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Next-generation Sequencing for *ALK* and *ROS1* Rearrangement Detection in Patients With Non–small-cell Lung Cancer: Implications of FISH-positive Patterns

Sergi Clavé,[1,2] Natalia Rodon,[3] Lara Pijuan,[1] Olga Díaz,[3] Marta Lorenzo,[1,2] Pedro Rocha,[4] Álvaro Taus,[2,4] Remei Blanco,[5] Joaquim Bosch-Barrera,[6] Noemí Reguart,[7] Noelia de la Torre,[8] Glòria Oliveras,[6] Blanca Espinet,[1,2] Beatriz Bellosillo,[1,2] Xavier Puig,[3] Edurne Arriola,[2,4] Marta Salido[1,2]

Abstract

Detection of *ALK* and *ROS1* rearrangements was assessed using next-generation sequencing and immunohistochemistry in a retrospective cohort of 40 patients with non–small-cell lung cancer with known fluorescence in situ hybridization (FISH) data. Comparison between methods revealed that isolated 3' signal FISH pattern might suggest a false-positive result. When used as a screening method, detailed reporting of FISH patterns should be strongly considered.

Background: Detection of *ALK* and *ROS1* gene rearrangements in non–small-cell lung cancer is required for directing patient care. Although fluorescence in situ hybridization (FISH) and immunohistochemistry have been established as gold standard methods, next-generation sequencing (NGS) platforms are called to be at least equally successful. Comparison of these methods for translation into daily use is currently under investigation. **Patients and Methods:** Forty non–small-cell lung cancer paraffin-embedded samples with previous *ALK* (n = 33) and *ROS1* (n = 7) FISH results were examined with the Oncomine Focus Assay and tested for ALK and ROS1 immunoreactivity. Clinical implications of concurrent molecular alterations and concordance between methods were evaluated. **Results:** NGS was successful in 32 (80%) cases: 25 *ALK* and 7 *ROS1*. Few concomitant alterations were detected: 1 *ALK* rearranged case had an *ALK* p.L1196M-resistant mutation, 4 had *CDK4*, *MYC*, and/or *ALK* amplifications, and 1 *ROS1* rearranged case showed a *FGFR4* amplification. Comparison between techniques revealed 5 (16%) discordant cases that had lower progression-free survival than concordant cases: 7.6 (95% confidence interval, 2.2-13) versus 19.4 (95% confidence interval, 10.1-28.6). Remarkably, 4 of these cases had isolated 3' signal FISH pattern (P = .026). **Conclusion:** Our data support that the identification of 3' isolated signal FISH pattern in *ALK* and *ROS1* cases might suggest a false-positive result. NGS seems a reliable technique to assess *ALK* and *ROS1* rearrangements, offering the advantage over immunohistochemistry of detecting other molecular alterations with potential therapeutic implications.

Clinical Lung Cancer, Vol. 20, No. 4, e421-9 © 2019 Elsevier Inc. All rights reserved.

Keywords: Concurrent alterations, Fluorescence in situ hybridization, Gene copy number variations, Resistance mutations, Targeted therapies

E.A. and M.S. contributed equally to this article as senior authors.

[1]Pathology Department, Hospital del Mar, Barcelona, Spain
[2]Cancer Research Program, Hospital del Mar Medical Research Institute, Barcelona, Spain
[3]BIOPAT Biopatologia Molecular, Grup Assistència, Barcelona, Spain
[4]Medical Oncology Department, Hospital del Mar, Barcelona, Spain
[5]Medical Oncology Department, Consorci Sanitari de Terrassa, Terrassa, Spain
[6]Medical Oncology Department, Catalan Institute of Oncology, Hospital Dr. Josep Trueta, Girona, Spain
[7]Medical Oncology Department, Hospital Clinic, Barcelona, Spain
[8]Pathology Department, Consorci Sanitari de Terrassa, Terrassa, Spain

Submitted: Nov 9, 2018; Revised: Feb 13, 2019; Accepted: Feb 17, 2019; Epub: Feb 26, 2019

Address for correspondence: Marta Salido, PhD, Passeig Marítim 25-29, 08003 Barcelona, Spain
E-mail contact: msalido@parcdesalutmar.cat

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https://doi.org/10.1016/j.cllc.2019.02.008

Introduction

ALK and *ROS1* gene fusions are important predictive biomarkers for targeted tyrosine kinase inhibitor (TKI) efficacy in non–small-cell lung cancer (NSCLC).[1,2] The development of TKIs such as crizotinib has led to a breakthrough in the treatment of *ALK*- and *ROS1*-rearranged patients that gain significant survival benefit.[3,4] More recently, second-generation drugs, such as alectinib and ceritinib, have provided improved outcomes and joined the armamentarium for the treatment of these patients.[5-7] Thus, incorporation of molecular analyses for detecting any of these rearrangements is considered a standard of care in the diagnostic management of patients with NSCLC with advanced disease.[8]

The first gold standard method for detecting these rearrangements was fluorescence in situ hybridization (FISH), adding on immunohistochemistry (IHC) when properly validated.[9] In 2018-updated guidelines, ALK IHC was considered an acceptable alternative to FISH, whereas ROS1 IHC was recommended as a screening test with need for confirmation by another cytogenetic method when positive.[10] Neither FISH nor IHC methods are perfect predictors of response to targeted therapies, and several studies have reported discordant results.[11-13] Next-generation sequencing (NGS) can detect multiple gene variants simultaneously, including *ALK* and *ROS1* rearrangements, enabling comprehensive biomarker testing in NSCLC. Consequently, NGS assays are being used in several diagnostic settings to test for gene fusions. Comparison of these molecular testing methodologies is now being reviewed for clinical application.[14,15]

In the current study, we analyzed a retrospective cohort of *ALK*- and *ROS1*-rearranged cases initially diagnosed by break-apart FISH and later assessed by NGS and IHC, in a single institution. Our aim was to investigate the performance of NGS for *ALK* and *ROS1* gene fusion detection, the clinical implications of concurrent molecular alterations, and the potential false-positive results that could appear when comparing with orthogonal evaluation methods.

Patients and Methods

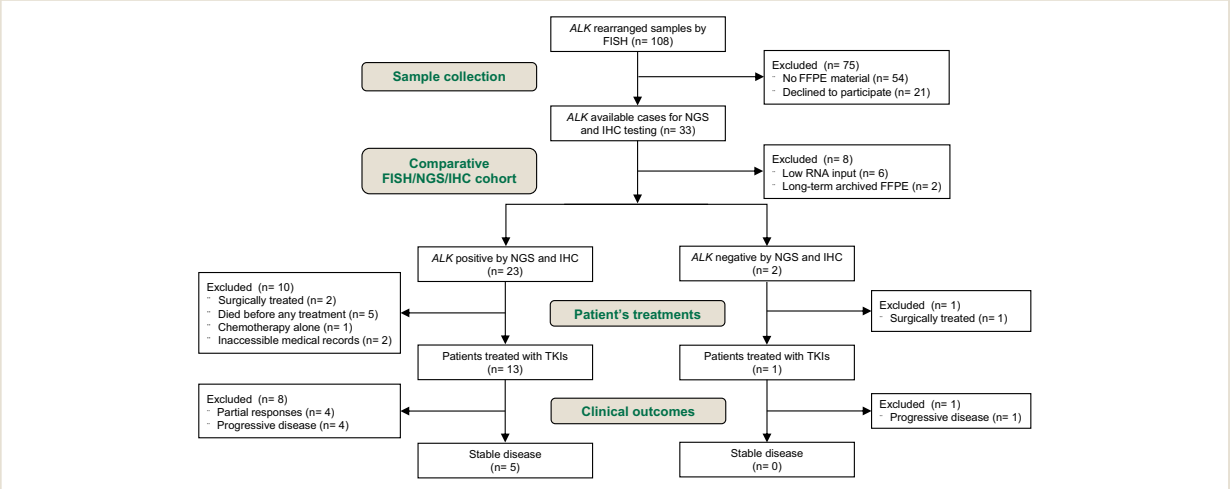
Study Population

Our institution, Hospital del Mar, has tested rearrangements over 3010 NSCLC samples since 2012 (2580 *ALK* and 1370 *ROS1* tests) as a referral center for *ALK* and *ROS1*. We screened all cases by FISH with Vysis *ALK* Break Apart FISH Probe Kit, Vysis 6q22 *ROS1* Break Apart FISH Probe (Abbott Molecular, Des Plaines, IL) or ZytoLight SPEC *ROS1* Dual Color Break Apart Probe (ZytoVision, Bremerhaven, Germany), considering positivity when the presence of $\geq$ 15% of nuclei with split or isolated 3′ signals. The percentage of rearranged cases in our series was 4.2% for *ALK* (n = 108) and 0.9% for *ROS1* (n = 12). For the present study, we have been able to retrospectively select 33 *ALK*- and 7 *ROS1*-rearranged samples (Figures 1 and 2), based on material availability and the presence of split signals, isolated 3′ signals (both positive patterns) or isolated 5′ signals (negative pattern). Samples and data from patients included in this study were provided by MARBiobanc, Biobanc IDIBGI, and Consorci Sanitari de Terrassa, integrated in the Xarxa de Bancs de Tumors de Catalunya (XBTC). This project was approved by the local ethics committee (CEIC-PSMAR: 2015/6336/I), and all patients provided written informed consent.

Sample Collection

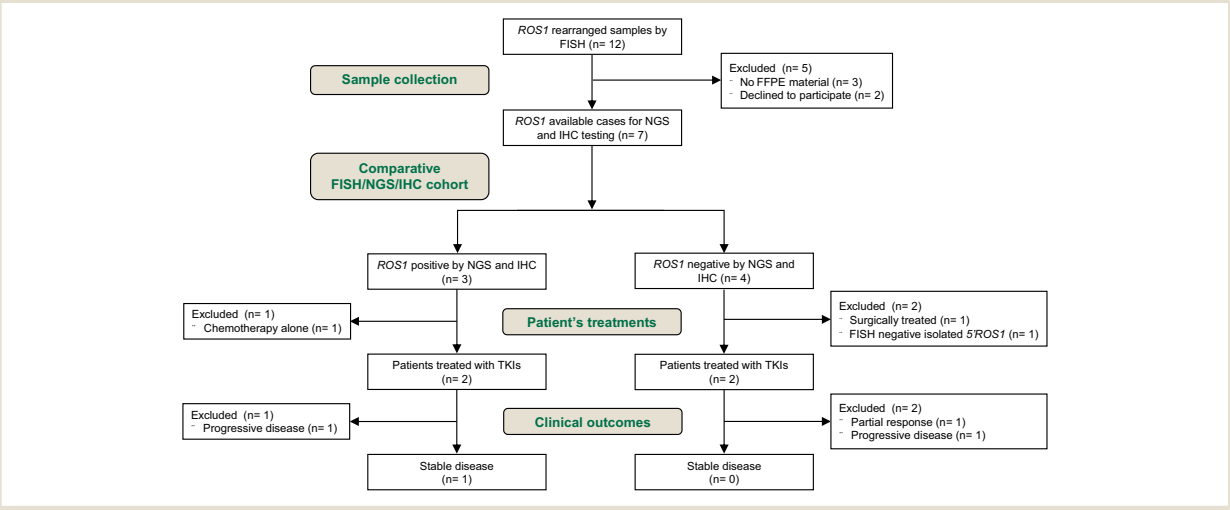
Formalin-fixed and paraffin-embedded (FFPE) tissue samples from all patients were collected and reviewed centrally in order to determine tumor content, percentage of infiltration, and microdissection area when appropriate. This initial sample evaluation and technical performance and interpretation was carried out by S.C.,

Figure 1 **Patients with *ALK* Rearrangement Detected by FISH and Selected for Further NGS and IHC Analysis. Flow Diagram Show Also Treatment Characteristics and Clinical Outcome of These Patients**



Abbreviations: FFPE = formalin-fixed, paraffin embedded; FISH = fluorescence in situ hybridization; IHC = immunohistochemistry; NGS = next-generation sequencing; TKIs = tyrosine kinase inhibitors.

Figure 2 Patients With *ROS1* Rearrangement Detected by FISH and Selected for Further NGS and IHC Analysis. Flow Diagram Show Also Treatment Characteristics and Clinical Outcome of These Patients



Abbreviations: FFPE = formalin-fixed, paraffin embedded; FISH = fluorescence in situ hybridization; IHC = immunohistochemistry; NGS = next-generation sequencing; TKIs = tyrosine kinase inhibitors.

N.R., L.P., and M.S. Four-micron sections of each case were stained with VENTANA ALK D5F3 CDx Assay (Ventana, Tucson, AZ) or ROS1 D4D6 Rabbit mAb (Cell Signalling Technology, Danvers, MA) following standard protocols. Four consecutive 10-micron FFPE sections were used to isolate DNA and RNA with the RecoverAll Total Nucleic Acid Isolation Kit for FFPE and quantified using a Qubit Quantitation Assay Kit in a Qubit Fluorometer (Thermo Fisher Scientific, San Francisco, CA).

NGS Assessment

A minimum of 10 ng of DNA and RNA were amplified using the NGS targeted panel Oncomine Focus Assay (OFA). This NGS assay enables simultaneous detection of 23 fusion genes: *ALK*, *RET*, *ROS1*, *NTRK1*, *NTRK2*, *NTRK3*, *FGFR1*, *FGFR2*, *FGFR3*, *MET*, *BRAF*, *RAF1*, *ERG*, *ETV1*, *ETV4*, *ETV5*, *ABL1*, *AKT3*, *AXL*, *EGFR*, *ERBB2*, *PDGFRA*, and *PPARG*; 35 targeted hotspot mutations: *AKT1*, *ALK*, *AR*, *BRAF*, *CDK4*, *CTNNB1*, *DDR2*, *EGFR*, *ERBB2*, *ERBB3*, *ERBB4*, *ESR1*, *FGFR2*, *FGFR3*, *GNA11*, *GNAQ*, *HRAS*, *IDH1*, *IDH2*, *JAK1*, *JAK2*, *JAK3*, *KIT*, *KRAS*, *MAP2K1*, *MAP2K2*, *MET*, *MTOR*, *NRAS*, *PDGFRA*, *PIK3CA*, *RAF1*, *RET*, *ROS1*, and *SMO*; and 19 copy number variations (CNVs): *ALK*, *AR*, *BRAF*, *CCND1*, *CDK4*, *CDK6*, *EGFR*, *ERBB2*, *FGFR1*, *FGFR2*, *FGFR3*, *FGFR4*, *KIT*, *KRAS*, *MET*, *MYC*, *MYCN*, *PDGFRA*, and *PIK3CA*. The sequencing was performed with an Ion PGM platform and analyzed with the Torrent Suite program v5.8. The detected variants were annotated and filtered with the Ion Reporter software and reviewed with the Integrative Genomics Viewer v2.4 (Broad Institute, Cambridge, MA). Fusion genes were considered positive when $\geq$ 20 fusion amplicon or when $\geq$ 0.0015 3'/5' imbalance value for *ALK* and $\geq$ 2.1 3'/5' imbalance value for *ROS1* were found. Regarding hotspot mutations, only those variants with a frequency $\geq$ 2% covered by more than 1000 reads were reported. CNVs were annotated when confidence interval (CI) at 5% was $\geq$ 4 gene copies with $\leq$ 0.5 median of the absolute values of all pairwise differences quality control measure. Cases with CNVs detected by OFA panel were validated using locus specific FISH probes. Both OFA library preparation, sequencing reagents, and analysis pipeline were from Thermo Fisher Scientific.

Statistical Analysis

Statistical associations were assessed using the Pearson χ^2 test or Fisher exact test, depending on the sample size. Survival curves were obtained with the Kaplan-Meier method, and the log-rank test was used for comparison between different groups of patients. All statistical tests were conducted at the 2-sided 0.05 alpha level of significance, carried out with SPSS Statistics software v25 (SPSS Inc, Chicago, IL).

Results

Demographic and Clinical Characteristics

Demographic and clinical characteristic of the study population are summarized in Table 1. Patients have been classified according to their FISH pattern (split vs. 3' isolated), and no significant differences were observed between them. ALK/ROS1 TKIs were given to 9 patients as first-line and to 13 as second-line treatment after standard chemotherapy, leading to 18 *ALK*- and 4 *ROS1*-treated patients. Four patients that received crizotinib at first-line switched to a second-generation TKI at second-line treatment. Seven (32%) patients experienced a stable disease, 8 (36%) a partial response, and 7 (32%) a progressive disease. The median progression-free survival (PFS) was 21.3 months (95% CI, 11.7-30.9 months). No differences in median PFS were found between TKI-treated patients with *ALK* positive FISH split (n = 14) or 3' isolated (n = 5) patterns (21.7 months [95% CI, 10.2-33.2 months] vs. 21.3 months [95% CI, 1.1-41.4 months], respectively). The median follow-up time of our series was 27 months (range, 2.3-96.4 months).

Table 1 Demographic and Clinical Data of Patients With *ALK*- and *ROS1*-Rearranged Tumors

	ALK (n = 32), n (%)[a]		*ROS1* (n = 6), n (%)[a]		Total (n = 38), n (%)
	Split (n = 17)	Isolated 3′ (n = 15)	Split (n = 3)	Isolated 3′ (n = 3)	
Age, y (range)	61 (30-85)	61 (36-86)	62 (42-76)	68 (67-71)	61 (30-86)
Gender					
Male	7 (41)	8 (53)	1 (33)	2 (67)	18 (47)
Female	10 (59)	7 (47)	2 (67)	1 (33)	20 (53)
Histology					
ADC	16 (94)	11 (73)	3 (100)	2 (67)	32 (84)
SCC	0	1 (7)	0	0	1 (3)
NSCLC NOS	1 (6)	3 (20)	0	1 (33)	5 (13)
Smoking status					
Never/light	10 (59)	9 (60)	3 (100)	1 (33)	23 (61)
Current/former	7 (41)	6 (40)	0	2 (67)	15 (39)
Stage					
I-II	3 (18)	2 (13)	1 (33)	0	6 (16)
III-IV	14 (82)	13 (87)	2 (67)	3 (100)	32 (84)
Biopsy site					
Lung parenchyma	12 (70)	7 (47)	1 (33)	2 (67)	22 (58)
Lymph node	1 (6)	1 (6)	1 (33)	1 (33)	4 (10)
Pleura	2 (12)	4 (27)	0	0	6 (16)
Other	2 (12)	3 (20)	1 (33)	0	6 (16)
First-line treatment[b]					
Chemotherapy	9 (64)	4 (37)	0	1 (34)	14 (45)
Crizotinib	3 (22)	3 (27)	2 (67)	1 (33)	9 (29)
Untreated[c]	2 (14)	4 (36)	1 (33)	1 (33)	8 (26)
Second-line treatment[d]					
Crizotinib	7 (70)	3 (60)	0	0	10 (59)
Alectinib	2 (20)	1 (20)	1 (50)	0	4 (23)
Ceritinib	1 (10)	1 (20)	1 (50)	0	3 (18)

Abbreviations: ADC = adenocarcinoma; FISH = fluorescence in situ hybridization; NSCLC NOS = non–small-cell lung cancer not otherwise specified; SCC = squamous cell carcinoma.
[a]The 2 patients with isolated 5′ *ALK* and *ROS1* FISH negative pattern were excluded from the analysis.
[b]We were unable to access the medical records of 7 patients, 3 *ALK* rearranged with split signals and 4 *ALK* rearranged with isolated 3′ signals.
[c]Untreated patients were those that died either before any treatment could be given, were surgically treated, or were treated with other targeted therapy for other driver alteration.
[d]Second-line treatment was available for 13 of the 14 patients that received first-line treatment with chemotherapy, and for 4 patients that switched upon first-line crizotinib progression.

Fusion Genes Detected by NGS

From the initial 40 cases, analysis by NGS was successful in 32 (80%): 25 *ALK* and 7 *ROS1*. Results could not be assessed in 8 cases owing to insufficient sequencing coverage: 6 of them were small biopsies with low RNA input (<0.02 ng/μL), and 2 were long-term archived FFPE samples. In 6 of the 32 successful samples (2 *ALK* and 4 *ROS1*), NGS did not detect any gene fusion leading to 23 *ALK* and 3 *ROS1* rearranged cases. Point out that one out of these six NGS-negative samples had isolated 5'*ROS1* signals, considered also negative by both FISH and IHC. Diagrams showing the number of *ALK*- and *ROS1*-rearranged patients included in the analysis are shown in Figures 1 and 2. Regarding the *ALK* fusions detected, 19 (83%) of 23 *ALK* rearranged corresponded to *EML4-ALK* inversions on chromosome 2 (variant 1 *EML4*(13)-*ALK*(20) in 14 cases; variant 3 *EML4*(6)-*ALK*(20) in 4 cases; and variant 5a *EML4*(2)-*ALK*(20) in 1 case). In addition, 1 case demonstrated the less common *KIF5B*(17)-*ALK*(20) fusion, and 3 were NGS-positive considering the ≥ 0.0015 3'/5' imbalance value but without detecting a specific fusion partner. The median PFS of the 8 TKI-treated patients with variant 1 *EML4*(13)-*ALK*(20) was 18.9 months (95% CI, 10.7-27 months). The low number of patients with the less common *EML4-ALK* variants did not allow us to compare clinical outcomes. Regarding the 3 *ROS1* fusions identified by NGS, 2 cases presented an *EZR*(10)-*ROS1*(34) and 1 case a *SDC4*(2)-*ROS1*(32) fusion. The unequal distribution of fusion variants owing to the low sample size precluded any statistical association regarding clinicopathologic features. Remarkably, all *ALK* and *ROS1* cases detected by NGS were also positive by IHC.

Concurrent Alterations Detected by NGS

According to hotspot mutations, we only detected a case with an *EML4*(13)-*ALK*(20) fusion coexisting with an *ALK* p.L1196M resistance mutation at 4% of allele frequency in the baseline biopsy. Regarding CNVs, NGS detected 1 *EML4*(13)-*ALK*(20) case with

CDK4 amplification (mean, 9.9 gene copies) and an *EML4*(6)-*ALK*(20) case with *MYC* amplification (mean, 5.3). Remarkably, 1 case presented both *MYC* and *ALK* amplifications (means, 16.6 and 10.6, respectively) at progression biopsy. In addition, a case with *SDC4*(2)-*ROS1*(32) harbored a *FGFR4* gene amplification (mean, 6.1). Clinical features and outcomes of patients with concurrent alterations are shown in Table 2.

Discordances Between FISH and NGS

The 5 discordant cases between FISH and NGS (2 *ALK* and 3 *ROS1*) are detailed in Table 3. Remarkably, only 1 *ROS1* case had FISH split signals, whereas 2 *ALK* and 2 *ROS1* had isolated 3′ signals ($P = .026$). IHC of these 4 cases was in agreement with NGS as illustrated in Figure 3. We confirmed FISH results in a new slide performed after FFPE sectioning for IHC, DNA, and RNA extractions. Clinically, all discordant 3' isolated signal cases were male with a current smoking habit and diagnosed with adenocarcinoma (ADC) except for 1 *ALK* rearranged case that presented a squamous histology. Interestingly, 2 of them had alternative driver alterations and received crizotinib, resulting in different responses (Table 3). The fifth discordant case with *ROS1* split signals was an Asian ethnicity 42-year-old non-smoking woman diagnosed with ADC with a previous detected *EGFR* insertion in exon 20. After gefitinib systemic progression, the patient was rebiopsied and screened for *ROS1* rearrangement. Particularly, this case exhibited *ROS1* tumor heterogeneity, showing 2 distinct FISH areas: 1 with *ROS1* gene deletion without rearrangement and the other with positive *ROS1* split signals. The patient received second-line crizotinib treatment, with a partial response lasting 11 months. Although numbers are small and could not be associated statistically, the median PFS in TKI-treated discordant patients was lower than the median PFS in concordant positive cases: 7.6 (95% CI, 2.2-13) versus 19.4 (95% CI, 10.1-28.6), respectively.

Discussion

Our study demonstrated that, although FISH is an accepted screening test for *ALK* and *ROS1* gene rearrangements, tumors with positive isolated 3′ signal patterns should be considered for confirmatory testing either by NGS or IHC. We found that 2 *ALK*- and 2 *ROS1*-positive cases with isolated 3′ signals were negative by both NGS and IHC. It is known that *ALK*/*ROS1* FISH signal patterns may vary from a split signal to complex patterns.[16] The 3′ isolated FISH pattern may reflect a gene rearrangement with deletion of the DNA sequences adjacent to the breakpoints and has to be considered positive.[17,18] However, large deletions and structural variants affecting 5′ probe-binding site without an *ALK* or *ROS1* rearrangement may result in an identical FISH pattern that would be a false-positive. A previous study by Gao et al identified 3 (14%) of 21 cases with 3′ isolated FISH pattern that were NGS- and IHC-negative. In 2 of them, concurrent alternative driver mutations were described conferring poor response to crizotinib: an *EGFR* p.L858R and a *KRAS* p.Q61L mutations.[19] In our 4 3′ isolated discordant cases we saw other molecular alterations in 2 cases: an *ERBB2* amplification concurrent with a *CDK4* p.R24H mutation in a *ROS1*-rearranged patient, and a *MET* low-amplification in an *ALK*-rearranged patient. In their study, patients with 3' isolated signals were older

Table 2 Clinical Details of Patients With Concurrent Alterations Detected by NGS

Patient Data				NGS			Clinical Outcome			
Case No.	Gender	Age	Smoking Status	Fusion	Hotspot Mutations	CNVs	First-line Treatment	PFS[a]	Second-line Treatment	PFS[a]	OS[a]
1	Male	60	Never	*EML4*(13)-*ALK*(20)	*ALK* p.L1196M	None identified	Cis-Pem	21.8	Crizotinib	21.2	46.5+
2	Female	83	Never	*EML4*(13)-*ALK*(20)	None identified	*CDK4* 9.9	None[b]	N/A	N/A	N/A	2.3
3	Male	63	Never	*EML4*(6)-*ALK*(20)	None identified	*MYC* 5.3	None[b]	N/A	N/A	N/A	0.7
4	Male	71	Former	*EML4*(2)-*ALK*(20)	None identified	*ALK* 16.6 *MYC* 10.6[c]	Crizotinib	1.8	Alectinib	2.1	15.3
5	Female	76	Never	*SDC4*(2)-*ROS1*(32)	None identified	*FGFR4* 6.1	Crizotinib	4.4	N/A	N/A	4.9

Abbreviations: Cis-Pem = cisplatin and pemetrexed; CNVs = copy number variations; N/A = non-applicable; OS = overall survival; PFS = progression-free survival.
[a]Expressed in months.
[b]Both patients had poor performance status and died after diagnosis.
[c]Detected in a rebiopsy sample after crizotinib progression.
+Patients alive.

Table 3 Clinicopathologic Details of the 5 Patients With Discordant *ALK* or *ROS1* Rearrangements Between FISH and NGS

Case No.	FISH[a]	IHC	NGS			Clinical Outcome				
			Fusion	Hotspot Mutations	CNVs	First-line Treatment	PFS[b]	Second-line Treatment	PFS[b]	OS[b]
1	*ALK* 73% isolated 3′	–	None identified	None identified	*MET* 4.8	Carbo-Tax	4.4	Crizotinib	0.8	7.3
2	*ALK* 66% isolated 3′	–	None identified	None identified	None identified	Surgery	N/A	N/A	N/A	78.4+
3	*ROS1* 46% isolated 3′	–	None identified	None identified	None identified	Surgery	N/A	N/A	N/A	29.9+
4	*ROS1* 85% isolated 3′	–	None identified	*CDK4* p.R24H	*ERBB2* 7.9	Crizotinib	6.6	N/A	N/A	9.2
5	*ROS1* 75% split	+	None identified	*EGFR* p.(D770_N771insNPH)[c]	None identified	Gefitinib	4.3	Crizotinib	11	18

Abbreviations: Carbo-Tax = carboplatin and paclitaxel; CNVs = copy number variations; FISH = fluorescence in situ hybridization; N/A = non-applicable; OS = overall survival; PFS = progression-free survival.
[a]Gene rearranged, FISH percentage of positive nuclei, and FISH positive pattern.
[b]Expressed in months.
[c]Hotspot mutation detected in previous sample.
+Patients alive.

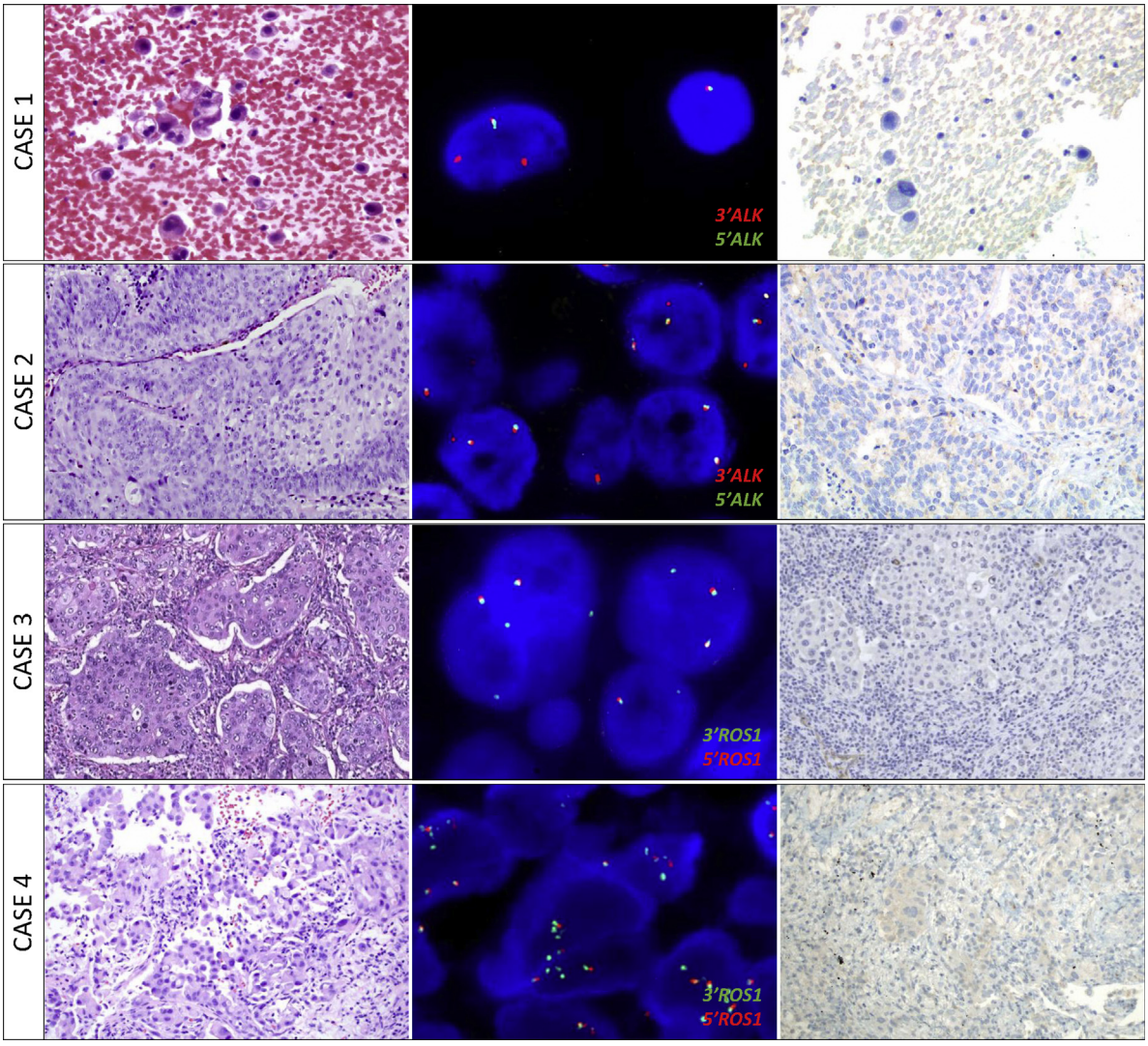
and tended to have more extended smoking histories than those with split signals.[19] We did not find any clinicopathologic feature associated with a specific FISH pattern. Consistent with our results, another recent publication by Dacic et al demonstrated that cases with 3′ isolated pattern are more prone to be FISH false-positive, and they also did not find demographic differences between both groups.[20] As our 3′ isolated FISH discordant cases showed other driver alterations and had lower PFS to TKIs than concordant cases, we might suggest that these were not true *ALK*/*ROS1* rearrangements but cases with structural alterations in the 5′ probe-binding site that give rise to FISH false-positives.

We found a fifth discordant case with *ROS1*-positive split signals that also harbored an *EGFR* mutation. Patients who have both alterations are extremely rare, and only 6 cases have been reported.[21] Clinicopathologic characteristics of our patient match with all these 6 described previously: Asian ethnicity non-smoker young woman diagnosed with a lung ADC. As NGS did not detect both alterations in the same sample, we assumed that there was no *ROS1*-rearranged cells in the FFPE sections used for this analysis. This case could be an example of spatial heterogeneity within the tumor, evidencing that subpopulations of cells with distinct genomic alterations can coexist.[22] In this patient, initial diagnosis by NGS would have allowed us to detect both molecular alterations at the same time for better monitoring during the treatment.

In the same way, another patient in our series with concurrent alterations could benefit from better clinical management if NGS would have been applied at diagnosis. The 60-year-old man with an *EML4*(13)-*ALK*(20) coexisting with an *ALK* p.L1196M resistance mutation at diagnosis was second-line treated with crizotinib. Although he obtained a prolonged partial response of 21.2 months, he could have benefited from a more selective TKIs such as alectinib, which has demonstrated better activity against cases with this *ALK* gatekeeper mutation.[23] Another relevant case with concomitant alterations was the *EML4*(2)-*ALK*(20) with both *ALK* and *MYC* amplification detected after crizotinib progression. *ALK* amplification is described to occur less frequently than secondary mutations, but recognized as a cause of acquired resistance to crizotinib.[24] A previous report showed that *MYC* amplification leads to increased expression of EML4-ALK within the cells, causing resistance to ALK inhibitors.[25] Moreover, a recent publication in preclinical models demonstrated that reduction of MYC increases sensitivity of TKIs in *ALK*-rearranged cell lines, and the authors hypothesized that both MYC and ALK could be cooperating in oncogenic signaling deriving in therapy resistance.[26] Heterogeneous resistant mechanisms including *ALK* activating mutations or gene amplifications have been identified in correlation with diverse treatment responses.[27] The detection of concurrent alterations by NGS approach at the time of diagnosis would allow us to better evaluate the best therapeutic approach for each patient.

In the present study, we included 2 patients with a negative isolated 5′ signal FISH pattern. Although initial recommendations for *ALK* FISH interpretation considered such cases as negative,[18] there are a few case reports that showed crizotinib response in these patients.[28,29] In our hands, both cases were IHC-negative, and only 1 case had available NGS data that confirmed the absence of fusion gene. A recent article discussed that these 5′ isolated FISH

Figure 3 Representative Images of the 4 Discordant Isolated 3' *ALK* (Cases 1 and 2) and *ROS1* (Cases 3 and 4) Samples. Case 1: Pericardial Effusion Aspirate Sample Showing Metastatic Lung Adenocarcinoma (ADC) Cells. Fluorescence in Situ Hybridization (FISH) With *ALK* Break-Apart Probe Revealed an Isolated 3′ Signal Pattern (1F1O and 1F2O) and No Immunoreactivity for ALK Protein was Detected by Immunohistochemistry (IHC). Case 2: Right Upper Lobe Lung Resection With a Squamous Cell Carcinoma Showing Isolated 3′ Signal FISH Pattern (2F1O) With Negative ALK IHC. Case 3: Left Upper Lobe Lung Resection Diagnosed as an ADC. FISH Images Showed an Isolated 3′ *ROS1* Signal Pattern (2F1G and 1F2G). No Immunoreactivity for ROS1 Protein Was Detected by IHC. Case 4: Endobronchial Ultrasound Bronchoscopy Sample From Right Upper Lobe Showing Adenocarcinoma Cells Showing Gained Isolated 3′ *ROS1* Signal Pattern and Negative IHC. Magnification 20× for Hematoxylin and Eosin and IHC and 100× for FISH Images



cases should be considered to carry a rare genomic event that could lead to protein expression and therefore become TKI-treatable.[30] Given the low frequency of such cases, confirmation analysis would be necessary to ensure the diagnosis.

Previous studies that have compared FISH, IHC, and NGS approaches for *ALK* and *ROS1* testing have come to the conclusion that, although concordance between the assays was generally good, many discrepancies were observed. For *ALK* testing is being accepted that screening with IHC followed by a molecular technique to identify the fusion variant is more appropriate than screening by FISH.[31,32] McLeer-Florin et al compared RNA sequencing (RNA-seq), IHC, and FISH in 53 samples initially diagnosed as *ALK* rearranged. Similar to our findings, they described 2 discordant cases that were FISH-positive and RNA-seq/IHC-negative, and both cases received crizotinib and experienced progressive disease.[33] For *ROS1* testing, there is currently a wide variety of testing approaches available, each with its interpretative limitations. Davies et al compared NGS DNA and RNA testing approaches with FISH, but none of them achieve 100% in terms of sensitivity.[15]

The success rate of NGS technology is closely related to the correct evaluation of the initial amount of tumor tissue, particularly in small biopsies. The main limitation of this retrospective study was the scarce FFPE material that did not allow us a complete characterization of the NGS findings. We were unable to characterize the fusion genes of the 3 positive cases with imbalanced 3'/5' and the putative deletions that generate the 3' isolated false-positive FISH cases. In addition, the correlation between *ALK* and *ROS1* detection techniques requires further studies in larger prospective cohorts.

In conclusion, the identification of *ALK* and *ROS1* rearrangements with 3' isolated signal FISH pattern might suggest a false-positive result. Although this observation had already been described for the *ALK* gene, to the best of our knowledge, this is the first report showing the same challenge for *ROS1* FISH interpretation. NGS seems a reliable technique to assess *ALK* and *ROS1* rearrangement and in complex or rare findings, validation through orthogonal methods should be considered.

Clinical Practice Points

- NGS assays are being used in several diagnostic settings to test for *ALK* and *ROS1* gene fusions. Comparison with widely accepted FISH and IHC testing methodologies is now being reviewed for clinical application.
- Discrepancies between techniques have been found, especially in *ALK*- and *ROS1*-rearranged cases with isolated 3′ signal FISH pattern. Differences in targeted treatment response and a higher number of concurrent alterations have been identified in these FISH-positive/NGS-negative tumors.
- As the effectiveness of therapies targeting ALK and ROS1 is highly dependent upon appropriate selection of patients, we propose a detailed reporting of positive FISH patterns and validation of samples with isolated 3′ signals through orthogonal methods (NGS or IHC).

Acknowledgments

The authors want to thank Dr Javier Gimeno for helping with image acquisition, Dr Anna Puiggros for statistical assistance, and Silvia Menéndez for technical support in immunohistochemistry; all from the Pathology Department at Hospital del Mar, Barcelona, Spain. We want to particularly acknowledge the patients and Xarxa de Bancs de Tumors de Catalunya (XBTC) financed by Pla Director d'Oncologia de Catalunya for their collaboration and MarBiobanc financed by ISCIII (PT17/0015/0011).

This work was partially supported with unrestricted research grants from Pfizer and Roche pharmaceuticals, and grants from Agència de Gestió d'Ajuts Universitaris i de Recerca (2017-SGR-507), and Fondo de Investigación Sanitaria-Instituto de Salud Carlos III/CIBERONC (CB16/12/00241 and PI16/00591).

Disclosure

The authors have stated that they have no conflicts of interest.

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