990s and this histotype is significantly correlated with molecular alterations (*EGFR*, *BRAF*, and *HER2/neu* mutations, *ALK* fusion), which represent targets for selective inhibitors; (3) it is necessary today to formulate an increasingly precise diagnosis of the histotype, and yet optimize the management of the tumor material (often very limited) to provide all the information necessary for the best therapeutic option.

What has emerged from the recent classification of pulmonary adenocarcinomas is that it is necessary to manage each case through a constant multidisciplinary integrated approach.[41] For an accurate diagnosis, it is essential that the pathologist is placed in the best conditions to define the morphological aspects and if need be, request appropriate ancillary investigations. The essential data that should be present in the request for a cytohistological test include the smoker's status, significant medical history data, and the results of laboratory or radiology investigations. For the diagnosis of lung cancer (primary versus metastatic; epithelial versus nonepithelial) and the subtyping of NSCLC, it is fundamental to conduct a thorough morphological investigation accompanied by immunohistochemical analyses.[41]

In the subtyping of poorly differentiated NSCLC in which the morphology is insufficiently informative, the first antibody panel to be used is represented by a marker of adenocarcinoma phenotype, thyroid transcription factor-1 (TTF-1), and one of squamous or epidermoid histotype, p63.[41–49] TTF-1 and p63 negativity can represent a serious diagnostic challenge. In this case, it is advisable to evaluate the possibility of a poorly differentiated adenocarcinoma (e.g., using a second marker of adenocarcinoma profile–napsin A–and one of squamous phenotype–p40 and/or desmocollin-3 and/or CK5/6), sarcomatoid carcinoma (if spindle and/or giant neoplastic cells are present), another form of rare lung tumor, or a neoplasia metastatic to the lung. In the case of a poorly differentiated lung carcinoma in which both the structure and the immunohistochemistry yield inconclusive results, it is advisable to retain the term NSCLC-not otherwise specified.[41]

ALK Analysis

To guide therapeutic decisions in NSCLC patients, the genetic analysis of *ALK* runs alongside *EGFR* gene mutation research.[50,51] Various technologies have been developed to study this marker.

Fluorescence In Situ Hybridization

FISH is currently the elective method for the analysis of *ALK* gene rearrangements. This method was in fact used in the clinical trials, which led to the approval for treatment with crizotinib. The technology is available in commercial kits developed for diagnostic use, among which is the diagnostic kit certified by the FDA, Abbott Vysis (*ALK* Break Apart FISH Probe Kit, Abbott Molecular Inc.). This kit is accompanied by a detailed kit on the technical procedures and interpretation of data.[52] Other commercial kits are available, which are not yet FDA-approved (e.g., ZytoLight SPEC *ALK/EML4* TriCheck Probe, ZytoVision, Bremerhaven, Germany). In each case, the determination of *ALK* rearrangements must be accurately validated in the individual diagnostic laboratories, with the analysis of a suitable number of positive and negative controls, before implementing clinical activity.

Immunohistochemistry

The expression of ALK protein could represent a potential marker indicating gene rearrangement or response to ALK inhibitors. Introducing a user-friendly and cost-friendly screening method such as immunoblotting, into anatomic pathology laboratories, is highly desirable. To this end, three monoclonal antibodies have been developed and commercially available for research purposes, clone 5A4 (Leica Biosystems Newcastle Ltd, Newcastle Upon Tyne, UK/Novocastra Laboratories Ltd., Newcastle Upon Tyne, UK, and prediluted Abcam, Cambridge, UK), clone ALK1 (Dako Denmark A/S, Glostrup, Denmark), and clone D5F3 (Cell Signaling Technology Inc., Danvers, MA). Results obtained with these antibodies in comparative studies, using the FISH method, are promising, particularly those obtained with clones 5A4 and D5F3 which recognize recombinant proteins.[30–32] However, results are insufficient to draw definitive conclusions at this time.

Reverse Transcription-Polymerase Chain Reaction Analysis

Reverse transcription-polymerase chain reaction (RT-PCR) can be performed on complementary DNA obtained by messenger RNA synthesis to highlight directly the process of fusion of *ALK* with *EML4* or other proteins, using dedicated primers. The technology is extremely sensitive and highly specific. Therefore, it is used as the reference procedure in studies to assess the sensitivity of FISH analysis and immunohistochemistry. Nonetheless, RT-PCR has numerous disadvantages in its application to clinical practice: (1) messenger RNA of a high degree of quality is required, which is unobtainable from tissues fixed in formalin and embedded in paraffin; (2) complex PCR multiplex amplification systems are required because of the wide variability of fusion types; (3) only the known alterations are recognized by the test.[33,53] Pros and cons of the different ALK assays are reported in Table 1.

Preparation of Samples for FISH Analysis

The biological material on which FISH is conducted for studying *ALK* gene fusion may comprise both histological and cytological samples representative of the primary tumor or metastasis. However, the diagnostic kits were developed and validated only on formalin-fixed paraffin-embedded histological samples. The best fixation time for tissue samples is between 6 and 48 hours. It is advisable to cut sections slightly thicker (5 microns) than those used for histopathology to obtain better results when reading. Before FISH evaluation, it is necessary to examine a section, contiguous with that to be analyzed, stained with hematoxylin-eosin, to recognize and evaluate the neoplastic areas of interest. In cases where the neoplastic component of the sample is scarce, it is advisable that the area examined be marked on the slide with a diamond-tip pencil so that it may be readily identifiable for the FISH examination under the fluorescence microscope.

As regards cytological preparations, samples may be mounted optimally and aimed at the FISH technique, otherwise, premounted stained smears can be used, which are then unmounted and destained. The routine cytological preparations from smears, alcohol-fixed and stained with Papanicolaou, Diff-Quick o May-Grünwald-Giemsa, are often the only material available. In this case, the slide areas containing a larger number of neoplastic cells must be preventively marked out, using a diamond-tip pencil.

Preparing the sample for hybridization involves a pretreatment of the sections in two distinct phases: the first with heat, and the second, enzymatic. Because the pretreatment depends on the nature of the tissue, and in particular, on the quantity of connective tissue present in the sample, the use of kits designed for the pretreatment of lung cancers is recommended (e.g., Abbott Vysis paraffin pretreatment 4, Abbott Molecular Inc.). The pretreatment procedure must be setup and validated in each laboratory, as it is strictly correlated with the procedure of fixation and inclusion.

TABLE 1. Pros and Cons of ALK Assays

	FISH	IHC	RT-PCR
Advantages	Availability of validated kit with standard procedures. Reliability (used in clinical trial)	User-friendly. Cost-friendly	High sensitivity and high specificity
Disadvantages	Technically challenging and costly	Lack of dedicated kits and standard procedures	High-quality mRNA needed (obtainable from frozen sample). Complex multiplex PCR amplification systems required. Only known variations identified

ALK, anaplastic lymphoma kinase; RT-PCR, reverse transcription-polymerase chain reaction; IHC, immunohistochemistry; FISH, fluorescence in situ hybridization; mRNA, messenger RNA.

TABLE 2. Major Recommendations for ALK Gene Testing in NSCLC Patients

Clinical Indications for Patient Selection	
Stage	Stage III B and IV NSCLC
Histotypes	Adenocarcinoma, large-cell carcinoma, mixed tumors with adenocarcinoma, NOS NSCLC
Other mutations	Mutational status of EGFR and KRAS (potentially useful)
Biological material	
Type of samples	surgically resected tissues, biopsies, or cytological samples from primary tumor and/or metastases
Rebiopsy	Recommended in patients with the highest probability of ALK alterations, i.e., nonsmokers, light smokers, and ex-smokers with the abovementioned histotypes, for which adequate material is not available
Methods for the analysis of ALK rearrangement in clinical practice	
FISH	Currently the elective method
IHC	Potentially useful for an initial selection of cases to be investigated in-depth through FISH
RT-PCR	In case of availability of frozen material

NOS, not otherwise specified; NSCLC, non–small-cell lung cancer; EGFR, epidermal growth factor receptor; IHC, immunohistochemistry; RT-PCR, reverse transcription-polymerase chain reaction; FISH, fluorescence in situ hybridization; ALK, anaplastic lymphoma kinase.

FISH Procedure

The FISH technique for diagnostic purposes is generally performed by means of dedicated commercial kits which have the advantage of standardizing the procedures, thereby reducing intralaboratory and interlaboratory differences. Because the kits contain detailed protocols for the technical procedures, these procedures will not be discussed in detail here. The training of staff involved in the work phases (from the selection of material, mounting, reading, and report writing), play a key role in the accuracy of the examination. It is absolutely inadvisable that resources be allocated if diagnostic requests are sporadic as the risk, that one can incur great expenses while not reaching sufficient operating standards, is high. It has been estimated that a laboratory can guarantee technological adequacy only if a minimum of 150 FISH assessments are carried out annually.[54]

Types of Probe and FISH

Different probes are commercially available for the 2p23 locus containing the *ALK* gene. In the United States, the Vysis LSI *ALK* Break Apart Rearrangement Probe, within the diagnostic kit, was approved by the FDA as a companion test. This comprises two DNA probes marked with two different fluorochromes (orange and green), premixed and optimized in a hybridization buffer. The probes recognize specific DNA sequences located upstream and downstream of the breakpoint.

Interpretation of the Specimen

The analysis is conducted with a fluorescence microscope. The first observation must be carried out with a DAPI filter, at 20×, identifying the neoplastic areas on which the gene is to be assessed. At high magnification (×100), the intranuclear fluorochrome signals in these areas are observed and the break identified. In fact, if the gene is intact, the fluorochromes are near and are seen as paired or fused, whereas, if the gene is interrupted, these become separate and are clearly seen as distanced from one another (more than two diameters of the fluorescent light signals). Also the isolated presence of fluorochrome orange in the absence of green, and not vice versa, must be considered as an index of rearrangement. The nuclei carrying *ALK* rearrangements are counted and the data tabulated. The examination is carried out on the entire section, evaluating at least 50 neoplastic nuclei to establish the percentage of positive nuclei.[52] The current diagnostic-therapeutic protocol sets a cutoff of 15% rearranged nuclei, to consider a case as *positive* and the patient as a candidate for treatment. This cutoff corresponds to that used in the seminal article by Kwak et al.[21] In 5% to 10% of cases, no rearrangement is observed, but rather an increase in the copies of the gene. The meaning of this alteration in terms of treatment with ALK inhibitors is not yet known.

Writing the Report

Writing the report is an integral part of the diagnostic procedure and should contain the following information: (a) identification of the patient and of the doctor or center requesting the analysis, (b) material used for the analysis, (c) the methodology used for the analysis and the type of commercial test used, (d) the results of the test, expressed in terms of negativity or positivity for the rearrangement of the *ALK* gene. In the case of positivity, the percentage of the number of nuclei with gene rearrangements over the total number of nuclei submitted for analysis must be stated, (e) the report must be written on an agreed form and signed by the anatomical pathologist or by the person who conducted the molecular test, (f) considering the impact on the therapeutic strategy, the timescale for producing the report must not exceed 2 weeks from the request for the analysis.

CONCLUSIONS

The clinicopathologic guidelines produced by AIOM and SIAPEC have been devised to guide physicians in the management of NSCLC patients who need to be tested for *ALK* rearrangements, before treatment with ALK inhibitors. The major recommendations for ALK gene testing in NSCLC patients are summarized in Table 2. These early recommendations may allow facilitation of the assessment of *ALK* alterations in NSCLC patients. However, further clinical studies

devoted to the identification of the best technical procedures required and an accurate validation of guidelines in daily clinical practice are needed.

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