Translational Oncology
www.transonc.com

Volume 12 Number 2 February 2019 pp. 389–395 389

Discordance between FISH, IHC, and NGS Analysis of ALK Status in Advanced Non–Small Cell Lung Cancer (NSCLC): a Brief Report of 7 Cases[1]

Anna Scattone[*], Annamaria Catino[†], Laura Schirosi[*], Lucia Caldarola[‡], Stefania Tommasi[§], Rosanna Lacalamita[§], Elisabetta Sara Montagna[†], Domenico Galetta[†], Gabriella Serio[¶], Francesco Alfredo Zito[*] and Anita Mangia[#]

[*] Pathology Department, IRCCS Istituto Tumori "Giovanni Paolo II" di Bari, viale Orazio Flacco 65, 70124 Bari, Italy; [†] Medical Oncology Unit, IRCCS Istituto Tumori "Giovanni Paolo II" di Bari, viale Orazio Flacco 65, 70124 Bari, Italy; [‡] Pathology Department, Hospital "SS Annunziata", via Bruno 1, 74121 Taranto, Italy; [§] Molecular Genetic Laboratory, IRCCS Istituto Tumori "Giovanni Paolo II" di Bari, viale Orazio Flacco 65, 70124 Bari, Italy; [¶] Pathology Department, DETO, University of Bari, piazza Giulio Cesare, Bari 70124, Italy; [#] Functional Biomorphology Laboratory, IRCCS Istituto Tumori "Giovanni Paolo II" di Bari, viale Orazio Flacco 65, 70124 Bari, Italy

Abstract

BACKGROUND: Anaplastic lymphoma kinase (ALK) rearrangement represents a landmark in the targeted therapy of non–small cell lung cancer (NSCLC). Immunohistochemistry (IHC) is a sensitive and specific method to detect ALK protein expression, possibly an alternative to fluorescence *in situ* hybridization (FISH). In this study, the concordance of FISH and IHC to determine ALK status was evaluated, particularly focusing on discordant cases. *MATERIALS AND METHODS:* ALK status was tested by FISH and the IHC validated method (Ventana ALK (D5F3) CDx Assay) in 95 NSCLCs. Discordant cases were analyzed also by next-generation sequencing (NGS). The response to crizotinib of treated patients was recorded. *RESULTS:* Seven (7.3%) discordant cases were ALK FISH positive and IHC negative. They showed coexistent split signals pattern, with mean percentage of 15.4%, and 5′ deletions pattern, with mean percentage 31.7%. Two cases had also gene amplification pattern. In three cases (42.8 %), the polysomy was observed. The NGS assay confirmed IHC results. In these patients, the treatment with crizotinib was ineffective. *CONCLUSIONS:* In our discordant cases, a coexistent complex pattern (deleted, split, and amplified/polysomic) of *ALK* gene was observed by FISH analysis. These complex rearranged cases were not detectable by IHC, and it could be speculated that more complex biological mechanisms could modulate protein expression. These data highlight the role of IHC and underscore the complexity of the genetic pattern of *ALK*. It could be crucial to consider these findings in order to best select patients for anti-ALK treatment in daily clinical practice.

Translational Oncology (2019) 12, 389–395

Introduction

During the past decade, the molecular characterization of advanced non–small cell lung cancer (NSCLC) has led to the use of targeted therapies in clinical practice. A driver gene alteration is present in 64% of lung adenocarcinoma cases. The anaplastic lymphoma kinase (*ALK*) rearrangement is detectable in a subset of 4%-6% of NSCLCs, and it is characterized by *ALK* gene inversion or translocation on the

Address all correspondence to: Dr.ssa Laura Schirosi, Pathology Department, IRCCS Istituto Tumori "Giovanni Paolo II" di Bari, viale Orazio Flacco 65, 70124 Bari, Italy.
E-mail: l.schirosi@oncologico.bari.it

[1] Competing Interests: The authors declare no conflict of interest, financial or otherwise.
Received 26 September 2018; Revised 13 November 2018; Accepted 13 November 2018

1936-5233/19
https://doi.org/10.1016/j.tranon.2018.11.006

short arm of chromosome 2. This rearrangement leads to the production of a chimeric protein, which has constitutive kinase activity [1–3]. In the last few years, crizotinib, a small molecule dual inhibitor of ALK receptor tyrosine kinase, showed a significant therapeutic activity in patients with NSCLC harboring *ALK* rearrangement [4,5]. Thus, accurate identification of *ALK* gene status is essential for the selection of appropriate therapy, and different technologies are available to assess it.

Until now, fluorescence *in situ* hybridization (FISH) has been the standard diagnostic tool since it has been used as a reference method in clinical trials or to confirm equivocal immunohistochemistry (IHC) results [6,7]. However, FISH is expensive and time consuming and requires specialized fluorescence microscopy equipment as well as a specific expertise. Moreover, the ALK FISH assay can be challenging due to the possibility of false-negative and false-positive results and also to a significant interobserver variability [8,9].

An alternative screening test for ALK protein in NSCLCs is IHC [10,11]. Recent regulatory changes have allowed the diagnostic use of IHC analysis for the identification of patients with NSCLC who are eligible for treatment with ALK inhibitors. This method is widely used in pathology laboratories and particularly useful in patients with advanced-stage carcinoma, for whom small biopsy specimens, with a limited number of neoplastic cells, are often available. Several studies showed that IHC is a sensitive and specific method to determinate ALK protein expression, with low cost, a short turnaround time, and ease of use [12].

Among different ALK antibodies (clones ALK-1, 5A4, and D5F3) investigated by IHC [13], the D5F3 clone has been included in the validated test by Ventana Medical System. Furthermore, the "Ventana ALK (D5F3) CDx Assay" has been approved by the FDA in 2015 as a companion diagnostics (CDx) test, reliable to identify patients eligible for treatment with crizotinib [14,15]. Staining of specimens with this system has been found to detect ALK protein with more sensitivity and specificity compared with FISH or other IHC assays [11]. This method allows a dichotomous result as positive or negative, without the need to perform further FISH confirmation test. In addition, in recent studies, the interpretation of Ventana IHC ALK staining showed excellent interreader agreement [16,17].

Generally, the FISH and IHC assays show a good level of correlation [18,19], and in a recent international interpretation study, they have demonstrated an overall sensitivity, specificity, and accuracy of 90%, 95%, and 93%, respectively [16]. However, latest large-scale studies have also found several cases with discordant results between IHC and FISH [9,20–23]. These discordant data have been mainly attributed to technical problems affecting FISH or IHC assays or to difficulties of signal interpretation. Nevertheless, biological issues, including the presence of ALK activating mutations/amplifications or posttranslational changes, could explain these findings. Based on these considerations, a testing algorithm with combination of FISH, IHC, and next-generation sequencing (NGS) might be a better approach to select the patients for ALK inhibitor therapy [24]. The use of alternative approaches, as reverse transcriptase polymerase chain reaction and/or NGS, in fact, has been recommended to resolve discordant or borderline cases [25,26].

In our study, we have analyzed the concordance between an automated standardized IHC assay, which detects the ALK protein, and FISH test, which directly identifies *ALK* gene rearrangement, in 95 samples of NSCLC. In this cohort of patients, however, we have evaluated *EGFR* status and ROS1 alteration. For the IHC/FISH discordant cases, we have also used a commercially available NGS assay, designed to identify mRNA produced by all *ALK* rearrangements, to explore *ALK* status. For these patients, the clinical response to crizotinib has been recorded.

Materials and Methods

From November 2015 to July 2016, 95 NSCLC cases, referred to the IRCCS Istituto Tumori "Giovanni Paolo II" of Bari, have been collected. Informed consent was obtained from all patients. The study was conducted in accordance with the ethical standards as outlined in the Declaration of Helsinki and in national and international guidelines.

The samples were obtained from transcutaneous fine needle aspiration, small biopsies, or surgical specimen. The procedures to make the formalin-fixed, paraffin-embedded (FFPE) cytological blocks from fine needle aspiration were the same as for histological samples. The cytological sample was centrifuged, fixed in 4%-10% neutral formalin (fixation time 24 hours), and embedded in paraffin. For pathological diagnosis, IHC antibodies were used. Antibodies CK7, TTF-1, napsin A, and p63 were used as markers for differentiation of lung adenocarcinoma from squamous cell carcinoma. Tumor samples were histologically classified as adenocarcinoma (93 cases) and squamous cancer (2 cases) on the basis of hematoxylin and eosin and IHC staining, according to the WHO [27] classification of lung tumors. Representative tumor areas were identified to perform molecular analyses.

Patients

Clinical data on the patients who resulted ALK IHC negative and FISH positive (n=7), treated with crizotinib on the basis of FISH result, were collected. The data included the patients' demographic and clinicopathologic tumor characteristics, and the clinical history of patients with their responses to chemotherapy, as assessed by the Response Evaluation Criteria in Solid Tumors and the National Cancer Institute Common Toxicity Criteria (for adverse events). The patients underwent clinical visits that included thoracic and abdominal computed tomography scans at baseline and after 6 to 8 weeks of crizotinib therapy. Responses were defined as the best response from the start of treatment until disease progression according to the Response Evaluation Criteria in Solid Tumors (version 1.1). Clinical and biologic data were collected by the oncologist and pathologists. Crizotinib was administered in a dosage of 250 mg twice daily. The occurrence of grade 4 and 5 adverse events during treatment was documented.

Detection of ALK Rearrangement by FISH

All the histological and cytological samples were tested by FISH ALK test, which was carried out using the Vysis ALK Break Apart FISH Probe kit (Abbott Molecular, Des Plaines, IL). Tissue sections 3 μm thick were prepared for FISH staining; the process and interpretation of the test were performed according to the manufacturer's instructions. Positive cases were defined as those exhibiting split signals [the 5′-part (green fluorescence) and 3′-part (red fluorescence) signals were regarded as split when the separation distance was greater than two fluorescence signal diameters] or an isolated red signal in more than 15% of tumor cells. At least 50 tumor cells for each section were analyzed. The interpretations of FISH stains for *ALK* rearrangement were made by two biologists.

In addition, gene amplifications or polysomy was evaluated. *ALK* amplification was defined by the presence of ≥6 copies of *ALK* per cell in ≥10% of analyzed cell [28], whereas for the ALK FISH/IHC discordant

Table 1. Clinical Characteristics and ALK Status of Seven Patients with Discordant FISH and IHC Results

Patient	Age	Sex	Histology	Sample	ALK FISH	FISH Pattern	% ALK Rearranged Positive Nuclei	ALK IHC	ALK NGS	Crizotinib Therapy	Overall Patient Response	Smoker
1	67	F	ADK	Cytology lung	Positive	Del 5′ 12% Split 14%	26	Negative	WT	Yes	No (PD, DOD)	N/A
2	77	M	SCC	Biopsy lung	Positive	Del 5′ 24% Split 26%	46	Negative	WT	Yes	No (PD, DOD)	N/A
3	76	M	SCC	Biopsy lung	Positive	Del 5′ 34% Split 20%	54	Negative	WT	Yes	No (PD, DOD)	N/A
4	74	M	ADK	Lung surgical specimen	Positive	Del 5′ 30% Split 16% Polysomy 83%	46	Negative	WT	Yes	PS (for 4 months then PD, ACHT, AWD)	yes
5	66	F	ADK	Pleural biopsy	Positive	Del 5′ 32% Split 16% Gains 64%	48	Negative	WT	Yes	No (PD, DOD)	yes
6	70	F	ADK	Pleural biopsy	Positive	Del 5′ 10% Split 10% Polysomy 70%	20	Negative	WT	Yes	No (PD, then ACHT,AWD)	no
7	67	M	ADK	Pleural biopsy	Positive	Del 5′ 80% Split 6% Gains 20% Polysomy 65%	86	Negative	NV	Yes	No (PD, DOD)	N/A

M, male; *F*, female; *ADK*, adenocarcinoma; *SCC*, squamous cell carcinoma; *NE*, not evaluable; *Del*, deletion; *DOD*, died of disease; *ACHT*, adjuvant chemotherapy; *PD*, progressive disease; *PS*, stable disease; *AWD*, alive with disease; *N/A*, not available.

cases, the chromosome 2 status was assessed by performing an additional FISH assay on adjacent section utilizing CEP2 probe (centromeric alpha-satellite specific for chromosome 2, from Abbott Molecular, Des Plaines, IL). Otherwise, the samples were considered FISH negative.

Detection of ALK and ROS1 Protein Expression by IHC

In the 95 NSCLC tissues, IHC assay for ALK has also been performed. Each case was stained with Ventana anti-ALK (D5F3) rabbit monoclonal primary antibody and a matched rabbit monoclonal negative control Ig antibody. Neoplastic cells labeled with the Ventana ALK (D5F3) CDx Assay were evaluated for presence or absence of the immunoreactivity signal. The matched negative control slide was used to assess nonspecific background staining. All cases were stained with the OptiView DAB IHC Detection kit and the OptiView Amplification kit (Ventana Medical Systems Inc., Tucson, AZ), in accordance with the manufacturing protocol. In detail, FFPE sections of 3-4 μm thick were prepared for IHC staining, which was performed automatically using the Ventana BenchMark XT Stainer (Ventana Medical Systems Inc., Tucson, AZ). Positive controls included lung tumor confirmed by FISH to be positive for *ALK* rearrangement. Negative controls included lung tumor confirmed by FISH to be negative for rearrangement as well as nontumor lung tissue. The expression of ALK on each tissue section was assessed and scored by a biologist and a pathologist who were trained to identify as a binary scoring system only, as follows: positive, presence of strong granular cytoplasmic staining in tumor cells (any percentage of positive tumor cells); negative, absence of strong granular cytoplasmic staining in tumor cells.

For the ROS1 immunostainig procedure, the OptivView DAB IHC Detection kit was used (Ventana Medical Systems Inc., Tucson, AZ), without amplification kit, using the anti-ROS1 rabbit monoclonal antibody (Clone D4D6, 1:20, dilution, Cell Signaling Technology, Danvers, MA). On the basis of the intensity of immunoreactivity, the samples were scored as negative, positive, or in doubt, and in this last case, an alternative reflex test, as FISH, should be considered.

Detection of EGFR

In all the 95 histologic and cytologic samples, also *EGFR* exons 18-21 have been analyzed. After DNA extraction from FFPE tissue using QIAamp DNA Micro kit (Qiagen, Hilden, Germany), the Therascreen EGFR RGQ PCR Kit (Qiagen, Hilden, Germany) was used to examine *EGFR* status, according to manufacturing protocol, with a limit of mutation detection of 0.5%-7%.

Detection of ALK Fusion by NGS

The ALK FISH/IHC discordant cases were also tested by another alternative molecular method. Only one case (patient 7) resulted not evaluable by NGS. In brief, RNA was isolated from FFPE tissue using Ambion RecoverAll Total Nucleic Acid Isolation Kit for FFPE. Ten nanograms of RNA for each sample was processed by using the Ion AmpliSeq RNA Library Kit and the Ion AmpliSeq RNA Lung Fusion Panel. This panel targets, in a single assay, four acceptor driver genes (*ALK*, *RET*, *ROS1,* and *NTRK1*) in addition to many donor genes and includes 5′ and 3′ *ALK*, *ROS1*, and *RET* gene expression assays, as an indicator of a translocation at these genes, and five expression control genes. Quantified libraries were sequenced on Ion 316 chip. Data were analyzed with integrated workflows in Ion Reporter Software 4.2.

Statistical Analysis

The sensitivity, specificity, and positive and negative predictive values for ALK IHC compared to the ALK FISH results were determined. Agreement between the IHC and FISH techniques was also calculated. The sensitivity of IHC was measured as the proportion of the IHC-positive cases in the FISH-positive cases, while the specificity was determined as the proportion of the IHC-negative cases in the FISH-negative cases. Positive predictive value (PPV) and negative predictive value (NPV) of the IHC as compared to FISH were also calculated. Concordance between IHC ALK expression and FISH analysis was performed using GraphPad QuickCalcs software. A kappa coefficient (κ) value of 0.41 to 0.6 indicates moderate agreement, 0.61 to 0.8 substantial agreement, and more than 0.8 almost perfect agreement (95% confidential intervals).

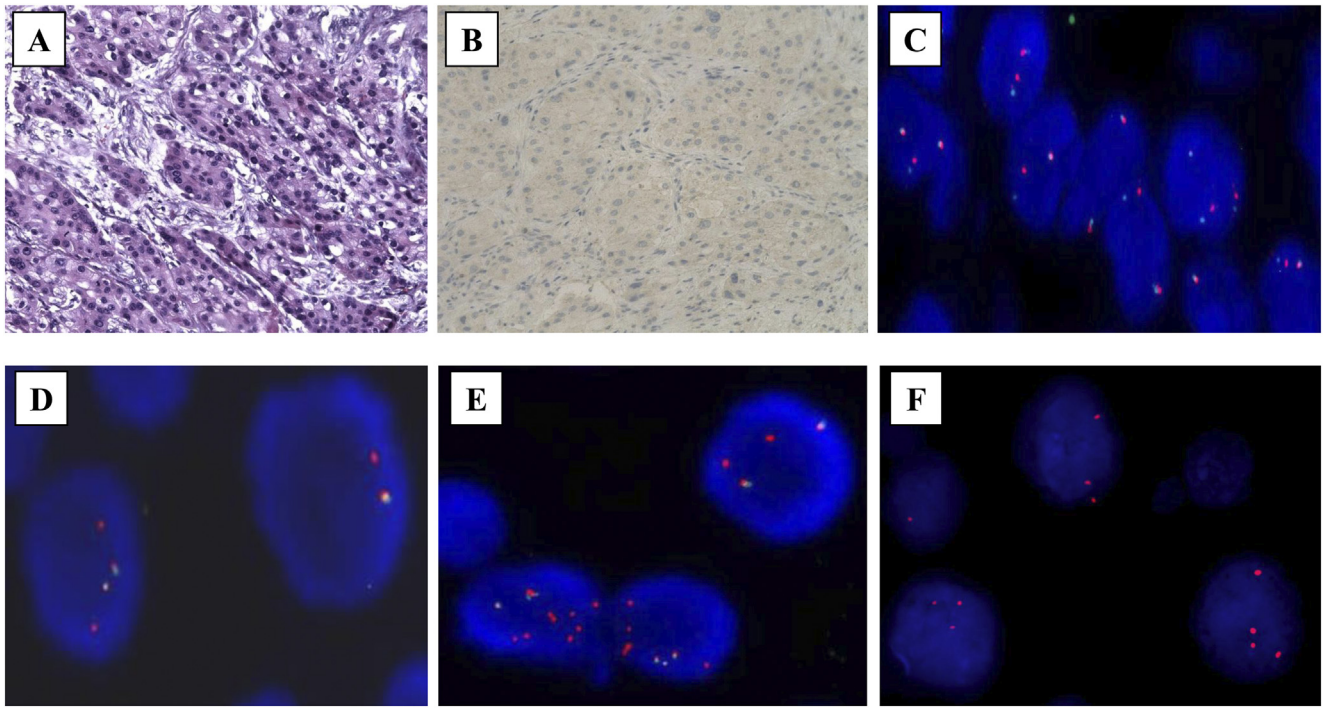

Figure 1. Representative images of one sample with discordant ALK IHC and FISH status. (A) Hematoxylin-eosin staining of a sample with adenocarcinoma histotype (Magnification ×20). (B) ALK IHC negative status (magnification ×20). (C) ALK FISH-positive case with typical split signals. (magnification ×60). (D) ALK FISH-positive case with 5' deletion pattern (magnification ×100). (E) ALK FISH-positive case with gene amplification pattern (magnification ×100). (F) Polysomy pattern of chromosome 2 (magnification ×100).

Statistical analysis was performed at the last study follow-up date (June 28, 2017). The patients' progression-free survival was calculated as the time from the date of beginning of crizotinib therapy to the first observation of disease progression, while overall survival was calculated as the time from the date of beginning of crizotinib therapy until death from any cause or until the last follow-up date.

Results

Our study included 95 patients with advanced NSCLC. Among them, 60 (63.1%) were females and 35 were male (36.8%), with a median age of 62 years (range, 33-82). In 21 cases (22%), an *EGFR* mutation was detected. ROS1 alteration investigated by IHC was found in two cases (2.2%). Thirteen of the 95 cases (13.6%) showed *ALK* translocation by FISH assay, whereas ALK testing by IHC analysis was positive in 6 cases (6.3%). ALK-IHC results correlated well with ALK-FISH in 88 cases (92.6%), of which 82 (86.3%) were both negative cases and 6 (6.3%) were both positive cases.

However, 7 (7.3%) discrepant cases were identified. The clinical and pathological characteristics, as well as the genetic pattern of them, are summarized in Table 1. In two cases, the histotype was squamous, while the other five were adenocarcinomas (Figure 1*A*). The discordant group consisted of three female and four male patients with mean age of 71.8 (range, 66-77 years). All were ALK-FISH positive and ALK-IHC negative (Figure 1*B*), whereas no case showed an opposite result. Among these cases, the mean percentage of FISH-positive rearranged nuclei was 46.5% (range 20%-86%). All positive cases showed coexistent split signals pattern (Figure 1*C*), with mean percentage of 15.4% (range 6%-26%), and 5′ deletions pattern (Figure 1*D*), with mean percentage 31.7% (range 12%-80%). Two cases (28%) had also *ALK* gene amplification pattern (Figure 1*E*) with mean percentage of 42% (range of 20%-64%). The amplified cells were always found as intermingled to cells carrying *ALK* rearranged. Moreover, FISH with CEP2 identified polysomy (Figure 1*F*) in three cases (42.8 %) with mean percentage of 72.6% (range of 65%-83%). None of these discordant cases expressed ROS1 or other driver alterations (*EGFR*). Moreover, none of them showed the most frequent *EML4-ALK* fusion transcript as revealed by NGS analysis (Table 1). These seven patients received crizotinib and were evaluable for response. A disease progression was observed in all patients, and five of them died after a very short period (progression-free survival <2 months).

Finally, in our study, the sensitivity and specificity of IHC method compared to the FISH test were analyzed, and they were 46.2% and 100%, respectively, with a PPV of 100% and an NPV of 92.1%. The concordance rate between the two techniques was 92.6% (κ = 0.597) (Table 2).

Discussion

The second most common gene abnormality associated with targeted therapy in NSCLC is the *EML4-ALK* rearrangement [29]. Patients

Table 2. Accuracy of FISH and IHC for ALK Status Based on 95 NSCLC Cases

		FISH		
		Positive	Negative	Total
IHC	Positive	6	0	6
	Negative	7	82	89
Total		13	82	95
Concordance		**92.6% (κ=0.597)**		
IHC sensitivity		46.2%		
IHC specificity		100%		
IHC PPV		100%		
IHC NPV		92.1%		

harboring this fusion transcript are usually younger than patients with the wild-type (WT) *ALK* gene and patients with *EGFR* mutation; generally, these patients are male and never smokers or light smokers with adenocarcinoma histotype [2]. Neverthless, the *ALK* fusion has also been detected in older patients (aged 76 years) with a smoking history [30] and in squamous cell and adenosquamous carcinoma cases [31,32].

In our study, the rate of ALK FISH-positive NSCLC cases was higher (13.6%) than the frequency reported in literature (3%-6%) [33], while ALK IHC-positive cases (6.3%) were almost consistent with published data [21,34].

In our series, the ALK-positive patients are elderly, predominantly male, and someone smoker, with adenocarcinoma or squamous NSCLC histotypes. Therefore, as already stated, the clinicopathologic characteristics are not sufficient to identify patients with *ALK* rearrangements, so a diagnostic test is necessary to determine *ALK* status [35].

Crizotinib is an approved molecule for the treatment of advanced *ALK* rearranged NSCLC [33,36,37], able to rapidly induce tumor regression in most of patients, while only a minority of NSCLC ALK-positive patients (in most studies <10%) do not respond to this drug [38]. Some authors [9,20–22] reported clinical progressive disease, under crizotinib therapy, in patients with ALK IHC-negative but ALK FISH-positive tumors, hypothesizing that the FISH assay positivity could include cases not responsive to this treatment. However, the reported IHC-negative/FISH-positive tumors represent mostly cases with a borderline FISH result near the cutoff, as a number of positive *ALK* rearranged nuclei fall into a so-called gray zone (around 15%-20%) [9,11,13,21,26,39–41].

In our series of NSCLCs, we had seven ALK FISH-positive cases with both negative ALK IHC and NGS results. Interestingly, in these cases, crizotinib therapy was ineffective. The discrepancies observed between the IHC and FISH data could be due to biological events, with a potential impact on the therapeutic outcome. In fact, in discordant cases, a coexistent complex pattern of rearrangements (deleted, split, and amplified/polysomy patterns) of ALK-positive cells has been detected by FISH analysis. The lack of *ALK* fusion transcripts observed in these cases by NGS might explain the absence of the ALK protein. We observed higher percentage of nuclei positive for *ALK* rearrangement in 5′ deletions (31.7%, range 12%-80%) as compared to the percentage of split signals, with mean value of 15.7% (range 6%-26%). As reported by Gao et al. [42], tumors that predominantly harbor isolated 3′ *ALK* pattern may be frequently associated with false-positive pattern. In addition, we could attribute this different findings to other reasons: the fusion of *ALK* gene to a region that cannot be transcribed, the breakpoint occurring outside of the *ALK* gene, and posttranscriptional mechanisms deleting the ALK mRNA product. Thus, these aberrations might represent late-stage events or simultaneous co-proliferation of different clones within the tumor. In our series, we found two cases with a low rate of mixed-pattern *ALK* rearranged cells (20% and 26%, respectively). Recent studies have considered as borderline the cases with a value around 20% (15% ± 5%) [8,43,44] and not showing immunoreactivity [41]. Similarly, in the study of Thorne Nuzzo et al. [23], in a discordant group of 25 cases, ALK FISH positive and ALK IHC negative, the discrepancies were detected in the cases which had a percentage of positive nuclei near the FISH cutoff. Literature data about ALK IHC negative and FISH-positive cases have reported a poor response to crizotinib (46% patients.) [22]. Ilie et al. [41] have suggested that this finding could be linked to an unclear biological mechanisms or technical reasons (i.e., false-positive FISH test). In their paper, five patients with ALK FISH-positive tumors were slightly above the cutoff; all tumors show no expression of the ALK protein by companion validated IHC [41,45]. Three of these cases responded (to varying degrees) to crizotinib, but they showed MET overexpression, and this offers an alternative explanation for the therapeutic drug response, originally developed as a MET inhibitor. Other authors [9,13] have reported similar findings: false-positive FISH results for interpretation errors with negative IHC at rates of 13.8% (4/29) and 13.3% (2/15). Cabillic et al. [21] reported, in a study of 3244 consecutive NSCLC cases, global ALK discordant FISH and IHC results in 46.6% of samples, and both types of discordance (FISH positive/IHC negative and FISH negative/IHC positive) responded to crizotinib. In this series, these results are not comparable due to the fact that no companion validated IHC approach was used. As previously hypothesized by Zito Marino et al. [46], discordant data could not be attributable only to technical problems affecting FISH or IHC assays and to the difficulty in signal interpretation for FISH analysis, but possibly to biological issues, including the presence of ALK activating mutations/amplifications or transcriptional and/or posttranslational changes.

Interestingly, we found by FISH unexpected *ALK* gene amplifications (mean value 42%) in two cases and high prevalence of polysomy in three cases (mean value 72.6%) together with *ALK* rearrangement. These complex rearranged cases were not detectable by IHC probably because of a lack of fusion protein expression. Considering that crizotinib inhibits the ALK protein and not specifically *ALK* rearrangements, it is possible that NSCLCs without IHC ALK expression may not respond to this therapy. Moreover, literature data [28,46] suggest that the amplification of the *ALK* fusion gene could cause the ALK inhibitor resistance [47]. A critical issue is the correlation between the *ALK* gene amplification, gene translocation, and the lack of fusion protein expression. Several possible mechanisms, such as the transcriptional or posttranslational events, could explain it. The clinical significance of *ALK* amplification and polysomy is not largely known and explored in lung cancer, and so it remains an interesting challenge that needs to be fully understood.

Conclusion

Our data suggest that concomitant complex *ALK* genetic pattern detected by ALK FISH in NSCLCs could be associated to a loss of the fusion protein. It could be hypothesized that this phenomenon may be related to more complex biological mechanisms, modulating *ALK* fusion protein expression, possibly linked to transcriptional or posttranslational regulations mechanisms, rather than to technical critical issues. These concepts, however, warrant further investigation.

IHC analysis has an important role in the selection of patients for anti-ALK treatment. Therefore, the cases that do not meet the current FISH break-apart positivity criteria but show complex rearrangements should be considered for additional evaluation with a second ALK diagnostic test.

In our series, the coexistence of complex genetic findings as *ALK* amplification and polysomy could represent a prognostic biomarker associated with poor outcome and aggressive behavior. Therefore, in order to better select the patient that are candidates for crizotinib therapy, these specimens should be considered for additional ALK diagnostic method as a companion diagnostic validated IHC test.

Acknowledgements

Special thanks to Dr.ssa Stella Petroni and Dr. Giovanni Simone,

who recently retired from the work, for their important contribution. The authors thank also Grazia Giannone and Milena Zambetti for technical assistance.

References

[1] Soda M, Takada S, Takeuchi K, Choi YL, Enomoto M, Ueno T, Haruta H, Hamada T, Yamashita Y, and Ishikawa Y, et al (2008). A mouse model for EML4-ALK positive lung cancer. *Proc Natl Acad Sci U S A* **105**, 19893–19897.

[2] Shaw AT, Yeap BY, Mino-Kenudson M, Digumarthy SR, Costa DB, Heist RS, Solomon B, Stubbs H, Admane S, and McDermott U, et al (2009). Clinical features and outcome of patients with non–small-cell lung cancer who harbor EML4-ALK. *J Clin Oncol* **27**, 4247–4253.

[3] Soda M, Choi YL, Enomoto M, Takada S, Yamashita Y, Ishikawa S, Fujiwara S, Watanabe H, Kurashina K, and Hatanaka H, et al (2007). Identification of the transforming EML4-ALK fusion gene in non small cell lung cancer. *Nature* **448**, 561–566.

[4] Shaw AT, Kim DW, Nakagawa K, Seto T, Crinó L, Ahn MJ, De Pas T, Besse B, Solomon BJ, and Blackhall F, et al (2013). Crizotinib versus chemotherapy in advanced ALK positive non small cell lung cancer. *N Engl J Med* **368**, 2385–2394.

[5] Camidge DR, Bang YJ, Kwak EL, Iafrate AJ, Varella-Garcia M, Fox SB, Riely GJ, Solomon B, Ou SH, and Kim DW, et al (2012). Activity and safety of crizotinib in patients with ALK positive non small cell lung cancer. updated results from a phase 1 study. *Lancet Oncol* **13**, 1011–1019.

[6] Kwak EL, Bang YJ, Camidge DR, Shaw AT, Solomon B, Maki RG, Ou SH, Dezube BJ, Jänne PA, and Costa DB, et al (2010). Anaplastic lymphoma kinase inhibition in non–small-cell lung cancer. *N Engl J Med* **363**(18), 1693–1703.

[7] Lindeman NI, Cagle PT, Beasley MB, Chitale DA, Dacic S, Giaccone G, Jenkins RB, Kwiatkowski DJ, Saldivar JS, and Squire J, et al (2013). Molecular testing guideline for selection of lung cancer patients for EGFR and ALK tyrosine kinase inhibitors: guideline from the College of American Pathologists, International Association for the Study of Lung Cancer, and Association for Molecular Pathology. *J Mol Diagn* **15**, 415–453.

[8] Martin V, Bernasconi B, Merlo E, Balzarini P, Vermi W, Riva A, Chiaravalli AM, Frattini M, Sahnane N, and Facchetti F, et al (2015). ALK testing in lung adenocarcinoma: technical aspects to improve FISH evaluation in daily practice. *J Thorac Oncol* **10**(4), 595–602.

[9] Sholl LM, Weremowicz S, Gray SW, Wong KK, Chirieac LR, Lindeman NI, and Hornick JL (2013). Combined use of ALK immunohistochemistry and FISH for optimal detection of ALK-rearranged lung adenocarcinomas. *J Thorac Oncol* **8** (3), 322–328.

[10] Conklin CM, Craddock KJ, Have C, Laskin J, Couture C, and Ionescu DN (2013). Immunohistochemistry is a reliable screening tool for identification of ALK rearrangement in non small cell lung carcinoma and is antibody dependent. *J Thorac Oncol* **8**, 45–51.

[11] Minca EC, Portier BP, Wang Z, Lanigan C, Farver CF, Feng Y, Ma PC, Arrossi VA, Pennell NA, and Tubbs RR (2013). ALK status testing in non small cell lung carcinoma: correlation between ultra sensitive IHC and FISH. *J Mol Diagn* **89**, 104–109.

[12] Ying J, Guo L, Qiu T, Shan L, Ling Y, Liu X, and Lu N (2013). Diagnostic value of a novel fully automated immunochemistry assay for detection of ALK rearrangement in primary lung adenocarcinoma. *Ann Oncol* **24**(10), 2589–2593.

[13] Savic S, Diebold J, Zimmermann AK, Jochum W, Baschiera B, Grieshaber S, Tornillo L, Bisig B, Kerr K, and Bubendorf L (2015). Screening for ALK in non small cell lung carcinoma: 5A4 and D5F3 antibodies perform equally well, but combined use with FISH is recommended. *Lung Cancer* **89**, 104–109.

[14] Department of Health and Human Services (). Letter to Ventana Medical Systems on approval of premarket authorization application for the VENTANA ALK (D5F3) CDx Assay. http://www.accessdata.fda.gov/cdrh_docs/pdf14/P140025a.pdf, Accessed date: 1 June 2015.

[15] Italian Medicines Agency (). Eligibility data sheet for crizotinib treatment. http://www.agenziafarmaco.gov.it/sites/default/files/Xalkori_NSCLC_20150219.zip, Accessed date: 1 February 2015.

[16] Wynes MW, Dietel M, Schuuring E, Tsao MS, Yatabe Y, Tubbs RR, and Hirsch FR (2014). An international interpretation study using the ALK IHC antibody D5F3 and a sensitive detection kit demonstrates high concordance between ALK IHC and ALK FISH and between evaluators. *J Thorac Oncol* **9**(5), 631–638.

[17] Wang W, Tang Y, Li J, Jiang L, Jiang Y, and Su X (2015). Detection of ALK rearrangements in malignant pleural effusion cell blocks from patients with advanced non–small cell lung cancer: a comparison of Ventana immunohistochemistry and fluorescence in situ hybridization. *Cancer Cytopathol* **123**(2), 117–122.

[18] Yi ES, Boland JM, Maleszewski JJ, Roden AC, Oliveira AM, Aubry MC, Erickson-Johnson MR, Caron BL, Li Y, and Tang H, et al (2011). Correlation of IHC and FISH for ALK gene rearrangement in non–small cell lung carcinoma: IHC score algorithm for FISH. *J Thorac Oncol* **6**, 459–465.

[19] Savic S, Bode B, Diebold J, Tosoni I, Barascud A, Baschiera B, Grilli B, Herzog M, Obermann E, and Bubendorf L (2013). Detection of ALK-positive non-small-cell lung cancers on cytological specimens: high accuracy of immunocytochemistry with the 5A4 clone. *J Thorac Oncol* **8**, 1004–1011.

[20] Wallander ML, Geiersbach KB, Tripp SR, and Layfield LJ (2012). Comparison of reverse transcription-polymerase chain reaction, immunohistochemistry, and fluorescence in situ hybridization methodologies for detection of echinoderm microtubule-associated proteinlike 4-anaplastic lymphoma kinase fusion-positive non–small cell lung carcinoma: implications for optimal clinical testing. *Arch Pathol Lab Med* **136**(7), 796–803.

[21] Cabillic F, Gros A, Dugay F, Begueret H, Mesturoux L, Chiforeanu DC, Dufrenot L, Jauffret V, Dachary D, and Corre R, et al (2014). Parallel FISH and immunohistochemical studies of ALK status in 3244 non–small-cell lung cancers reveal major discordances. *J Thorac Oncol* **9**(3), 295–306.

[22] Marchetti A, Di Lorito A, Pace MV, Iezzi M, Felicioni L, D'Antuono T, Filice G, Guetti L, Mucilli F, and Buttitta F (2016). ALK protein analysis by IHC staining after recent regulatory changes: a comparison of two widely used approaches, revision of the literature, and a new testing algorithm. *J Thorac Oncol* **11**(4), 487–495.

[23] Thorne-Nuzzo T, Williams C, Catallini A, Clements J, Singh S, Amberson J, Dickinson K, Gatalica Z, Ho SN, and Loftin I, et al (2017). Sensitive ALK immunohistochemistry companion diagnostic test identifies patients eligible for treatment with crizotinib. *J Thorac Oncol* **12**(5), 804–813.

[24] Pekar-Zlotin M, Hirsch FR, Soussan-Gutman L, Ilouze M, Dvir A, Boyle T, Wynes M, Miller VA, Lipson D, and Palmer GA, et al (2015). Fluorescence in situ hybridization, immunohistochemistry, and next-generation sequencing for detection of EML4-ALK rearrangement in lung cancer. *Oncologist* **20**(3), 316–322.

[25] Chihara D and Suzuki R (2011). More on crizotinib. *N Engl J Med* **364**(8), 776–777.

[26] von Laffert M, Stenzinger A, Hummel M, Weichert W, Lenze D, Warth A, Penzel R, Herbst H, Kellner U, and Jurmeister P, et al (2015). ALK-FISH borderline cases in non–small cell lung cancer: implications for diagnostics and clinical decision making. *Lung Cancer* **90**, 465–471.

[27] Travis WD (2015). WHO. Tumours of the lung, pleura, thymus and heart. . 4th ed.Lyon: IARC Press; 2015 .

[28] Salido M, Pijuan L, Martinez-Aviles L, Galvan AB, Canadas I, Rovira A, Zanui M, Martinez A, Longaron R, and Sole F, et al (2011). Increased ALK gene copy number and amplification are frequent in non–small cell lung cancer. *J Thorac Oncol* **6**, 21–27.

[29] Wong DW, Leung EL, So KK, Tam IY, Sihoe AD, Cheng LC, Ho KK, Au JS, Chung LP, and Pik Wong M, et al (2009). The EML4-ALK fusion gene is involved in various histologic types of lung cancers from nonsmokers with wild-type EGFR and KRAS. *Cancer* **115**, 1723–1733.

[30] Rodig SJ, Mino-Kenudson M, Dacic S, Yeap BY, Shaw A, Barletta JA, Stubbs H, Law K, Lindeman N, and Mark E, et al (2009). Unique clinicopathologic features characterize ALK-rearranged lung adenocarcinoma in the western population. *Clin Cancer Res* **15**, 5216–5223.

[31] Dragnev KH, Gehr G, Memoli VA, and Tafe LJ (2014). ALK-rearranged adenosquamous lung cancer presenting as squamous cell carcinoma: a potential challenge to histologic type triaging of NSCLC biopsies for molecular studies. *Clin Lung Cancer* **15**(3), e37–e40.

[32] Kim H, Park E, Kim YJ, and Chung JH (2013). ALK rearrangement in a pure squamous cell carcinoma: the challenge of detection of ALK rearrangement. *Virchows Arch* **462**, 597–599.

[33] Bang YJ (2011). The potential for crizotinib is non small cell lung cancer: a perspective review. *Ther Adv Med Oncol* **3**, 279–291.

[34] Jung-Soo P, Guhyun K, and Jin Hee S (2016). ALK immunohistochemistry for ALK gene rear-rangement screening in non–small cell lung cancer: a systematic review and meta-analysis. *J Biol Markers* **31**(4), e413–e421.

[35] Kim H, Yoo SB, Choe JY, Paik JH, Xu X, Nitta H, Zhang W, Grogan TM, Lee CT, and Jheon S, et al (2011). Detection of ALK gene rearrangement in non–small cell lung cancer: a comparison of fluorescence in situ hybridization and chromogenic in situ hybridization with correlation of ALK protein expression. *J Thorac Oncol* **6**, 1359–1366.

[36] Christensen JG, Zou HY, Arango ME, Li Q, Lee JH, McDonnell SR, Yamazaki S, Alton GR, Mroczkowski B, and Los G (2007). Cytoreductive antitumor activity of PF-2341066, a novel inhibitor of anaplastic lymphoma kinase and c-Met, in experimental models of anaplastic large-cell lymphoma. *Mol Cancer Ther* **6**, 3314–3322.

[37] Zou HY, Li Q, Lee JH, Arango ME, McDonnell SR, Yamazaki S, Koudriakova TB, Alton G, Cui JJ, and Kung PP, et al (2007). An orally available small-molecule inhibitor of c-Met, PF-2341066, exhibits cytoreductive antitumor efficacy through antiproliferative and antiangiogenic mechanisms. *Cancer Res* **67**, 4408–4417.

[38] Scagliotti G, Stahel RA, Rosell R, Thatcher N, and Soria JC (2012). ALK translocation and crizotinib in non–small cell lung cancer: an evolving paradigm in oncology drug development. *Eur J Cancer* **48**, 961–973.

[39] von Laffert M, Warth A, Penzel R, Schirmacher P, Jonigk D, Kreipe H, Schildhaus HU, Merkelbach-Bruse S, Büttner R, and Reu S, et al (2013). Anaplastic lymphoma kinase (ALK) gene rearrangement in non small cell lung cancer (NSCLC): Result of a multicentre ALK-testing. *Lung Cancer* **81**(2), 200–206.

[40] McLeer-Florin A and Lantuéjoul S (2012). Why technical aspects rather than biology explain cellular heterogeneity in ALK-positive non–small cell lung cancer. *J Thorac Dis* **4**(3), 240–241.

[41] Ilie MI, Bence C, Hofman V, Long-Mira E, Butori C, Bouhlel L, Lalvée S, Mouroux J, Poudenx M, and Otto J, et al (2015). Discrepancies between FISH and immunohistochemistry for assessment of the ALK-status are associated with ALK 'borderline'-positive rearrangements or a high copy number: a potential major issue for anti-ALK therapeutic strategies. *Ann Oncol* **26**, 238–244.

[42] Gao X, Sholl LM, Nishino M, Heng JC, Jänne PA, and Oxnard GR (2015). Clinical implications of variant ALK FISH rearrangement patterns. *J Thorac Oncol* **10**(11), 1648–1652.

[43] Camidge DR, Theodoro M, Maxson DA, Skokan M, O'Brien T, Lu X, Doebele RC, Barón AE, and Varella-Garcia M (2012). Correlations between the percentage of tumor cells showing an anaplastic lymphoma kinase (ALK) gene rearrangement, ALK signal copy number, and response to crizotinib therapy in ALK fluorescence in situ hybridization-positive non small cell lung cancer. *Cancer* **118**(18), 4486–4494.

[44] Camidge DR, Skokan M, Kiatsimkul P, Helfrich B, Lu X, Barón AE, Schulte N, Maxson D, Aisner DL, and Franklin WA, et al (2013). Native and rearranged ALK copy number and rearranged cell count in non–small cell lung cancer: implications for ALK inhibitor therapy. *Cancer* **119**(22), 3968–3975.

[45] Illie M and Hofman P (2015). Reply to the letter to the editor "ALK FISH rearranged and amplified tumor with negative immunohistochemistry: a rare and challenging case concerning ALK status screening in lung cancer" by Urguen et al. *Ann Oncol* **26**(8), 1802.

[46] Zito Marino F, Rocco G, Morabito A, Mignogna C, Intartaglia M, Liguori G, Botti G, and Franco R (2016). A new look at the ALK gene in cancer: copy number gain and amplification. *Expert Rev Anticancer Ther* **16**(5), 493–502.

[47] Katayama R, Shaw AT, Khan TM, Mino-Kenudson M, Solomon BJ, Halmos B, Jessop NA, Wain JC, Yeo AT, and Benes C, et al (2012). Mechanisms of acquired crizotinib resistance in ALK-rearranged lung cancers. *Sci Transl Med* **4** (120)120ra17.