Validation of a new algorithm for a quick and easy RT-PCR-based ALK test in a large series of lung adenocarcinomas: Comparison with FISH, immunohistochemistry and next generation sequencing assays.

Antonio Marchetti[a], Maria Vittoria Pace[a], Alessia Di Lorito[a], Sara Canarecci[a], Lara Felicioni[b], Tommaso D'Antuono[a], Marcella Liberatore[a], Giampaolo Filice[a], Luigi Guetti[c], Felice Mucilli[c], Fiamma Buttitta[b,*]

[a] *Center of Predictive Molecular Medicine, Center for Excellence on Ageing and Translational Medicine (CeSI-MeT), University of Chieti-Pescara, Chieti, Italy*
[b] *Oncological and Cardiovascular Molecular Medicine Unit, Center for Excellence on Ageing and Translational Medicine (CeSI-MeT), University of Chieti, Chieti, Italy*
[c] *Department of Surgery, University of Chieti, Chieti, Italy*

a b s t r a c t

Objectives: Anaplastic Lymphoma Kinase (ALK) gene rearrangements have been described in 3–5% of lung adenocarcinomas (ADC) and their identification is essential to select patients for treatment with ALK tyrosine kinase inhibitors. For several years, fluorescent in situ hybridization (FISH) has been con- sidered as the only validated diagnostic assay. Currently, alternative methods are commercially available as diagnostic tests.

Material and methods: A series of 217 ADC comprising 196 consecutive resected tumors and 21 ALK FISH- positive cases from an independent series of 702 ADC were investigated. All specimens were screened by IHC (ALK-D5F3-CDx-Ventana), FISH (Vysis ALK Break-Apart-Abbott) and RT-PCR (ALK RGQ RT-PCR- Qiagen). Results were compared and discordant cases subjected to Next Generation Sequencing.

Results: Thirty-nine of 217 samples were positive by the ALK RGQ RT-PCR assay, using a threshold cycle (Ct) cut-off ≤35.9, as recommended. Of these positive samples, 14 were negative by IHC and 12 by FISH. ALK RGQ RT-PCR/FISH discordant cases were analyzed by the NGS assay with results concordant with FISH data. In order to obtain the maximum level of agreement between FISH and ALK RGQ RT-PCR data, we introduced a new scoring algorithm based on the ΔCt value. A ΔCt cut-off level ≤3.5 was used in a pilot series. Then the algorithm was tested on a completely independent validation series. By using the new scoring algorithm and FISH as reference standard, the sensitivity and the specificity of the ALK RGQ RT-PCR(ΔCt) assay were 100% and 100%, respectively.

Conclusions: Our results suggest that the ALK RGQ RT-PCR test could be useful in clinical practice as a complementary assay in multi-test diagnostic algorithms or even, if our data will be confirmed in independent studies, as a

standalone or screening test for the selection of patients to be treated with ALK inhibitors.

Keywords
NSCLC; Lung adenocarcinoma ALK expression; ALK translocation ALK RT-PCR; FISH NGS; Predictive biomarkers Translational research Molecular

∗ Corresponding author at: Oncological and Cardiovascular Molecular Medicine Unit, CeSI-MeT, G. D'Annunzio University-Foundation, via L. Polacchi, 13, 66100 Chieti, Italy.
E-mail address: fbuttitta@unich.it (F. Buttitta).

1. Introduction

Molecular characterization of NSCLC has led to the identification of patient subgroups which are proved to be sensitive to targeted therapy. The Echinoderm Microtubule-Associated protein-Like 4 (EML4)-Anaplastic Lymphoma Kinase (ALK) gene fusion has been found in approximately 3–5% of patients with lung adenocarcinoma (ADC), the most frequent histological subtype of NSCLC [1,2]. ALK gene rearrangements result in the overexpression of the EML4-ALK fusion protein with oncogenic activity [2,3]. Several EML4-ALK vari- ants have been identified, characterized by fusion of various EML4 exons with ALK exon 20 [4]. In addition, other fusion partners, including TFG [5] and KIF5B [6] have been reported.

ALK rearrangements define a distinct molecular subset of NSCLC patients who can benefit from treatment with Crizotinib, an ora small-molecule tyrosine kinase inhibitor (TKI) [7]. Crizotinib has been approved by the Food and Drug Administration (FDA) and the European Medicines Evaluation Agency (EMEA) for treatment of patients with locally advanced or metastatic NSCLCs [8]. More recently, additional ALK1 inhibitors, with greater potency and dif- ferent kinase selectivity have been developed, including ceritinib and alectinib [9,10].

For several years, fluorescent in situ hybridization (FISH) anal- ysis has been considered as the only validated method for the assessment of ALK gene status, on the basis of the results of the first clinical trials with crizotinib on ALK positive patients selected by FISH. However, FISH is expensive, particularly the cost per ALK pos- itive patient, considering the high incidence of NSCLC and the low frequency of ALK gene rearrangements. In addition, this method is not widely diffused in pathology laboratories, requires expertise and the interpretation of the results is complex and time consuming [11]. An alternative method for the detection of ALK rearrange- ments, based on protein overexpression of fusion molecules, is immunohistochemistry (IHC). Several anti-ALK monoclonal anti- bodies are now available for IHC assessment of ALK status. Recently, the VENTANA ALK (clone D5F3) CDx assay has been approved by the FDA as a companion diagnostics (CDx) test to aid in identify- ing patients eligible for treatment with crizotinib [12]. The main advantages of using IHC are that the method is fast, less expen- sive, widely diffused in pathology laboratories, and can detect ALK rearrangements independent of the fusion

partners. However, the sensitivity and reproducibility of this detection system may be crit- ical when the expression level of the fusion protein is low and raises questions about the optimal IHC protocol to avoid false-negative results [13]. Another available method is reverse transcription- polymerase chain reaction (RT-PCR) with specific fusion primers. However, this technical approach is quite challenging on RNA extracted from formalin fixed paraffin-embedded (FFPE) tissue sec- tions, since complex multiplex reactions are required, and the detection is restricted to the most common fusion variants [14,15]. These factors have limited the application of this technique in clin- ical practice. Very recently, a simple and highly sensitive RT-PCR
test that detects the overexpression of 3r ALK mRNA regions has
been developed: the ALK RGQ RT-PCR assay (QIAGEN Manchester, UK). The method is based on the fact that wild-type ALK is con- stitutively silent in normal adult tissue except in brain. ALK gene rearrangements lead to the overexpression of the 3r portion of ALK, encoding the kinase domain. This RT-PCR-based method can allow to detect the expression of even a few molecules of chimeric ALK transcripts and it can identify all ALK fusion variants, whatever the 5r ALK fusion partner [16].
In this study we tested the ALK RGQ RT-PCR assay in a large cohort of lung adenocarcinomas, comparing the results with those obtained by IHC, FISH and, in selected cases, next generation sequencing (NGS) analysis. The main aim was to assess the fea- sibility to adopt the ALK RGQ RT-PCR assay in clinical practice as a standalone test or as a complementary tool in new testing algo- rithms to select patients for anti-ALK therapy.

2. Materials and methods

2.1 Tumor samples

One-hundred and ninety-six lung adenocarcinoma samples, col- lected from 2009 to 2014, were included in this study. These cases were consecutively sent to the Molecular diagnostic laboratory of the SS. Annunziata Hospital, G. d'Annunzio University of Chieti, with written informed consents from all patients. In addition, 21 ALK positive cases selected from 702 lung adenocarcinomas previ- ously analyzed by fluorescence in situ hybridization (FISH) analysis,
were included in this study. All tumor samples were formalin-fixed and paraffin-embedded (FFPE). There were 195 resected samples and 22 biopsies; all cases ware histologically classified as adeno- carcinoma based on hematoxylin-eosin staining, according to the WHO classification of lung tumors with the IASLC/ATS/ERS recom- mended modifications [17].

2.2 RT-PCR

Total RNA was extracted from each samples without dissection, using the RNeasy FFPE kit (QIAGEN Manchester, UK), according to the manufacture's instructions. After RNA isolation, the set up of the ALK RT-PCR assay plate was performed following the ALK RGQ RT-PCR protocol.
Using Scorpions technology, the kit enables the detection of RNA transcripts

encoding the ALK tyrosine kinase domain and a control region in the ABL1 gene. The kit is designed to detect the aberrant expression of mRNA encoding the ALK tyrosine kinase domain, irrespective of the different fusion variants. The analysis and the expression calls were performed manually after analysis by the Rotor Gene Q software once the runs were completed. The run controls were assessed to ensure that acceptable cycle thresh- old (Ct) values were achieved and the reactions were performed correctly. The acceptance criteria of Ct for samples were according to the manufacturer's protocol. The quantity and quality of mRNA obtained from the 22 biopsies was suitable for the ALK RGQ RT-PCR test in all cases.

2.3 Immunohistochemistry

FFPE samples were cut at a thickness of 4 μm and stained using the VENTANA ALK (D5F3) CDx Assay (Ventana Medical System, Tucson, AZ, USA) based on an anti-ALK rabbit monoclonal primary antibody (Clone D5F3). The test was performed on the BenchMark XT immunostainer (Ventana) and it was used with the OptiView DAB IHC Detection Kit and OptiView Amplification Kit. The staining results were evaluated using a binary scoring system: positive or negative, following the manufacturer's instructions.

2.4 Fluorescence in situ hybridization

FISH was performed on unstained 3–4 μm FFPE tumor tis- sue sections, using the Vysis LSI ALK Dual Colour, Break-Apart Rearrangement Probe (Abbott Molecular, IL, USA) following the manufacturer's protocol. FISH results were obtained using a slide scanning system under a 63 oil immersion objective with a fluo- rescence microscope (Olympus BX61, Olympus Corporation, Tokyo, Japan) equipped with appropriate filters, a charge-coupled device camera, and the FISH imaging and capturing software SoloTouch (Bioview, DuetTM, BioView, Ltd, Rehovat, Israel). More than fifty cancer cells per case were scored and signals were analyzed with the imaging system. Samples were considered ALK FISH-positive if more than 15% of the tumor cells showed split red and green signals (signals separated by one or more signal diameters) and/or single red 3r signals (deleted green signal) in addition to fused and/or broken-apart signals. Otherwise, the samples were considered FISH negative.

2.5 Next generation sequencing (NGS)

The ArcherTM Universal RNA Reagent Kit v2 (Archer, Boulder, Colorado, USA) was used to detect genomic rearrangements in the ALK gene. This kit, utilized in conjunction with ArcherTM Fusion- Plex Assays and Molecular Barcode (MBC) Adapters, was applied on the Illumina MiSeq® instrument (Illumina, Inc., San Diego, CA, USA). Archer's Targeted Sequencing technology based on Anchored Multiplex PCR (AMPTM) allow to identify gene fusion without prior knowledge of breakpoints or fusion partners with high sensitivity and specificity [18]. At the end of the process, the Analysis Pipeline dedicated software was used to generate a report on the status of the targeted sequences.

2.6 Statistical analysis

The variables measured in the study were investigated for asso- ciation by logistic regression analysis to account for the effect of the different variables. Sensitivity and specificity of the different assays were calculated by contingency tables. Accuracy was also measured by the area under the Receiver Operating Characteristic (ROC) curve, which represents an average probability of correctly classifying a case chosen at random. A $P < 0.05$ was considered as significant. All statistical analyses were performed using SPSS ver- sion 22 (SPSS).

3. Results

A series of 217 cases, comprising 196 consecutive lung adeno- carcinomas and 21 ALK positive tumors from an independent series of 702 cases previously evaluated by FISH, were investigated by the ALK RGQ RT-PCR assay. All runs included a positive control for both ALK and ABL expression and a negative control (RNA-free sample). According to the manufacturer's instructions, ALK positivity was defined by an ALK Ct value 35.9 with an ABL Ct value comprised between 24 and 35. Thirty-nine (18%) of 217 samples were found to be positive by the ALK RGQ RT-PCR assay, with a an ALK Ct value ranging from 27.56 to 35.84 (Table 1). All cases were also evaluated by IHC and FISH, using the VENTANA ALK (D5F3) CDx Assay and the Vysis ALK Break Apart System, respectively. Twenty-six sam- ples (12%) were positive by IHC and 28 (13%) by FISH. Of the 39 RT-PCR positive samples with Ct lower than 35.9, 14 were negative by IHC and 12 by FISH. On the other hand, there was a case (#202) positive by IHC and FISH that was negative by the ALK RGQ RT-PCR assay (Table 1). Using FISH as reference, there was a false positive rate of 31% and a false negative rate of 2,6%.

Selected samples were analyzed by NGS: 2 FISH/IHC positive samples (#21, #46), 1 FISH/IHC negative sample (#190), and 13 cases with ALK RGQ RT-PCR/FISH discordant data (#28, #32, #35, #43, #48, #64, #69, #75, #83, #102, #118, #189, #202). In all cases tested, NGS data were in agreement with FISH and IHC results (see Table 1).

These data suggest that the Ct value is an unreliable parameter for the assessment of ALK gene rearrangement, due to the pres- ence of a substantial number of false positive results. Therefore, we decided to use the ΔCt value, resulting from the difference between ALK Ct and ABL Ct. ΔCt is commonly utilized to quantify mRNA expression by RT-PCR and it is widely considered a more accurate and reliable parameter than Ct, as it also takes into account the amplificability of the sample.

In order to obtain the maximum level of agreement between FISH data and ALK RGQ RT-PCR data we established a number of dif- ferent ΔCt cut-off on a series of 100 cases (pilot series) that included 13 samples positive by FISH. With a cut-off level $\leq$ 3.5 we observed a 100% agreement with FISH data. Then we tested the algorithm on a completely independent, randomly selected, series of 117 cases (validation series), containing 15 FISH positive samples. In this lat- ter series we found an absolute correlation between the ALK RGQ RT-PCR(ΔCt) and FISH data. The sensitivity and the specificity of the ALK RGQ RT-PCR(Ct) and the ALK RGQ RT-PCR(ΔCt) assays in the pilot and validation series are reported in

Total	15	102	117

Table 2.

Overall, by using the new proposed algorithm, the 28 FISH pos- itive cases were all positive by the ALK RGQ RT-PCR(ΔCt) assay and the 13 equivocal samples, found to be discordant with FISH and IHC by the Ct parameter, were scored concordant with FISH, IHC and NGS results (Table 1). A scatterplot of data clearly indi- cated the superiority of the ΔCt over the CT value on the whole series examined (see Fig. 1). Receiver operating characteristic (ROC) curve analysis revealed an area under the curve for the ALK RGQ RT-PCR(Ct) and the ALK RGQ RT-PCR(ΔCt) assays of 0.977 (95% CI 0.947–0.992) and 1 (95% CI 0.983–1.000), respectively, (Fig. 2).

Two of the 28 FISH/ALK RGQ RT-PCR(ΔCt) positive cases were found to be negative by IHC. These two cases corresponded to small biopsies with tissue alterations produced by electrosurgical machines (Fig. 3).

4. Discussion

ALK gene rearrangement is a quite uncommon event in NSCLC but extremely important for the selection of patients to be treated with ALK inhibitors. The detection of positive cases is still chal- lenging and it has been addressed with in situ (FISH, IHC) and not in situ (RT-PCR, NGS) assays. An ideal diagnostic method should be rapid, sensitive and applicable to small biopsies that are often the only material available for testing. Although in situ methods for ALK gene/protein analysis are common in pathology laboratories, not all diagnostic centers have the possibility of using technologi- cal platforms specifically devised for Companion Diagnostics (Cdx) assays to carry out these types of analysis in optimal conditions and the sensitivity of in situ techniques is, in some cases, insuffi- cient. In this study, we tested the ALK RGQ RT-PCR test (QIAGEN Manchester, UK), a new commercially available PCR-based method for the detection of ALK gene mRNA expression, in a large retro- spective series of lung adenocarcinomas. We first examined a series of 196 consecutive lung ADC, enriched with 21 ALK FISH-positive tumors from a larger cohort of patients, using the test as suggested by the manufacturer with an interpretative algorithm based on the Ct parameter. Results were compared with IHC and FISH data, using these latter as reference standard. The statistical analysis indicated a high sensitivity of the test, but the specificity was rather low, with an excessive number of false positives results. In order to exclude the possibility that cases evaluated by the ALK RGQ RT-PCR assay with a Ct 35.9 were indeed ALK positive cases not detected by in situ techniques, we decided to further investigate ALK gene sta- tus in these discordant cases by the ARCHER system, an extremely sensitive NGS strategy, based on Anchored Multiplex PCR (AMP), a variant of RACE-PCR. A case negative by ALK RGQ RT-PCR and positive by FISH and IHC was also subjected to NGS analysis. NGS confirmed FISH and IHC data in all cases found to be discordant with the ALK RGQ RT-PCR assay, clearly indicating that they were false positive or false negative results and that a scoring system based on the Ct level, is not accurate and therefore not suitable for clinical practice.

Based on our previous experience in gene expression analysis in FFPE samples by highly sensitive RT-PCR tests [19], we decided to adopt a different algorithm that uses the ΔCt parameter which takes into account the amplificability of cDNA obtained from mRNA. To this purpose, we divided our cases in two series, a pilot one of 100 cases, including 13 ALK FISH-positive samples, which was uti- lized

to define the ΔCt cut-off level giving the highest degree of agreement with FISH data, and a validation series of 117 cases, com- prising 15 ALK FISH-positive samples, to test the new algorithm. By using the ΔCt parameter and a cut-off level 3.5 we obtained results absolutely concordant with FISH data in both series. Our results suggest that the ALK RGQ RT-PCR(ΔCt) test could be ≤use- ful in clinical practice for the selection of patients to be treated with anti-ALK therapies. In this study, the assay has been tested mainly on resected samples in order to have enough tissue for com- parison of the data with those obtained with other technological approaches, including NGS. However, 22 small biopsies were also tested with comparable results. The quantity and quality of mRNA recovered from archival biopsies was more than enough in all cases. In particular, it is to point out that in two biopsies with clear tissue artifacts induced by electrosurgery the IHC staining for ALK was negative, whereas the ALK RGQ RT-PCR(ΔCt) assay and FISH were clearly positive.

Our data indicate that the ALK RGQ RT-PCR assay is potentially applicable in routine diagnostics. This test may be considered as an alternative to FISH or IHC in cases in which a diagnostic center doesn't have dedicated platforms for in situ tests, or as a comple- mentary technology in new testing algorithms. Considering that in large studies it has emerged that there are cases in which FISH and IHC data are discordant [20,21], in order to ensure that no ALK-positive patients are left behind, new testing algorithms based on the use of multiple diagnostic approaches have been developed [20]. In some of these algorithms, a cascade of progressively more complex and costly technologies is proposed, in order to make an accurate diagnosis in most cases, saving time, tissue and money. We have recently reported a testing algorithm based on a first immuno- histochemical screening, followed by a confirmatory FISH assay in equivocal cases and a subsequent non-in situ technique (RT-PCR or NGS) in selected cases [21]. The algorithm was found to be effective in a large series of consecutive lung adenocarcinomas. On the basis of the results of the present study, we suggest a new algorithm in which, after a first IHC screening, the ALK RGQ RT-PCR assay may be used, in alternative to FISH, as a confirmatory method (as shown in Fig. 4). However, if the data reported in this study will be con-firmed in independent series, the ALK RGQ RT-PCR test could even become a standalone test or a good option in the first screening of patients for anti-ALK treatment.

5. Conclusions

In conclusion, we have validated, on a large series of NSCLCs, a new interpretation algorithm for a commercially available non- "in situ" test for the detection of ALK alterations. Comparison with FISH, IHC, and NGS data indicates that the ALK RGQ RT-PCR assay can identify ALK rearrangements, with sensitivity and specificity similar or greater than those of well recognized in situ diagnostic techniques. Since the ALK RGQ RT-PCR assay is rapid and easy to perform, even on small biopsies, we think that it has the poten- tial to become a diagnostic method to identify patients eligible for treatment with anti-ALK therapies.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgment

Dr. Marchetti is currently receiving a grant from the Italian Min- istry of University.

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Fig. 1. Scatter plot showing the distribution of RT-PCR/Ct values and RT-PCR/DELTA Ct values by FISH positivity. The cut-off for Ct values (horizontal line) and DELTA Ct values (vertical line) are reported. The correlation coefficient for Ct and DELTA Ct wete -0.053 and -0.048, respectively.

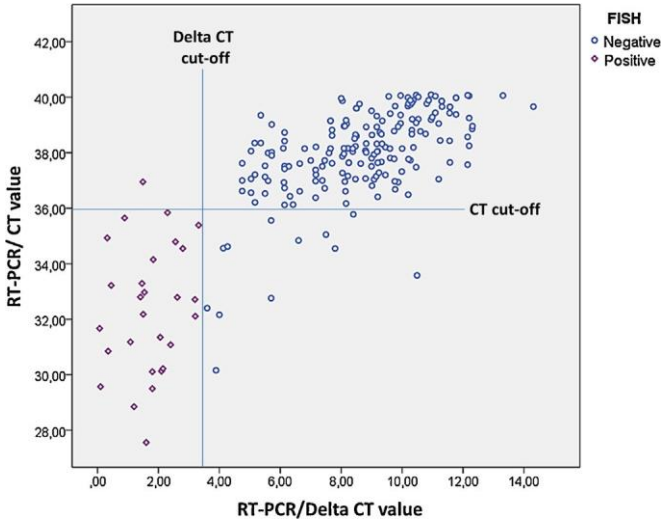

Fig. 2. ROC curves for DELTA Ct and Ct using the FISH assay as standard (positivity vs negativity).

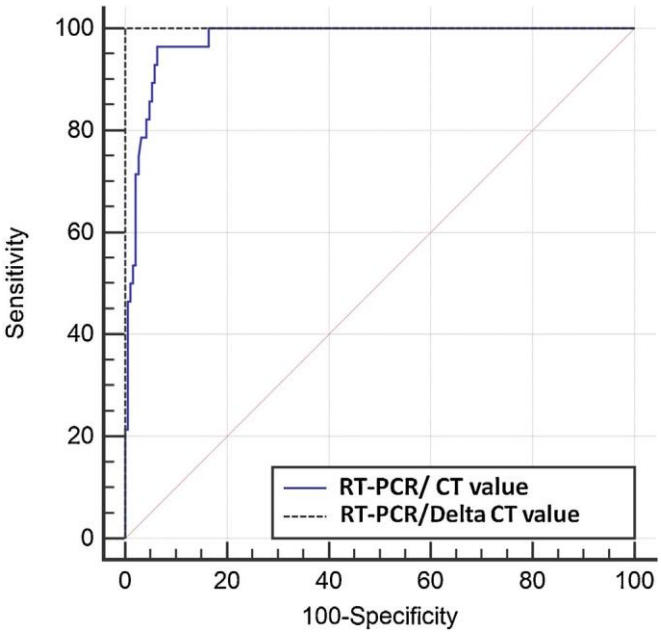

Fig. 3. Two small biopsies A (#175) and B (#184) found to be positive by FISH and negative by Immunohistochemistry (Hematoxyllin-eosin staining—40× magnification). Further magnification (400×) of the frames is reported to show severe cauterization artifacts.

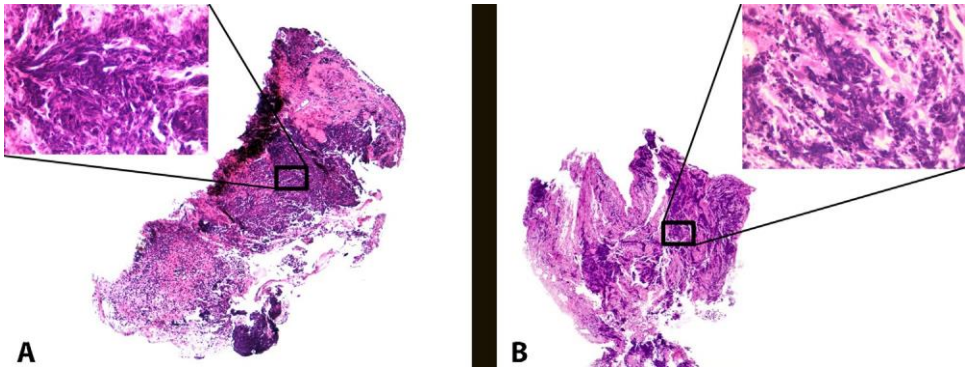

Fig. 4. Comprehensive algorithm for the selection of patients with non-small cell lung cancer to be treated with anti-anaplastic lymphoma receptor tyrosine kinase (ALK) therapy. A first step by immunohistochemical (IHC) staining is suggested. Fluorescence in situ hybridization (FISH) analysis, RT-PCR or other non-in situ assays are reserved for cases scored equivocal by IHC or negative cases with clinicopathological parameters more frequently reported in ALK-positive patients (green box). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

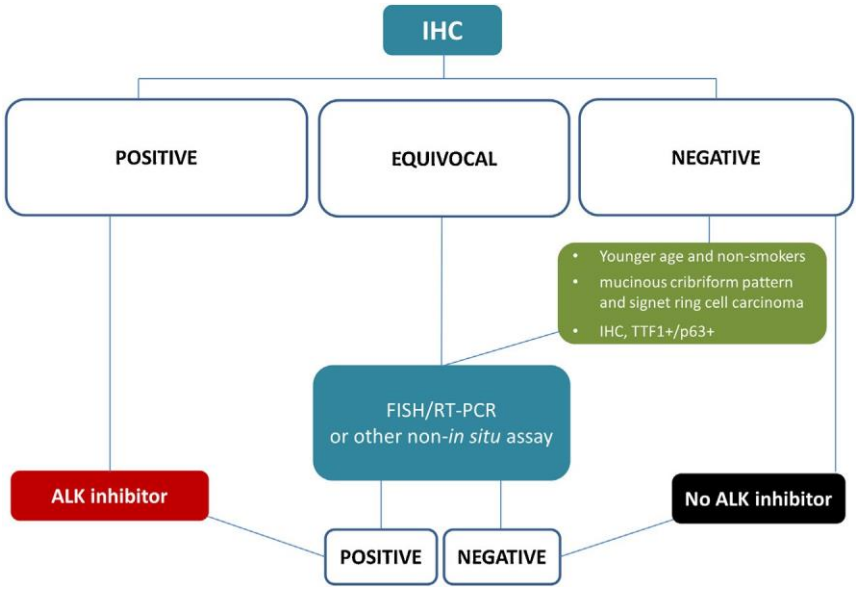

Table 1
Comparison of RT-PCR with threshold cycle (Ct) values, RT-PCR with delta Ct (ΔCt) values, fluorescence in situ hybridization (FISH), immunohistochemistry (IHC), and next generation sequencing (NGS) data.

CASES	RT-PCR score (Ct ≤35.9)	RT-PCR score (ΔCt ≤3.5)	FISH	IHC	NGS Fusion type
#21	+ (28.85)	+ (1.2)	+	+	+ (EML4-ALK)
#46	+ (27.56)	+ (1.6)	+	+	+ (EML4-ALK)
#53	+ (32.18)	+ (1.5)	+	+	NA
#56	+ (34.55)	+ (2.8)	+	+	NA
#59	+ (30.13)	+ (2.1)	+	+	NA
#62	+ (30.11)	+ (1.8)	+	+	NA
#65	+ (32.71)	+ (3.2)	+	+	NA
#71	+ (29.50)	+ (1.8)	+	+	NA
#81	+ (32.80)	+ (1.41)	+	+	NA
#84	+ (32.11)	+ (3.21)	+	+	NA
#87	+ (31.08)	+ (2.4)	+	+	NA
#91	+ (31.35)	+ (2.06)	+	+	NA
#95	+ (30.22)	+ (2.15)	+	+	NA
#98	+ (33.22)	+ (0.45)	+	+	NA
#100	+ (31.18)	+ (1.08)	+	+	NA
#104	+ (31.67)	+ (0.07)	+	+	NA
#107	+ (34.15)	+ (1.83)	+	+	NA
#109	+ (34.79)	+ (2.56)	+	+	NA
#130	+ (32.97)	+ (1.54)	+	+	NA
#138	+ (35.84)	+ (2.3)	+	+	NA
#143	+ (30.85)	+ (0.35)	+	+	NA
#147	+ (35.65)	+ (0.89)	+	+	NA
#150	+ (34.93)	+ (0.32)	+	+	NA
#161	+ (35.39)	+ (3.32)	+	+	NA
#166	+ (29.57)	+ (0.1)	+	+	NA
#175	+ (32.79)	+ (2.62)	+	–	NA
#184	+ (33.29)	+ (1.46)	+	–	NA
#28	+ (32.16)	— (4.0)	–	–	–
#32	+ (34.55)	– (7.8)	-	-	-
#35	+ (34.84)	– (6.6)	-	-	-
#43	+ (32.76)	– (5.7)	-	-	-
#48	+ (35.78)	– (8.4)	-	-	-
#64	+ (35.05)	– (7.5)	-	-	-
#69	+ (34.56)	– (4.13)	-	-	-
#75	+ (35.56)	– (5.7)	-	-	-
#83	+ (34.62)	– (4.27)	-	-	-
#102	+ (33.58)	– (10.49)	-	-	-
#118	+ (30.16)	– (3.89)	-	-	-
#189	+ (32.40)	– (3.6)	-	-	-
#202	– (36.95)	+ (1.49)	+	+	+ (EML4-ALK)
#190	– (39.76)	– (8.6)	-	-	-

Table 2
Sensitivity and Specificity of RT-PCR analyses performed on the Pilot series (A) and Validation series (B) with threshold cycle (Ct) and delta Ct (ΔCt) values versus FISH data as reference standard.

(A)

RT-PCR/CT score	FISH +	FISH −	Total
Positive	13	7	20
Negative	0	80	80
Total	13	87	100

Sensitivity = 100% (95% confidence interval: 75.29–100.00%)
Specificity = 92% (95% confidence interval: 84.12–96.70%)

RT-PCR/Delta CT score	FISH +	FISH −	Total
Positive	13	0	13
Negative	0	87	87
Total	13	87	100

Sensitivity = 100% (95% confidence interval: 75.29–100.00%)
Specificity = 100% (95% confidence interval: 95.85–100.00%)

(B)

RT-PCR/CT score	FISH +	FISH −	Total
Positive	14	5	19
Negative	1	97	98
Total	15	102	117

Sensitivity = 93.3% (95% confidence interval: 68.05–99.83%)
Specificity = 95.1% (95% confidence interval: 88.93–98.39%)

RT-PCR/Delta CT score	FISH +	FISH −	Total
Positive	15	0	15
Negative	0	102	102
Total	15	102	117

Sensitivity = 100.00% (95% confidence interval: 78.20–100.00%)
Specificity = 100.00% (95% confidence interval: 96.45–100.00%)