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Clinical significance of ROS1 5' deletions in non-small cell lung cancer

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ABSTRACT

Objectives: Patients harboring rearrangements of the ROS1 gene are eligible for first-line therapy with Crizotinib, which represents the best available treatment option. Diagnostic criteria, based on break-apart fluorescence in situ hybridization, were mirrored from ALK by analogy and include tumors with 5' deletions. However, the probability of response to Crizotinib in patients with 5' deletion in ROS1 is unknown given the rarity of this condition.

Materials and methods: We hereby describe clinical outcome of 8 NSCLC patients harboring a 5' deletion at FISH treated with Crizotinib

Results: Three out of 4 cases whose 5' deletion was confirmed by NGS as a ROS1/EZR fusion displayed an objective response to Crizotinib while a case with ROS1/SDC4 fusion did not. By contrast, among the 4 cases where NGS did not detect ROS1 gene fusions only 2 patients responded to crizotinib therapy with one also harboring a concomitant EML4-ALK rearrangement.

Conclusion: 5' ROS1 deletions detected by FISH are associated with a high chance of response to Crizotinib in NSCLC, similarly to canonical ROS1 split-apart FISH rearrangements. However, the confirmation of the ROS1 gene fusion with at least another method, such as NGS, seems beneficial in order to define the ROS1 fusion partner and to avoid possible false positive results.

1. Introduction

The *ROS1* gene is located on chromosome 6q22 and belongs to the insulin receptor family controlling proliferation through various cell pathways including STAT3 and PI3K/AKT/mTor. [1] Approximately 1–3% of non-small cell lung cancer (NSCLC) harbor ROS1 gene fusions, mainly with the *CD74*, *EZR* and the *SCD4* genes, that are oncogenic. [1,2] Given the high sequence homology between *ALK* and *ROS1* genes, inhibitors of the tyrosine kinase (TKI) catalytic domain of ALK can be utilized successfully also in tumors harboring *ROS1* fusions. Therefore the use of Crizotinib has been approved for first-line therapy of NSCLC patients with confirmed *ROS1* rearrangement. [3] Several methods including next-generation sequencing (NGS) can be utilized to assess *ROS1* fusions. [4] Break-apart fluorescent in situ hybridization (FISH) still represents the gold standard method for the detection of *ROS1* rearrangements on histological and cytological material. [5] This test is based on two probes specific for the 6q22 region. [6] In case of translocation between *ROS1* and a partner gene, the FISH will show at least one split and one merged signal. Conversely, in case of 5' deletion at the 6q22 region the FISH will reveal at least one merged signal and one single signal referred to the 3' residual probe. Both the latter conditions are generally diagnosed as positive for gene fusion and lead to therapy with a TKI. NGS technologies can detect *ROS1* fusions using imbalance assays after retro-transcription of fusion RNA transcript to cDNA and can provide information regarding the partner of the fusion. [7]

Objective tumor response to TKI in case of 5' deletion at FISH has been reported for *ALK*-positive patients but not for *ROS1* so far. Therefore, we have investigated the clinical and molecular significance of *ROS1* 5' deletions in a consecutive series of patients with advanced NSCLC submitted to Crizotinib treatment

2. Patients' population

We retrospectively studied 8 patients with a diagnosis of advanced NSCLC with positive FISH result of 5' *ROS1* deletion at the Sant'Orsola University Hospital of Bologna. The 8 patients were selected from a

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pool of 485 consecutive patients with advanced NSCLC, and from the total number of 18 patients with *ROS1* rearrangements who presented ad our Institution between 2016 and 2018. Eligibility criteria were: *i*) diagnosis of 5' *ROS1* deletion by FISH; *ii*) availability in the samples of 50 ng of RNA with at least 50% tumor cell enrichment; *iii*) treatment with Crizotnib for at least 4 weeks following the diagnosis of *ROS1* fusion; *iv*) availability of clinical and radiological response data after therapy with TKI;

All cases were diagnosed as NSCLC with adenocarcinoma histotype. Crizotinib was administered to all patients at the dose of 250 mg BID as per clinical practice [8]. One patient received Crizotinib as first line of therapy (case #2) while the others received it as second line treatment. All patients signed an informed consent to therapy and privacy management. Radiological response to Crizotinib was assessed using the RECIST criteria after comparison of the CT scans before therapy and after at least one month of treatment.

3. Methods

Samples consisted of small formalin-fixed and paraffin-embedded biopsies in 5 cases and cytological smears obtained during bronchoscopy in 3 cases.

FISH assay was performed using the Zytolight SPEC ROS1 Dual Color Break Apart Probe (ZytoVision, Germany). This break-apart FISH test is based on a mixture of two probes hybridizing to the proximal (3', green-labeled probe) and distal (5', orange-labeled probe) to the ROS1 breakpoint cluster region. At least 50 non-overlapping tumor nuclei were scored for each specimen by a trained technologist and a pathologist. Cells positive for rearrangement were defined by two main patterns: i) a "split pattern", with 3' and 5' break apart signals at a distance of two times the diameter of the largest signal; ii) a "5' deletion pattern", showing one fusion signal and an isolated 3'green signal (without the corresponding 5' orange signal). A case was considered FISH positive for ROS1 rearrangements when at least 15% of tumor cells showed any split or any 5' deletion pattern.

NGS was performed starting from 10 μm-thick serial sections or cytological smears including the tumor area of interest with at least 50% tumor cell enrichment. Slides were manually macrodissected, and the RNA was isolated. Library were prepared using the Oncomine™ Focus Assay, 318 Solution (Thermo Fisher Scientific) using a total of 10 ng input RNA per sample. The RNA panel can identify rearrangements in 23 genes including ROS1. Sequencing was performed using the Ion PGM™ Hi-Q™ View Sequencing Kit on the Ion Torrent Personal Genome Machine (Thermo Fisher Scientific). Fusions were detected using the fusion detection module within the Ion Reporter workflow, in particular 20,000 was the minimum number of total valid mapped reads required to qualify a sample as valid and to proceed with the analysis.

4. Results

The mean age of the 8 patients was 56,5 years (range 46–67), 5 were males and 3 females. At diagnosis, none of the patients presented with brain metastasis. 2 were in stage IIIB, 5 in stage IVA for bilateral lung nodules and pleural effusion, and one in stage IVB with multiple liver lesions. 5 patients were never-smokers, two light former smokers, one patient (case # 1) current heavy smoker (50 pack/year).

Both FISH and NGS analyses were successfully carried out in all patients. Table 1 summarizes the clinical and the laboratory findings in the eight patients. In particular, the break-apart FISH test revealed a 5' *ROS1* deletion in all the 8 cases, characterized by the presence of isolated green signals indicating the 3'probe (Fig. 1). The percentage of rearranged nuclei ranged from 30 to 60%. Concomitant "split" signals were encountered sporadically and never exceeded the 15% of cancer nuclei. In 4 of 8 cases (cases # 2,3,7,8) the NGS analysis confirmed a *ROS1* fusion; in three cases with the partner *EZR* and in one with *SDC4*

Table 1
Clinical-biological patients' characteristics.

Patient	Age	Smoking habits	Stage at diagnosis	FISH		NGS		Outcome					
				break-apart status	% of rearranged nuclei	Presence of gene fusion	ROS1 partner gene	Line of crizotinib administration	Response to crizotinib	Status D = dead A = Alive	Survival after crizotinib	Still on treatment	Time on treatment
1	52	Current	IVA	5' deletion	41%	NO		Second	NO (PD)	D	4.5	NO	3.5
2	67	Never	IIIB	5' deletion	60%	YES	EZR	First	YES (CR)	A	31.1	YES	31.1
3	60	Never	IVA	5' deletion	52%	YES	EZR	Second	YES (CR)	A	19.8	YES	19.8
4	54	Never	IVA	5' deletion	44%	NO *		Second	YES (PR)	A	15.7	YES	15.7
5	58	Former	IVA	5' deletion	32%	NO *		Second	YES (PR)	A	16.7	YES	16.7
6	53	Never	IVA	5' deletion	30%	NO		Second	NO (PD)	D	2.1	NO	0.7
7	62	Former	IVA	5' deletion	48%	YES	EZR	Second	YES (PR)	A	6.4	YES	6.4
8	46	Never	IVB	5' deletion	56%	YES	SDC	Second	NO (PD)	A	2.1	NO	2.1

CR: Complete Response; PR: Partial Response; PD Progressive Disease.
* ALK rearrangement detected.

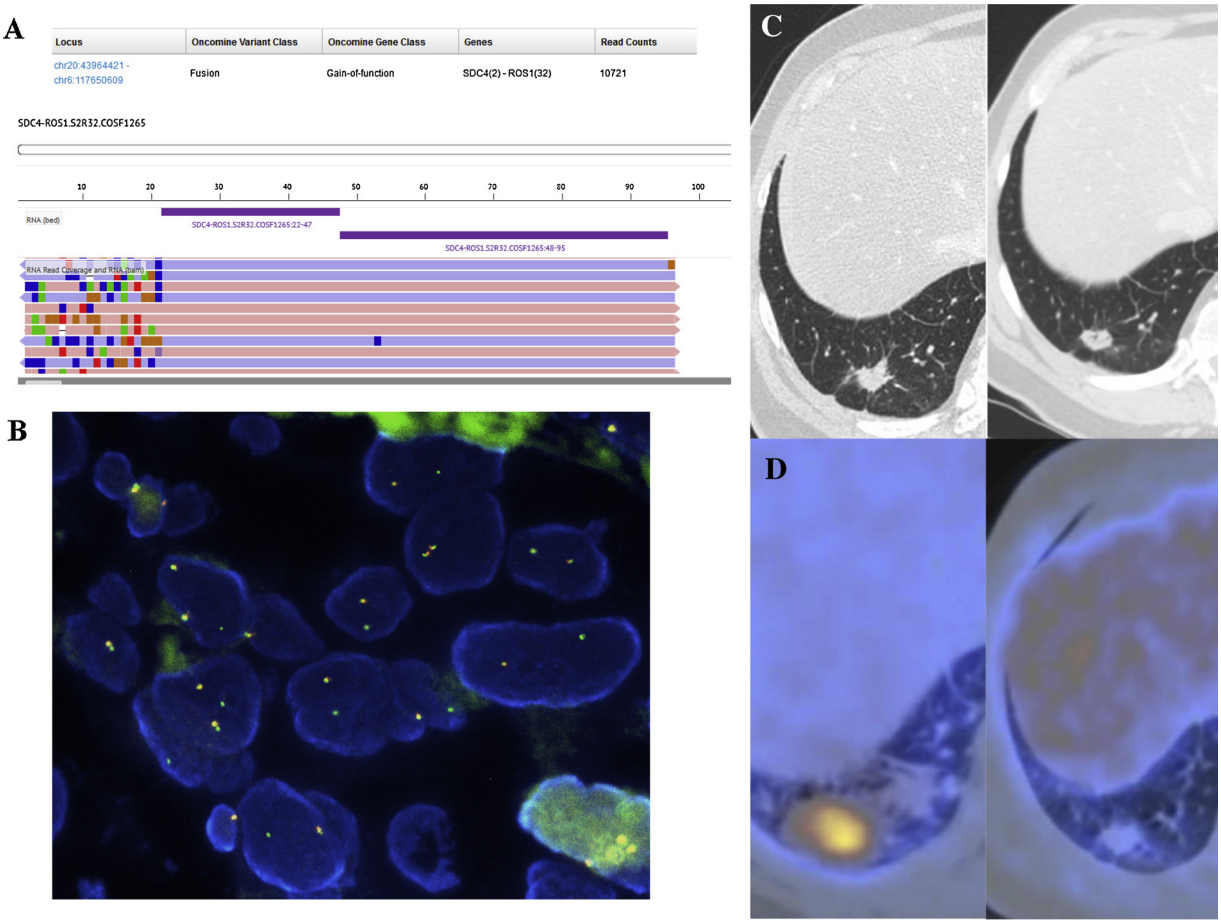

Fig. 1. NGS and FISH results and response to therapy related to case #8: A) NGS analysis report describing the fusion of *ROS1* with *SDC4* (Ion Reporter software and IGV visualization); B) FISH image showing the 5' *ROS1* deletion pattern (DAPI 100X); C) CT and (D) PET scan before (first column) and after (second column) therapy with Crizotinib.

(case #8). In the other four patients (cases # 1,4,5,6) the NGS analysis failed to detect *ROS1* fusions (Fig. 2). One case (#5) showed a concomitant *EML4-ALK* rearrangement that was confirmed by FISH (data not shown).

Therapy with Crizotinib was started in all patients and lasted for a mean of 11.0 months (range 2–31). The median overall survival was not reached at a median follow up of 11.1 months (15.7 months censored only). Objective responses were observed in 5 of 8 patients, two with complete response, while the remaining three faced rapid disease progression (Fig. 1). All the patients with confirmed *ROS1/EZR* rearrangement at NGS displayed an objective response to Crizotinib and are still alive at the time of last available follow-up. The three patients who experienced rapid progressive diseases were either negative for *ROS1* fusions (#1 and 6) or displayed a fusion with a gene different from EZR at NGS (#8). Two patients (#4 and 5) showed an objective response to Crizotinib despite absent detection of *ROS1* fusions at NGS but one of them (#5) harbored a concomitant *ALK* fusion.

5. Discussion

Consistently with previous reports, our NSCLC *ROS1*-positive patients were young and almost never/light smokers. All except one had no extra-thoracic disease and none had brain metastasis at diagnosis. The criteria for treating patients with *ROS1* rearrangement with Crizotinib were mirrored from *ALK* by analogy. Therefore, patients with either split signals or 5' deletions at FISH were considered candidate for Crizotinib. [9] To our knowledge, this is the first report on the efficacy of Crizotinib in a subset of patients harboring the 5' deletion FISH pattern. Our results indicate that half of the patients with *ROS1* 5' deletion at FISH also harbored a *ROS1* gene-fusion at NGS and three of them were high responders to Crizotinib treatment, similarly to those with canonical *ROS1* split rearrangements. Conversely, in patients whose 5' deletion FISH pattern was not associated with detection of a gene-fusion at NGS the response to Crizotinib was lower. In addition, two patients without evidence of *ROS1* fusion and one with detected fusion at NGS did not respond at all to Crizotinib.

These findings suggest that 5' deletion might not always represent a biologically relevant molecular event since most of the ROS1 rearrangements confirmed by sequencing analysis as true fusions lead to effective *ROS1* TKI targeting. In fact, the detection of single 3'FISH signals does not necessarily mean that such deletion is associated with *ROS1* protein overexpression. Two patients with 5' deletion at FISH and no fusion at NGS actually responded to Crizotinib. In one case, the possible explanation is the concomitant ALK fusion that was confirmed by FISH (with a typical split break-apart pattern) and NGS (EML4-ALK fusion). In the other case, no other genetic alterations were detected, at least with our 50-gene panel. The occurrence of a rare *ROS1* fusion with a gene not included in our NGS gene panel represents a possible explanation of the response. In fact, the NGS fusion panel used in this study covers the most frequent fusion partners of *ROS1* such as CD74, SDC4 and EZR). A fusion of ROS1 with a different gene would not have been detected by our NGS panel but would have caused a deletion visible at FISH with single 3' signals. The single case harboring *ROS1* fusion with *SDC4* was the only confirmed by NGS who did not respond to Crizotinib. Fusion of *ROS1* with *SDC4* is less common than the fusion with *EZR* but even this rearrangement is considered oncogenic. [10]

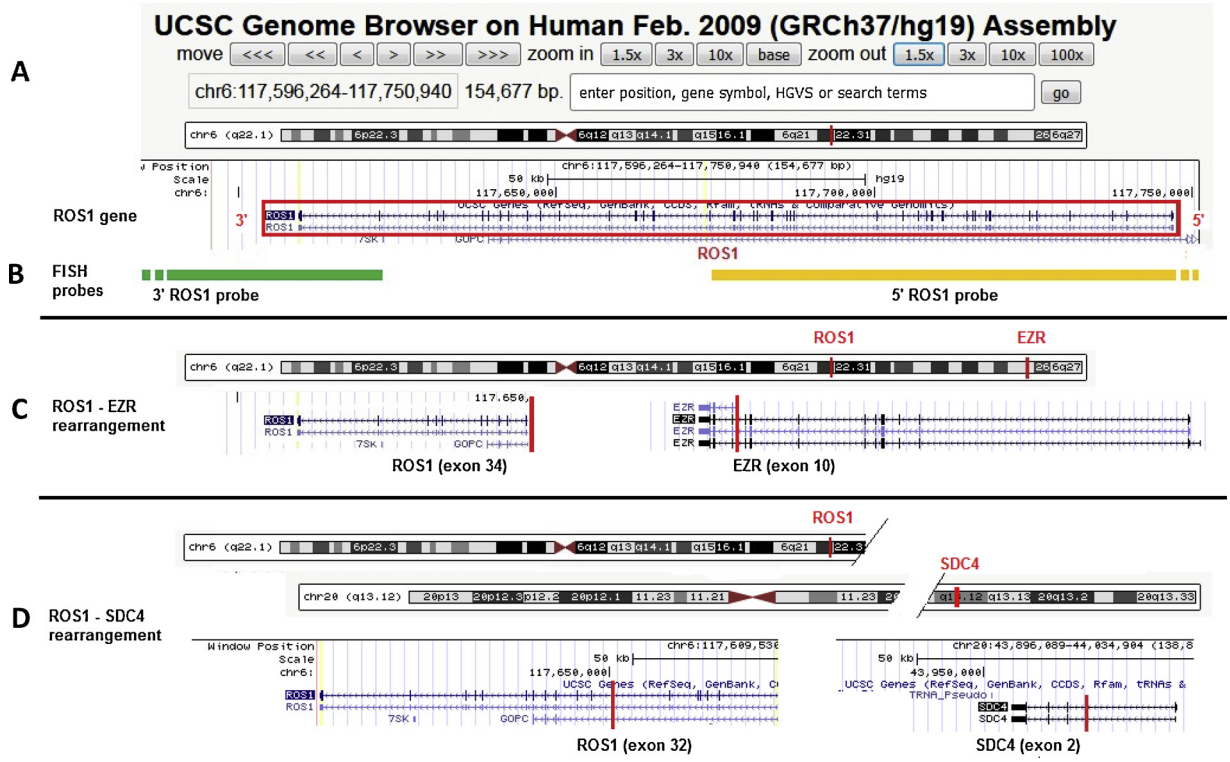

Fig. 2. Visual comparison of the full-length *ROS1* gene (A); the hybridization regions of the 3' (orange) and 5' (green) break-apart probes (B); and the sites of rearrangement between ROS1 and EZR (C) or ROS1 and SDC4 (D). Data are based on the UCSC Genome Browser on Human February 2009 (GRCh37/hg19) Assembly (http://genome.uscs.edu) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Further studies on larger series are required to assess whether this fusion confers less sensitivity to Crizotinib or not.

A major limitation of the present study is the lack of confirmation of ROS1 protein overexpression by immunohistochemistry. Unfortunately, three of our cases were cytological smears unsuitable for ROS1 immunohistochemistry and in other three cases, the histological material was exhausted, preventing to run immunohistochemistry. The remaining two cases came as consults to our laboratory and had had immunohistochemistry done in the original institution with positive results. One of the two revealed the fusion at NGS but the other did not. Therefore, our study is not informative on the predictive role of ROS1 protein expression in tumors with 5' deletions at FISH.

We provide confirmation that patients with 5' *ROS1* deletions might respond to anti-ALK/ROS1 therapy by analogy with *ALK*. However, response to Crizotininb in this subset of patients is variable. Before starting treatment, we recommend a confirmation of the rearrangement with at least another method. NGS is the most comprehensive and efficient method to detect fusions in archival tumor tissue specimens and the use of NGS cancer panels is becoming the technology of choice for molecular predictive characterization of NSCLC.

Declaration of Competing Interest

None.

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