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Review

ALK in Non-Small Cell Lung Cancer (NSCLC) Pathobiology, Epidemiology, Detection from Tumor Tissue and Algorithm Diagnosis in a Daily Practice

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Abstract: Patients with advanced-stage non-small cell lung carcinoma (NSCLC) harboring an *ALK* rearrangement, detected from a tissue sample, can benefit from targeted *ALK* inhibitor treatment. Several increasingly effective ALK inhibitors are now available for treatment of patients. However, despite an initial favorable response to treatment, in most cases relapse or progression occurs due to resistance mechanisms mainly caused by mutations in the tyrosine kinase domain of *ALK*. The detection of an *ALK* rearrangement is pivotal and can be done using different methods, which have variable sensitivity and specificity depending, in particular, on the quality and quantity of the patient's sample. This review will first highlight briefly some information regarding the pathobiology of an *ALK* rearrangement and the epidemiology of patients harboring this genomic alteration. The different methods used to detect an *ALK* rearrangement as well as their advantages and disadvantages will then be examined and algorithms proposed for detection in daily routine practice.

Keywords: *ALK* rearrangement; lung cancer; biology; immunohistochemistry; FISH; molecular biology

1. Introduction

Among the genomic alterations present in non-small cell lung carcinoma (NSCLC) the *ALK* rearrangement is one that results in targeted therapy and in most cases gives a therapeutic response that prolongs the life of patients [1]. Thus, several molecular therapies targeting the *ALK* rearrangement have been developed or are being developed and are often effective, but obtain variable results for survival [2,3]. For example, crizotinib is a potent and selective ATP-competitive inhibitor of ALK tyrosine kinases. It received Food and Drug Administration (FDA) approval in the USA in 2011, and European Medicines Agency approval in 2012 [4]. However, despite its clinical efficacy, resistance to crizotinib invariably develops [5]. There is now a next generation of ALK inhibitors, including two that have been approved-ceritinib and alectinib-and others that are in development such as brigatinib and lorlatinib [3,6–10]. Moreover, ceritinib and the other next-generation ALK inhibitors are more potent than crizotinib and can overcome tumor cell resistance mechanisms [5]. Taken altogether, these results highlight the need to perform rapid and highly sensitive screening for an *ALK* rearrangement in NSCLC patients, so that new drugs can be appropriately administered.

An *ALK* rearrangement is detected in 3 to 7% of patients with stage IIIB/IV NSCLC, depending on the series and also probably according to the selection of the patients for molecular testing; in most cases it concerns an adenocarcinoma [11–15]. The frequency of this genomic alteration is higher if only non-smoker patients are considered, from 17 to 20%, depending on the series [11,12]. Relapse or tumor progression is systematically noted at a more or less short term, which may lead to a change in the

targeted therapy [16,17]. The major cause of this change results from the emergence of mutations in the *ALK* gene though other mechanisms are possible [18–20].

In view of the therapeutic consequences of the detection of an *ALK* rearrangement several methodological approaches using tissue or cell samples have progressively been developed. Fluorescence in situ hybridization (FISH) was the first to be described. Immunochemistry and molecular biology approaches such as reverse transcriptase-polymerase chain reaction (RT-PCR) or new generation sequencing (NGS) were then developed. The use in clinical practice of one or several of these methods for a single patient raises a number of questions, in particular the sensitivity and specificity of the method. In fact, the reduced size of tissue samples obtained for morphological diagnosis of NSCLC and the increase in the number of cytological samples (associated or not with tissue biopsies) has led to new strategies for optimal handling of biological material and to methods of detection with these samples. The emergence of *ALK* mutations during treatment also raises the question of access to new tissue and/or cell samples for its detection, in particular by methods in molecular biology.

After briefly covering the biology of lung cancers associated with an *ALK* rearrangement, the consequences of this rearrangement in cells, the epidemiology of lung cancer, this review will examine the different analytical methods that detect this genomic alteration, as well as their advantages and limits, and will present algorithms for diagnosis in daily practice.

2. The *ALK* Rearrangement in Lung Cancer: Mechanism and Consequences

The *ALK* rearrangement leads to constitutive expression of an oncogenic fusion protein, initially detected in NSCLC [21–23]. At the cellular level, ALK regulates canonical signaling pathways that are shared with other receptor tyrosine kinases including RAS-mitogen-activated protein kinase, phosphoinositide 3-kinase-AKT, and JAK-STAT pathways. When there are some *ALK* rearrangements, 5′ end partners such as EML4 and NPM are fused to the intracellular tyrosine kinase domain of ALK. The consequence is aberrant expression of the ALK fusion protein in the cytoplasm. The different domains in the partner proteins promote dimerization and oligomerization of the fusion proteins, inducing constitutive activation of the ALK kinase and its downstream signaling pathways [21–23]. The consequence is uncontrolled cellular proliferation and survival. More than twenty *ALK* fusion partners have been described [24]. The breakpoints on the *ALK* gene almost always occur in intron 19 and, rarely, in exon 20, resulting in a constant inclusion of the *ALK* kinase domain in the fusion gene. A common feature of the fused partner genes is the presence of a basic coil-coil domain, which allows the dimerization of the fusion proteins. Moreover, *EML4-ALK*, the most common *ALK* fusion found in NSCLC, is formed by an inversion occurring on the short arm of chromosome 2 involving the genes encoding *ALK* (2p23) and *EML4* (2p21) with variants 1, 2, and 3a/3b being the most frequent fusion patterns [25,26]. The three major variants (v1: E13; A20, v2: E20; A20, and v3; E6; A20) account for more than 90% of lung cancers associated with *EML4-ALK*. In *ALK* translocated NSCLC, *EML4* does not appear to be the exclusive fusion partner with *ALK*. Two other fusions have been described, *TFG* and *KIF5B* [27,28]. Both were identified as an *ALK*-fusion partner from NSCLC tumor samples and the two proteins also fuse with the intracellular domain of ALK [27,28]. It is noteworthy that the presence of these non-*EML4* fusion partners for *ALK* can have implications for the method used for detection of *ALK* translocated NSCLC in daily practice. Because the gene rearrangement involves a large chromosomal inversion and translocation, FISH was the first method used for detection of *ALK* rearrangement. Mutations in the *ALK* gene result in decreased binding of an inhibitor, such as crizotinib, or increased ATP binding affinity [29]. Moreover, other resistance mechanisms have been identified such as the activation of EGFR and KRAS pathways by their respective mutations, *ALK* and *KIT* gene amplification, and more recently *YES1* mutations [29,30].

3. Epidemiology of Lung Cancers with an *ALK* Rearrangement

The percentage of patients with an *ALK* rearrangement varies from 1% to 5% depending on the population, and if all the histological types of NSCLC are taken into consideration or not for evaluation of the presence of an *ALK* rearrangement, while disregarding the smoking status [11,31–34]. In general, this genomic alteration if more often detected in young and non or past smokers [35,36]. So the frequency of an *ALK* rearrangement can be higher than 17% for non-smoker and young patients [11,37]. It is a biomarker of poor prognosis in a population of non-smoker patients [38]. This genomic alteration is essentially detected in adenocarcinomas and only very occasionally in epidermoid carcinomas or other rare histological types of NSCLC [11,39–41]. *ALK* rearrangement is more frequent in acinus forms of adenocarcinomas of Asian patients and in signet-ring cell adenocarcinomas of Caucasian patients [28,37]. Most *ALK*-positive lung adenocarcinomas show areas of solid tissue with 10% signet-ring cells [42]. The fact that an *ALK* rearrangement can exist in certain patients with an epidermoid carcinoma raises the question of systematically analyzing for this modification in this histological type [43]. The presence of an *ALK* rearrangement almost always excludes the presence of other genomic alterations associated in particular with mutations in *KRAS* and *EGFR* [44]. However, exceptional cases associated with other mutations have been reported [39]. It is noteworthy that some studies reported *ALK* translocation in non-tumor tissue, but below the accepted thresholds for determined the *ALK* status positivity [45].

4. Methods for Detection of an *ALK* Rearrangement

Different methods can be used to define the *ALK* status in tumor tissues. The most frequently employed methods include immunohistochemistry (IHC) and immunocytochemistry (ICC), FISH, real-time polymerase chain reaction (RT-PCR) and NGS. A number of studies, described in detail below, have shown discordant results when comparing these approaches, which can limit systematic use [45–48]. The FISH reasons for this are numerous. The first concerns the sample itself and the variable pre-analytical handling of the sample. Thus, bad fixation (delay in fixation, hyper or hypofixation, inappropriate fixative solution) can have a substantial and variable impact on the detection level of the expression of the ALK protein and/or the quality of the ALK RNA. One other reason lies in the heterogeneity of the expression of this genomic alteration within the tissue [49]. However, the most frequent cause of discrepancies between the different techniques concerns the variable sensitivity of these approaches, notably linked for the quality and quantity of the RNA obtained after extraction.

4.1. Immunohistochemistry and Immunocytochemistry Methods

IHC is a method used in all pathology laboratories and thus highly accessible [50]. It is possible to rapidly evaluating the *ALK* status with IHC on formalin fixed tissue sections, which is an easy approach that does not require a lot of technical or medical expertise. Several antibodies for the detection of the expression of ALK in tissues have been commercialized, in particular the 54A (Novocastra, Leica Biosystems, Buffalo Grove, IL, USA) and D5F3 (Cell Signaling Technology, Danvers, MA, USA) clones, which are the most used and reliable [51–54]. Certain antibodies, such as ALK1 (Dako) are not recommended and others like 1A4 (Origene, Rockville, MD, USA) must be used with precaution due to their variable specificity [55,56]. Overall, the D5F3 clone seems to give the most satisfactory results and is now approved by the FDA as a companion diagnostic test for ALK inhibitors treatment. Thus, patients with a positive ALK IHC can be treated with any specific ALK inhibitor. Several studies have also validated the use of anti-ALK antibodies with cytological material. ICC has essentially been developed with formalin fixed samples embedded in paraffin, as cellblocks [57–60].

Thus, IHC with anti-ALK holds several advantages. Aside from those described above, it is accepted that even after a too long fixation time, the stability of the protein allows detection of most cases harboring an *ALK* rearrangement.

To obtain a reliable interpretation certain pitfalls must be avoided such as false positive results close to necrotic zones, in the event of tyramide signal amplification and if positive alveoli macrophages are mistaken for tumor cells. Heterogeneous labeling can also be seen with certain surgical resected specimens, in particular in the center of large tumors, subsequent to a delay in fixation. A delay in fixation also has an impact on the interpretation of ALK IHC when performed on tissue microarrays with cores removed at random from the center of a tumor that has had a delay in fixation with formalin. Thus, within the context of a quality process and accreditation of an IHC test, the validation of the method and inter-laboratory controls, which includes external evaluation of the quality, are important for this predictive marker of therapeutic response [61].

4.2. The FISH Method

FISH was the first method used to detect an *ALK* rearrangement, foremost in tissue biopsies and then on cytological material. Initially it was considered to be the only gold standard method. Several probes can be used to visualize this rearrangement. Regardless of the probe, the cutoff for a positive result is 50 tumor cells with the rearrangement. FISH was the most frequently used method until the commercialization of anti-ALK antibodies and most laboratories continue to use this approach, either as a first-line test or to validate a positive IHC or ICC result [62,63]. However, compared to IHC this method has a number of disadvantages. It requires more time to perform and necessitates special equipment in addition to substantial technical and analytical expertise [64]. The observed signal is sometimes difficult to interpret or ambiguous and, in principle, more than a hundred tumor cells are required to obtain a reliable result, which may not be the case for small-sized tissue or cytological samples [65]. Not all pathology laboratories have a fluorescence microscope or the equipment for the hybridization steps. Additionally, rare false negative and false positive results can occur with FISH for detection of *ALK* rearrangements. In this case, certain rearrangements give rare and particular patterns [66]. In the event of an ambiguous FISH analysis, some complementary probes can be used [67]. False negative results can arise from a small number of tumor cells in the sample for analysis (in particular those with less than 50 tumor cells), from reactive epithelial cells or normal or abnormal cells mistaken for cancer cells or from difficult to interpret signals resulting from formalin hyper- or hypo-fixation. False positive results arising from atypical patterns due to multiple fusion of signals or a single green signal with 5′ centromeric probes.

4.3. RT-PCR Method

An approach using RT-PCR for detection of an *ALK* rearrangement in tissue and cell samples has been proposed [68,69]. The sensitivity and specificity varies depending on the different variables of the detection panel used but also on the quantity and quality of the extracted RNA. This method requires technical and medical expertise to obtain and interpret the results. Moreover, to eliminate sampling error and potential false negative results, visual control of the morphology is required to ensure the presence and percentage of tumor cells in the tissue or cytological sample. A multiplex RT-PCR-based assay can detect with sensitivity certain *ALK* fusion gene variants, although reproducible RT-PCR results are difficult to obtain with FFPE tissue sections [70,71]. RT-PCR is a sensitive approach for detection of some *EML4-ALK* variants but not all. In particular, this technique allows the detection of the *EML4-ALK* variant 1, 2 and 3a/b. One advantage of RT-PCR, in contrast to IHC and FISH, is that this technique is free from subjectivity in assessment of the analysis. Moreover, identification of the specific variant can be determined using RT-PCR, which could be important in predicting patient response to ALK inhibitors. In this context, different additional primer sets can be added to the RT-PCR methodology to detect some rare reported variants. Therefore RT-PCR analysis must definitively be multiplexed. For this there are at least 13 variants *EML4-ALK* fusions and some non-*EML4* translocation partners [26,68]. In this context, any PCR based strategy must incorporate validated primer pairs for all known *ALK* fusions to avoid false negative results. Moreover, more than 20 putative fusion partners of ALK have been described so fare. Therefore, the diagnosis can be certainly difficult to interpret

in some cases when using PCR methods. A few publications show that RT-PCR based detection of *EML4-ALK* can yield positive results in the absence of detectable *ALK*-rearrangements in both tumor and non-tumor tissues [45]. Finally, the clinical utility of RT-PCR should always be evaluated with regard to the treatment response in clinical studies.

4.4. NGS Approach

Analysis by NGS in molecular pathology laboratories has revolutionized the care of advanced-stage NSCLC. A single analysis is capable of detecting a substantial number of mutations on variable panels of genes [72,73]. Thus, due to the potential of the NGS technique it is now present in most molecular pathology laboratories but certain hurdles continue to be present and discussed [74–77]. It is indeed important to consider not only the quantity and the percentage of tumor cells used for this type of analysis, but also the total amount of tissue and the total number of cells present in the sample. Depending on the NGS technique used, the minimal amount of nucleic acid varies from 5 to 10 ng (around 1000 tumor cells) for the NGS technique based on PCR, and, 100 to 200 ng for the NGS technique based on hybrid capture. In fact, since this latter technique requires at least 100 ng of RNA of good quality more than a third of the samples cannot be used to give reliable results [76]. The detection of an *ALK* rearrangement can be associated with examination for fusions on the *ROS1* and *NTRK* genes, which avoids sequential analyses that are costly, time consuming and require additional biological material [47,78]. Several recent studies describe the potential of the NGS technique for the detection of an *ALK* rearrangement in routine practice, but a number of [79,80]. As an example, Qiagen (Hilden, Germany) recently launched the GeneReader NGS system offering soon a complete solution (called the GeneRead™ QIAact Lung All-in-One Assay) for both detection of mutation and different rearrangements. In particular, a targeted panel will cover the fusion genes of interest for NSCLC (including *ALK*, *ROS1*, *NTRK1*, *RET*) will be launched at the end of 2017 [81]. However a number of pitfalls and/or limitations of NGS approach for *ALK* rearrangement detection need to be pointed out. The vast majority of lung cancer biopsy specimens from patients are stored in formalin fixed paraffin embedded (FPPE) tissues. As for RT-PCR, the quality of the RNA extracted from material fixed in formalin needs to be considered since too much degradation can lead to false negative results. As seen above, NGS can also require a certain amount of extracted RNA depending on the approach and the panel of analyses. These techniques require strong technical and medical expertise and in general need more time than other methods, thus leading to variable delays in obtaining a response, which depends on the laboratory and the method. In fact, we have to thinks that presently it is recommended that an *ALK* result be provided for the physicians within 10 days after the performed biopsy [82]. An additional advantage of NGS is the detection of potential presence of some *ALK* mutations, in particular when considering survival of patients on ALK inhibitors. These mutations are the main reason for progression or relapse of patients on specific ALK inhibitors. It is of interest to note that depending on the inhibitor used certain mutation in the *ALK* gene emerge more frequently and resistance to a specific inhibitor requires a quick change in inhibitor [18].

4.5. Other Methods

A multiplexed transcript-based assay (nCounter, nanoString, Seattle, WA, USA) has been developed for simultaneous detection of multiple gene fusions. In particular, this approach has been developed for detection of an *ALK* rearrangement [83]. Briefly, this assay is based on the dual hybridization of a capture and a molecularly bar-codes reporter probe complementary to a contiguous target sequence [84]. Moreover, a single-tube test for *ALK, ROS1* and *RET* fusions has been recently developed [85]. This approach seems to have the potential for a cost-effective assay in daily practice. Moreover, the nanoString nCounter can detect targetable gene fusions on a variety of specimens from surgical resection to small biopsies or cytology cell blocks. Globally, around 20% tumor cellularity is required for detection, but the detection of an *ALK* fusion transcript could be possible in samples with as low as 5% tumor cellularity. Moreover, the fixation method does not influence the performance of

the nanoString approach. Finally, nanoString might provide additional information complementary to IHC and FISH. In situ hybridization with RNA has also been described for evaluation of the tissue expression of an *ALK* rearrangement [86–88]. In fact, the RNA ISH method (RNAscope) is a rapid technique, with quite easy handling, similar to IHC. It can semi quantitatively assess the mRNA signal in target cells with conventional light microscopy [88]. Finally, multiplex IHC analyses are also being developed to analyze in parallel the *ALK* status and the expression of certain checkpoint inhibitors, and these analyses can participate in studies into novel therapeutic strategies [89].

4.6. Comparatives Analyses

Taken the number of different approaches available for the evaluation of the *ALK* status in the daily practice, several comparative studies have evaluated their sensitivity and specificity. Other considerations such as the costs, the procedural difficulties and the resolution should be also part of these comparisons (Table 1). Several studies have compared IHC, in particular using the D5F3 clone, and *ALK* FISH, which show very good concordant results with a sensitivity of 81 to 100% and a specificity of 82 to 100%, depending on the study [62,90–94]. However, these results must take into consideration the intensity of the label and only the strong label (3+) concords at 100% with the *ALK* results obtained with FISH [95]. Thus, discordant results have been also reported for these different methods [46,48,96–100]. These discordant results were ALK-positive with IHC and *ALK*-negative with FISH. This can be explained by false negative FISH results from difficult to interpret samples and a positive result from less than 20% tumor cells [65,99]. FISH does not detect all *ALK* rearrangements and certain complex rearrangements reduce the distance between the two FISH probes, which can give a false negative FISH image [46]. Aside from the difference in sensitivity and specificity the discord can sometimes be explained by the intra- and inter-tumor heterogeneity in terms of the *ALK* status, in particular when comparing IHC/ICC or FISH and methods using direct extraction of RNA without prior visual control of the lesion [49,101,102]. Certain isoforms of *ALK* arise from a mechanism of alternative initiation of transcription giving protein expression that is not *ALK*-positive with FISH [103]. It is important to mention that some patients ALK-positive with IHC and *ALK*-negative with FISH can respond to treatment with crizotinib [104,105]. A negative IHC result can be observed in cases of *ALK* amplification [106,107]. In the same way, negative FISH results can be associated with positive RT-PCR results [108]. Some studies have compared the specificity and sensitivity of IHC, FISH, RT-PCR and/or NGS [109–111]. Finally, other studies have shown very good concordance of the results obtained with the nanoString testing with IHC and FISH, while some discrepancy was observed in other studies [112–114].

In conclusion, although correlation between the results for ALK IHC and *ALK* FISH is excellent, as a predictive marker for response to ALK inhibitor therapy, IHC alone has recently been validated with the D5F3 IHC assay [115]. The RT-PCR method for fusion genes is a high-throughput screening tool with quite a rapid turnaround time. However, this method is not able to identify rearrangements involving unknown fusion partners. NGS probably represents a more practical and reliable *ALK* testing approach for use in clinical routine practice. Moreover, this method can assess genomic-related mechanisms of resistance to *ALK*-targeted therapies.

Table 1. Comparison of different methods for *ALK* testing in tissue sample.

Parameters	FISH	IHC	qRT-PCR	NGS	NanoString
Sample criteria					
RNA input required	NA	NA	+	+++	++
RNA quality	++	+++	+	+	++
% of tumor cells	+	+++	++	+	+
Sensitivity	+++	+++	++	++	++
Specificity	+++	+++	++	+++	++
Costs					
Reagents	++	+++	++	+	+++
Device/hardware/software	++	+++	++	+	+
Resolution					
Quantitative precision	+	+	++	+++	+++
Accuracy at low concentrations	NA	NA	++	+	+++
Variants detection	NA	NA	++	+++	++
TAT					
Hands on time	+	+++	++	+	+++
Results analysis	+	+++	++	+	++
Interpretation	+	+++	++	++	++
Throughput	+	++	++	+++	+++

TAT: turnaround time; NA: not applicable; +: worse approach; ++: intermediate option; +++: best approach.

5. Algorithms for Diagnosis of an *ALK* Rearrangement

The substantial number of technological approaches for detection of an *ALK* rearrangement raises the question of their complementarity or lack of complementarity and the necessity to see development of different approaches in the same laboratory so as to reply to the needs of physicians [116,117]. It is possible to question if these approaches can be used in a sequential fashion or in combination. In fact, the samples from NSCLC patients sent to the pathology laboratories are smaller and smaller and thus require strategies that economize the amount of biological material for the morphological, IHC and molecular biology analyses [118]. Moreover, it is important to rapidly transfer reliable results, which requires sensitive and specific tests [119]. Therefore, several algorithms have been proposed [53,104,120–125]. At present the extensively employed algorithm consists in first-line ALK IHC (or ICC) and if the result is positive FISH analysis is used for confirmation. A negative IHC does not lead to other analyses and the *ALK* status is considered to be negative. A second algorithm with first-line ALK IHC/ICC and then if positive NGS analysis can be proposed for confirmation [109,110]. Algorithm may also depend of the country, the local organization and the resources (personal, platform) available in each institution. One possible algorithm is shown in Figure 1. In this context, it is necessary to mention that companion diagnostic tests (CDx) are gatekeepers with respect to the treatment decision for patient with life-threating conditions [126]. Physicians must always require high standards for introduction of new analytical methods and technologies. Therefore, it is noteworthy to mention that so far, in USA, the ALK (D5F3) CDx IHC assay and the FISH *ALK* break apart assay are the only assays that have obtained regulatory approval. In this context, if other assays, such as those based on RT-PCR, NGS and the Nanostring approach, should be used as CDx for ALK inhibitor treatments, it will be mandatory to do extensive analytical and clinical validation studies as well as some ring studies with external quality assessment. However, CDx, which obtained regulatory approval, is not always perfect. As an example, for FISH analysis, the FDA recommended counting at least 50 tumor cells for *ALK* status assessment, and if 15–25 cells demonstrated an *ALK* rearrangement, an additional 50 tumor cells have to be counted by another pathologist. However, it is quite obvious that it is not an ideal approach and some pitfalls and/or errors may occur for different reasons [127].

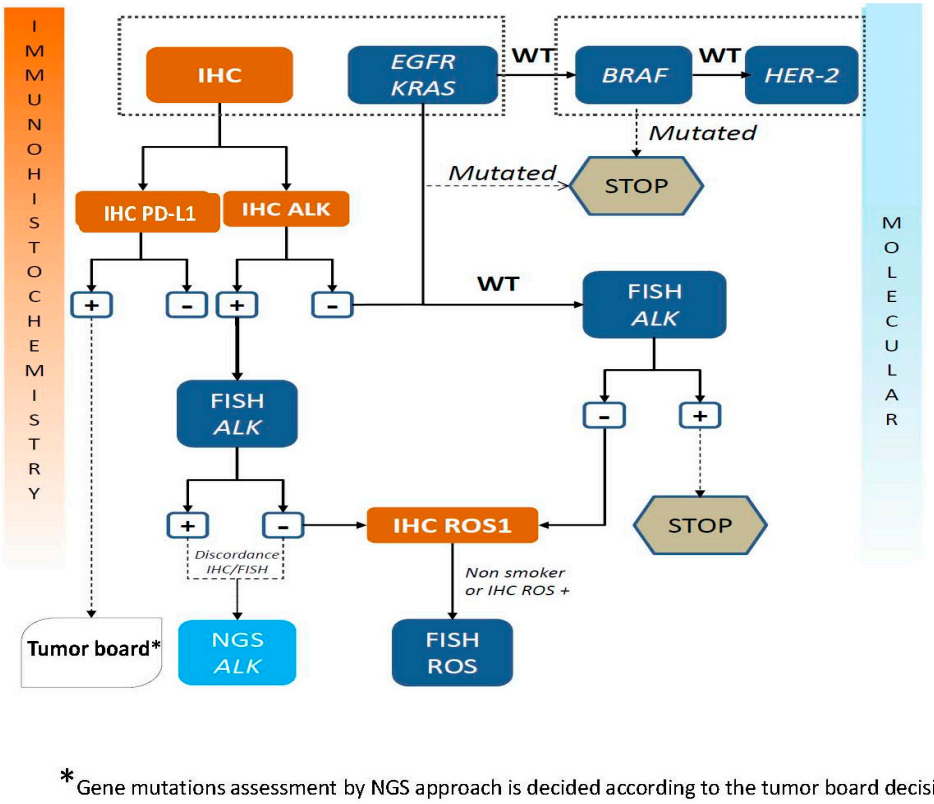

Figure 1. Current algorithm proposed at the LPCE (Nice Hospital, France) for *ALK* testing [incorporating into the standard of care *ROS1* status and *Epidermal Growth Factor Receptor* gene (*EGFR)* mutation testing in lung adenocarcinoma].

6. Conclusions

To provide patients with advanced-stage NSCLC with ALK inhibitors it is essential to systematically analyze for *ALK* rearrangements using a rapid, cost effective and reliable approach [1,3]. For a long time the FISH technique was the only method for evaluation of the *ALK* status. However, FISH analysis is now considered to be labor-intensive, quite expensive and difficult to implement systematically in all pathology laboratories as a screening and diagnostic assay. Moreover, the discordant results comparing IHC and FISH are problematic and are quite frequent in cases with borderline FISH positivity (15–20% split nuclei). At present, IHC/ICC is the first to be used, in particular when the sample is small and/or contains few cells [128]. Moreover, it has been suggested that ALK IHC is better than *ALK* FISH at predicting response to ALK inhibitors [123]. However, occasionally there is FISH-IHC discordance that may make difficult the determination of the *ALK* status. Confirmation of the IHC/ICC ALK result must be made by another method. The NGS approach, except for the *ALK* rearrangement, can detect other rearrangements including with *ROS1*, *RET* and *NTRK*, all in a single analysis, which avoids sequential investigation of these genomic alterations and thereby gains precious time for administration of effective treatment. However, depending on the amount of tumor cells and the quality of the nucleic acid, these techniques need to be discussed on a case-by-case basis, before arriving at a negative result. Finally, it is very important, disregarding the method used, to assure the quality of the results. Therefore, participation in external quality control and inter-laboratory control and ring studies is indispensable. This should lead to accreditation tests based on the norms of the country in which these theranostic tests are performed [61,129–132].

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