Introducing ROS1 copy number alterations as a frequent feature in non-small cell lung cancer

Sergi Clavé, Javier Gimeno, Ana M. Muñoz-Mármol, Joana Vidal, Noemí Reguart, Enric Carcereny, Lara Pijuan, Silvia Menéndez, Álvaro Taus, José Luis Mate, Sergio Serrano, Joan Albanell, Blanca Espinet, Edurne Arriola, and Marta Salido

1 Laboratori de Citogenètica Molecular, Servei de Patologia, Hospital del Mar, Barcelona, Spain
2 Programa de Recerca en Càncer, IMIM (Institut Hospital del Mar d’Investigacions Mèdiques), Barcelona, Spain
3 Servei de Patologia, Hospital del Mar, Barcelona, Spain
4 Servei d’Anatomia Patològica, Hospital Universitari Germans Trias i Pujol, Badalona, Spain
5 Servei d’Oncologia Mèdica, Hospital del Mar, Barcelona, Spain
6 Servei d’Oncologia Mèdica, ICMHO, Hospital Clinic Barcelona, Barcelona, Spain
7 Departament d’Oncologia Mèdica, Institut Català d’Oncologia (ICO), Hospital Universitari Germans Trias i Pujol, Badalona, Spain
8 Cancer Sciences Unit, University of Southampton. Southampton, United Kingdom

Correspondence to: Sergi Clavé, email: sclave@imim.es

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ABSTRACT

Objectives: We aimed to determine the prevalence and partners of ROS1 rearrangements, to explore the correlation between FISH and IHC assays, and to investigate clinical implications of ROS1 copy number alterations (CNAs).

Methods: A total of 314 NSCLC patients were screened using ROS1 FISH break-apart probes. Of these, 47 surgical tumors were included in TMAs to analyze ROS1 heterogeneity assessed either by FISH and IHC, and chromosome 6 aneusomy. To characterize ROS1 partners, probes for CD74, EZR, SLC34A2 and SDC3 genes were developed. ROS1 positive FISH cases were screened also by IHC.

Results: Five patients were ROS1 positive (1.8%). We identified two known fusion partners in three patients: CD74 and SLC34A2. Four out of five ROS1 rearranged patients were female, never smokers and with adenocarcinoma histology. Rearranged cases were also positive by IHC as well. According to ROS1 CNAs, we found a prevalence of 37.8% gains/amplifications and 25.1% deletions.

Conclusions: This study point out the high prevalence of ROS1 CNAs in a large series of NSCLC. ROS1 gains, amplifications and deletions, most of them due to chromosome 6 polysomy or monosomy, were heterogeneous within a tumor and had no impact on overall survival.

INTRODUCTION

Over the last decade there have been important advances in understanding the biology of non-small cell lung cancer (NSCLC), identifying oncogene-driven subtypes of tumors such as epidermal growth factor receptor (EGFR) mutations or anaplastic lymphoma kinase (ALK) and ROS proto-oncogene 1 (ROS1) rearrangements [1-3]. Targeted therapies based on molecular diagnostics have opened a new era of personalized medicine in NSCLC treatment, and during the last years new predictive biomarkers have emerged [4, 5].

ROS1 gene (located at chromosome 6q22) encodes for a receptor tyrosine kinase which belongs to the insulin receptor family [6]. In a similar way to ALK aberrant kinase activity, rearrangements involving the ROS1 gene lead to a constitutively activated downstream signaling of several oncogenic pathways [7, 8]. ROS1 rearrangements are rare events accounting for up to 0.6-1.8% of patients with NSCLC [9, 10]. Until now, several
fusión partners (n=12) have been described, being CD74, EZR, SLC34A2 and SDC3 the most prevalent [11, 12]. ROS1 rearrangements are more commonly found in never or mild smokers patients with adenocarcinoma (ADC) histology and in triple negative (EGFR/KRAS/ALK) population, similar to patients with ALK-rearranged NSCLC [13, 14].

Crizotinib (Xalkori®, Pfizer), approved by the US Food and Drug Administration (FDA) in treating ALK positive NSCLC, binds also with high affinity to ROSI-rearranged receptors [15]. In addition to previous case reports [2, 13], a recent study described marked antitumor responses to Crizotinib in patients harboring ROSI-rearranged NSCLC [11].

Adequate molecular-based selection is essential for NSCLC patients to achieve optimal results from targeted therapies. Fluorescence in-situ hybridization (FISH) with break-apart probes remains the gold standard method for detecting both ALK and ROSI rearrangements in lung clinical trials [11, 16]. However, other methods such as immunohistochemistry (IHC) using anti-ROS1 antibodies could provide a widely available alternative screening method. Despite previous manuscripts described a reasonable correlation between FISH and IHC, more information about IHC sensitivity and specificity is needed [17, 18].

In this study, we analyzed a Spanish cohort of NSCLC to determine the prevalence and specific features of ROSI rearranged patients and to explore the correlation between FISH and IHC assays. We also investigated ROSI copy number alterations (CNAs) to clarify the correlation between ROSI gene alteration and clinicopathological parameters.

RESULTS

Clinicopathological characteristics

The median age was 64 years, 69% were males and 71% were ever smokers with a median cumulative index of 52 packs per year (Table 1). Fifty-two per cent of the study population presented stage IV disease and 83% were ADC. The predominant histological patterns in ADC were acinar and solid (some of them mucinous type), whereas lepidic and micropapillary patterns were less common. Other histological subtypes were: squamous cell carcinoma (SCC), 3%; large cell carcinoma (LCC), 2%; and non-small cell lung carcinoma not otherwise specified (NSCLC NOS), 12% of patients. Patients with SCC were added in the analysis due to their clinical characteristics (non-smoking patients). KRAS and EGFR mutations were found in 17% and 6% of the samples respectively, whereas 1% (4 cases) presented ALK rearrangements.

ROS1 rearrangements

ROS1 FISH was evaluable in 283 cases (90.1%). The basket cases were due to insufficient tumor material or FISH assay failure. ROS1 rearrangements were found in five cases (1.8%); three of them showed a typical rearranged pattern with deletion of the fused allele (101G) and the other two cases had an atypical rearranged pattern with 5‘ROS1 deletion (1F1G) and with gains (1F2G) (Figure 1). Of note, one of the typical rearranged cases was heterogeneous showing a negative area with ROSI deletion. The mean percentage of positive cells in all cases was 70%. Regarding to ROSI partners, they were identified in three cases: two harbored a t(5;6) (q32;q22)with CD74-ROS1 fusion, one of them showing isolated 5‘CD74 signals; and the third presented a t(4;6) (q15.2;q22)with SLC34A2-ROS1 rearrangement.

Four of these five tumors presented ADC histology, the other one classified as NSCLC NOS. Two of the confirmed ADC had a predominantly solid pattern, whereas the two remaining cases had a predominantly papillary pattern. Regarding to ROS1 IHC pattern of staining, three out of the five cases presented a cytoplasmic predominant pattern whereas only one case presented a membranous predominant pattern (with mild +1 cytoplasmic associated ROS1 staining). Material was not available from the last case to perform ROSI IHC assay. Characteristics of ROSI positive cases are presented in Table 2.

We observed a significant association between ROSI rearrangement and female gender (p=0.039) as well as with non-smoking history (p=0.030).

ROS1 copy number alterations

In ROSI non-rearranged cases, gains were the most prevalent CNAs observed in 96 cases (33.9%) and in a high proportion of cells (mean percentage of 48%). Using CEP6 control probe, we confirm that gain of ROSI was due to chromosome 6 polysomy. ROSI amplifications were identified in eleven cases (3.9%), all of them with ADC histology. Amplified cases were classified in two groups: patients that had ROSI signal clusters (n=3) and patients with a chromosome 6 high polysomy (n=8) (Figure 2). In addition, 71 cases (25.1%) had ROSI deletions with a mean percentage of ROSI deleted cells of 42%. After CEP6 hybridization, heterogeneity in the enumeration of chromosome 6 was observed. Both monosomy and polysomy of CEP6 were observed in the same tumor sample.

All the specimens that were negative for the ROSI rearrangements by FISH presented negative IHC ROS1 staining. In some of them, mild (+1/+2) and diffuse cytoplasmic staining was detected (presenting H-score: <200). If only 3+ (diffusely) expressing tumors were
Table 1: Clinicopathological features of the 314 ROS1-screened NSCLC patients

<table>
<thead>
<tr>
<th>Feature</th>
<th>Global population</th>
<th>ROS1 positive (n = 5)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
<td></td>
</tr>
<tr>
<td>Median Age: y (range) (n = 307)</td>
<td>64 (25-91)</td>
<td>59 (42-74)</td>
<td>0.503</td>
</tr>
<tr>
<td>Sex (n = 309)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>212 (69)</td>
<td>1 (20)</td>
<td>0.039</td>
</tr>
<tr>
<td>Female</td>
<td>97 (31)</td>
<td>4 (80)</td>
<td></td>
</tr>
<tr>
<td>Smoking status (n = 287)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non smoker</td>
<td>82 (29)</td>
<td>4 (80)</td>
<td>0.030</td>
</tr>
<tr>
<td>Ever smoker</td>
<td>205 (71)</td>
<td>1 (20)</td>
<td></td>
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<tr>
<td>Stage (n = 284)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>63 (22)</td>
<td>0</td>
<td>0.646</td>
</tr>
<tr>
<td>II</td>
<td>23 (8)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>51 (18)</td>
<td>1 (20)</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>147 (52)</td>
<td>4 (80)</td>
<td></td>
</tr>
<tr>
<td>Histology (n = 309)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADC¹</td>
<td>256 (83)</td>
<td>4 (80)</td>
<td>0.827</td>
</tr>
<tr>
<td>SCC²</td>
<td>11 (3)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>LCC³</td>
<td>6 (2)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>NSCLC NOS⁴</td>
<td>36 (12)</td>
<td>1 (20)</td>
<td></td>
</tr>
<tr>
<td>KRAS (n = 210)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>174 (83)</td>
<td>5 (100)</td>
<td>1.000</td>
</tr>
<tr>
<td>Mutated</td>
<td>36 (17)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>EGFR (n = 276)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>260 (94)</td>
<td>5 (100)</td>
<td>1.000</td>
</tr>
<tr>
<td>Mutated</td>
<td>16 (6)</td>
<td>0</td>
<td></td>
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<tr>
<td>ALK (n = 311)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-rearranged</td>
<td>309 (99)</td>
<td>5 (100)</td>
<td>1.000</td>
</tr>
<tr>
<td>Rearranged</td>
<td>4 (1)</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

¹Adenocarcinoma
²Squamous cell carcinoma
³Large cell carcinoma
⁴Non-small cell lung carcinoma not otherwise specified

Table 2: Histopathological features of ROS1-rearranged specimens

<table>
<thead>
<tr>
<th>Case</th>
<th>FISH pattern¹</th>
<th>Fusion partner</th>
<th>H-Score</th>
<th>Staining pattern</th>
<th>Sample</th>
<th>Histology, predominant</th>
</tr>
</thead>
<tbody>
<tr>
<td>ROS1 1</td>
<td>1O1G</td>
<td>SLC34A2</td>
<td>400</td>
<td>Cytoplasmic</td>
<td>Visceral pleura</td>
<td>NSCLC NOS², N/A³</td>
</tr>
<tr>
<td>ROS1 2</td>
<td>1F2G</td>
<td>CD74</td>
<td>300-400</td>
<td>Cytoplasmic</td>
<td>Lung</td>
<td>ADC⁴, acinar</td>
</tr>
<tr>
<td>ROS1 3</td>
<td>1O1G</td>
<td>N/A</td>
<td>400</td>
<td>Membranous</td>
<td>Lung</td>
<td>ADC, solid</td>
</tr>
<tr>
<td>ROS1 4</td>
<td>1O1G</td>
<td>CD74</td>
<td>300-400</td>
<td>Cytoplasmic</td>
<td>Lung</td>
<td>ADC, papillary</td>
</tr>
<tr>
<td>ROS1 5⁵</td>
<td>1F1G</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>Lung</td>
<td>ADC, papillary</td>
</tr>
</tbody>
</table>

¹ FISH pattern indicates the result of the ROS1 break-apart probe used, labeled 5’ROS1 Spectrum orange (O) and 3’ROS1 Spectrum green (G).
² Non-small cell lung carcinoma not otherwise specified
³ Not available
⁴ Adenocarcinoma
⁵ Material was not available to perform FISH fusion partner studies and ROS1 IHC assay.
Figure 1: Representative images from case 2 ROS1-rearranged: A. Hematoxylin and eosin, 20x. Adenocarcinoma showing a predominantly acinar pattern. B. ROS1 IHC, 20x. Cytoplasmatic +3/+4 staining pattern. C. ROS1 FISH, 100x. Break-apart probe showing an atypical rearranged pattern with 5'ROS1 deletions (1F2G). D. CD74 FISH, 100x. Break-apart probe showing a typical rearranged pattern with fused and split signals (2F2O2G).

Figure 2: Representative images from ROS1 copy number alterations detected by FISH (ROS1 break-apart probe (spec.orange/green fusion signals) and CEP6 probe (spec.aqua signals). A. ROS1 cluster amplification (arrows) in a nuclei with two copies of CEP6. B. ROS1 amplification with chromosome 6 high polysomy (15 ROS1 and 15 CEP6 copies). C. Polysomic nuclei with 3-4 copies of chromosome 6 and 3-4 copies of ROS1. D. Case showing heterogeneity of ROS1 deletion: nuclei with ROS1 monosomy (1 ROS1/1 CEP6) and nuclei with ROS1 deletion and chromosome 6 disomy (1 ROS1/2 CEP6).
considered positive, ROS1 IHC is 100% sensitive and 87.5% specific for the presence of ROS1 rearrangements by FISH. Of note, in some cases mild (+1) ROS1 cytoplasmic staining was detected in benign type II pneumocytes.

Heterogeneity assessment

Focusing on heterogeneity in TMAs, 161 out of 192 cores were assessable for ROS1 FISH analysis and 174 for ROS1 IHC (Figure 3). As no positive ROS1 cases were found in the TMAs, the heterogeneity assessment was performed considering only ROS1 CNAs. When evaluating CNAs as a categorical variable (gain, amplification, deletion and disomic), Kappa agreement index for ROS1 FISH status between cores A and B was 0.24. When ROS1 IHC status was analyzed considering 0, +1, +2 and +3 score criteria, no differences in classification between cores A and the remaining three cores (B to D) were observed. The result changed from negative to borderline +2 only in four cases.

Survival analysis

We sought to explore the potential impact on survival of ROS1 alterations, analyzed only in patients with advanced disease (stages III-IV). Out of 144 cases with available data, 94 death events occurred during the follow-up period. The median follow-up time for the whole series was 15.2 months (95% CI 12.18-18.3) and median survival time was 26.2 month. One, two and three-year survival rates were 59.1%, 33% and 21.6%, respectively. The median survival time in ROS1-positive patients was significantly higher: 69.8 month vs. 13.7 months (95% CI 9.9-17.5) in the ROS1-negative group ($p = 0.028$) (Figure 4). All positive patients received Crizotinib treatment. Setting survival analysis according to CNAs, neither copy number gains nor deletions of ROS1 gene had impact on survival.

DISCUSSION

We examined ROS1 status in a cohort of patients with NSCLC. The main findings of our study are the following: (1) the prevalence of ROS1 gene rearrangement in a Spanish cohort is 1.8%; (2) ROS1-rearranged cases are significantly associated with female gender and never smoker patients; (3) IHC correlates with FISH in ROS1-rearranged cases; (4) ROS1 CNAs are frequent, with a remarkably 33.9% of gains, 3.9% of amplifications, and 25.1% of deletions; (5) ROS1 CNAs had no impact on overall survival.

In previous studies, ROS1 rearrangements were detected in 0.8 to 2.5% of unselected NSCLC patients [12, 19]. Here we present a cohort of cases evaluated for ROS1 status using FISH that confirms the low frequency of ROS1 rearrangements in a Caucasian population. Similar to prior reports, we found an association of ROS1 rearrangement with non-smoking history [19, 20]. Moreover our study reports an association with female patients. The clinical profile of these patients is remarkably similar to that of ALK-rearranged, including features like gender and non-smoking history [13] and also advanced stage [21]. As recent publications demonstrate, the frequency of ROS1 rearrangements in clinically selected patients is higher, reaching values of 7.4% [18]. Giving that fact, a properly clinical selection can significantly enrich for the incidence of ROS1 rearrangements in the tested population.

ROS1 rearranged cases were detected also with ROS1 D4D6 IHC. In previous studies, FISH and IHC had a good correlation although optimal IHC criteria need to be established [9, 17]. As other authors mentioned, ROS1 is expressed in NSCLC without concomitant translocation [22] and also some cases with ROS1 rearrangements detected by FISH have low protein expression suggesting a low sensitivity of IHC [9, 23]. In our hands, IHC analysis optimally detects ROS1 positive cases although additional studies are needed to consider this method as a screening routine test.

Regarding FISH characterization of positive cases, we identify two known fusion partners: CD74 and SLC34A2. Clinically, the small numbers of cases preclude any correlation between the specific fusion partner and outcome [12, 24] as well as to date, no correlation has been observed between the type of ROS1 rearrangement and clinical response to Crizotinib [11]. Next generation sequencing (NGS) techniques could be a better approach to identify all ROS1 fusion genes, due to the high number of fusion partners described. Still, the main limitation of these techniques is to have sufficient material to be studied [25].

Regarding ROS1 CNAs, we found a high prevalence of ROS1 gene copy number gains without impact in overall survival. Jin et al. (2015) recently reported that ROS1 gains were significantly associated with both shorter disease-free survival and shorter overall survival [19]. It is remarkable that they only found 4.8% of cases with ROS1 gains whereas in our cohort the percentage of samples with gains/amplifications was significantly higher (37.8%). They studied unselected cohort of surgically resected NSCLC patients, and found that copy number gains are more common in male patients with squamous histology. Most of the patients included in our study had a stage III-IV (70%) with a low representation of squamous histology (3%). Differences in patient selection could explain discrepancies. In our series, amplifications were identified in only eleven specimens, all of them presenting signal clusters or chromosome 6 high polysomy in large cells. Remarkably, ROS1 protein expression was not detectable in any of these cases suggesting that amplification might not be a biologically relevant event or predict response to...
Figure 3: Intra-tumor heterogeneity regarding ROS1 status by FISH and IHC in one TMAs case. A. ROS1 FISH, 100x. Core 1: Negative ROS1 presenting cells with focal amplifications. B. ROS1 FISH, 100x. Core 2: Disomic ROS1 cells and nuclei with ROS1 gene gains without amplification. C. ROS1 IHC, 20x. Core 1: ROS1 +1/+2 predominantly acinar. D. ROS1 IHC, 20x. Core 2: ROS1 0 discohesive (sarcomatoid).

Figure 4: Kaplan-Meier univariate analysis of survival rates among patients with different ROS1 FISH results (rearranged, gains/amplifications, deletions and disomic cases) ($p = 0.087$).
ROS1 inhibitors [26].

Additionally, the current study demonstrates that ROS1 gene copy number is heterogeneous within surgical NSCLC specimens. This finding has potential diagnostic implications considering that in our routine clinical practice endoscopic, core-needle biopsies or cytology samples containing only a small part of tumor were used to make treatment decisions.

Interestingly, our study is the first to report a high prevalence of ROS1 deletions. Heterogeneity in the enumeration of chromosome 6 was observed, being 6q deletion or monosomy or both, the cause of ROS1 loss. Previous cytogenetic studies demonstrated that loss of 6q, where ROS1 gene is located, is one of the commonest chromosomal aberrations in lung ADC [27, 28]. So, as shown in our cohort, it is expected to observe ROS1-positive samples with concomitant deletion of ROS1 non-rearranged allele. Particularly, one of the ROS1 positive tumors exhibits two distinct areas, one with ROS1 gene deletion without rearrangement and the other with positive ROS1 10:1G pattern suggesting that even though ROS1 rearrangements are driver mutations, 6q deletion/monosomy could occur as a primary event in NSCLC. Opposed to the other ROS1 positive patients that elicited a partial response to Crizotinib, this patient did not respond, suggesting that heterogeneity of genetic alterations could explain differences in response to treatments.

Our study demonstrates a significant increased median overall survival time for ROS1-positive compared to ROS1-negative NSCLC patients (69.8 vs. 13.7 month) (p=0.028). This result is consistent with recently data reported by Scheffler et al. (2015): ROS1 seems to be one of the best prognostic molecular markers in NSCLC and stays in line of recent reports pointing the high efficacy of Crizotinib treatment for these patients [29, 30].

In conclusion, our study confirms the low prevalence of ROS1 rearrangements in Spanish NSCLC patients and shows a high prevalence of ROS1 CNAs that should be taken into account when assessed by FISH although do not present associated clinical implications. ROS1 break-apart FISH is herein validated as a reliable method to diagnose ROS1 rearrangements and also as a detailed method that helps us to understand the biology of NSCLC tumors more deeply.

MATERIAL AND METHODS

Study population

A total of 314 formalin-fixed and paraffin-embedded (FFPE) samples referred between 2012 and 2014 were available for this study. Material confirmed for NSCLC (surgical specimens, core-needle biopsies or cytological cell-blocks) was screened for ROS1 rearrangements using FISH in two Spanish centers: Hospital del Mar, Barcelona (n=247), and Hospital Germans Trias i Pujol, Badalona (n=67). ROS1 FISH was performed in sequence to EGFR, KRAS and ALK testing. This project was approved by the local ethics committee (CEIC-IMAS) and all patients provided written informed consent. Clinical data were extracted from medical records and included age, sex, smoking history, tumor disease stage and clinical follow-up information.

Tissue microarray construction

Based on tissue availability, 47 surgically resected tumors from our Institution were selected to construct three tissue microarrays (TMAs) [31]. Briefly, original hematoxylin and eosin-stained sections were reviewed from each patient to determine different malignant areas and to identify benign lung tissue. Three tissue cores were obtained from each patient, two of them containing different histological areas of the carcinoma (named A and B) and the third containing benign lung parenchyma. The cores then were brought into a new recipient and a paraffin block was constructed using a tissue micro arrayer (Beecher Instruments, Sun Prairie WI). To assess heterogeneity three other TMAs were constructed with different replicas of each tumoral area (named C and D).

ROS1 fluorescence in situ hybridization (FISH)

FISH was conducted on FFPE tissues from the whole tumor samples and the TMAs sections as previously described [32], using commercially break-apart ROS1 locus probes (Abbott Molecular, Des Plaines, IL and/or ZytoVision, Bremerhaven, Germany). A minimum of fifty non-overlapping cells with hybridization signals were examined for each case with a BX51 fluorescence microscope (Olympus, Tokyo, Japan). Samples were considered positive if more than 15% of cells showed split 5′ROS1 (Spec. Orange) and 3′ROS1 (Spec. Green) signals (typical rearranged pattern) or isolated 3′ROS1 signals (atypical rearranged pattern). The FISH rearrangement patterns are described in the text as the number of fusion signals (F) followed by the number of 5′ROS1 signals (O) and 3′ROS1 signals (G). ROS1 CNAs such as gains/amplifications and deletions were also recorded. Gain was defined as a mean copy number greater than 3 fused signals in >40% of nuclei, and amplification as the presence of >15 copies of ROS1 per cell (high polysomy) or clusters, in a minimum of 15% of analyzed cells. Cut-off for ROS1 deletion was assessed in benign lung parenchyma from TMAs cores and established at 1 copy in >30% of nuclei. Moreover, in order to determine the cause of ROS1 CNAs, TMAs and ROS1 amplified cases were
analyzed with CEP6 control probe (Spec. Aqua; Abbott Molecular, Des Plaines, IL).

Positive cases were screened for the detection of fusion partners using break-apart non-commercial probes selected from the Human 32K BAC Re-Array Library (BACPAC Resource Center, Oakland, CA). The following clones were selected: CD74 (5q32); RP11-725L10 and RP11-690J03; EZR (6q25.3): RP11-654E18 and RP11-268N15; SLC34A2 (4p15.2): RP11-659J14 and RP11-790A19; and SDC4 (20q12): RP11-69D17 and RP11-220B01.

**ROS1 immunohistochemistry (IHC)**

ROS1 IHC was performed using the commercially available clone D4D6 (Cell Signalling Technology, Danvers, MA) on unstained FFPE tissue sections from the whole tumor of ROS1 positive and the TMAs samples. The primary antibody was manually applied at 1:50 dilution and incubated at 37°C for 1 hour. Samples were then revealed using the FlexPlus DAB Detection Kit in a Dako Autostainer Plus (Dako, Carpinteria, CA). ROS1 IHC staining was evaluated by two independent pathologists. Staining was graded semiquantitatively as follows: 0 for absent expression or nuclear expression only, 1+ for cytoplasmic faint, barely perceptible staining not exceeding background in any percentage of cells, 2+ for cytoplasmic staining exceeding background in 0 to 50% of tumor cells, and 3+ for cytoplasmic staining exceeding background in >50% of tumor cells [33]. Moreover, H-score was recorded as initially described to evaluate EGFR expression [34]. Briefly, this score ranges from 0 to 400 that result from the combination of the staining intensity (0-4) and the percentage of positive tumor cells (0-100%) in each sample.

**Statistical analysis**

The whole series of cases were studied in order to characterize associations between clinicopathological variables and FISH data (ROS1 rearrangement and ROS1 CNAs). From TMAs cases, the core with the most prevalent CNA was selected. Statistical associations were assessed using Pearson’s χ2 test or Fisher’s exact test, depending on the sample size. All statistical tests were conducted at the two-sided 0.05 alpha level of significance. Survival curves were obtained with the Kaplan-Meier method, and log-rank test was used for comparison of survival curves between different groups of patients. For each patient, cumulative survival was calculated from the date of diagnosis to death (from any cause) or last follow-up. Heterogeneity of FISH and IHC data of TMAs was assessed using Kappa agreement index for categorical variables. All statistical analyses were carried out with SPSS version 15 (SPSS Inc., Chicago, IL).

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**CONFLICT OF INTERESTS**

There is no conflict of interest.

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