Rajiv Gandhi Cancer Institute and Research Centre

A Unit of Indraprastha Cancer Society
Registered under "Societies Registration Act 1860"
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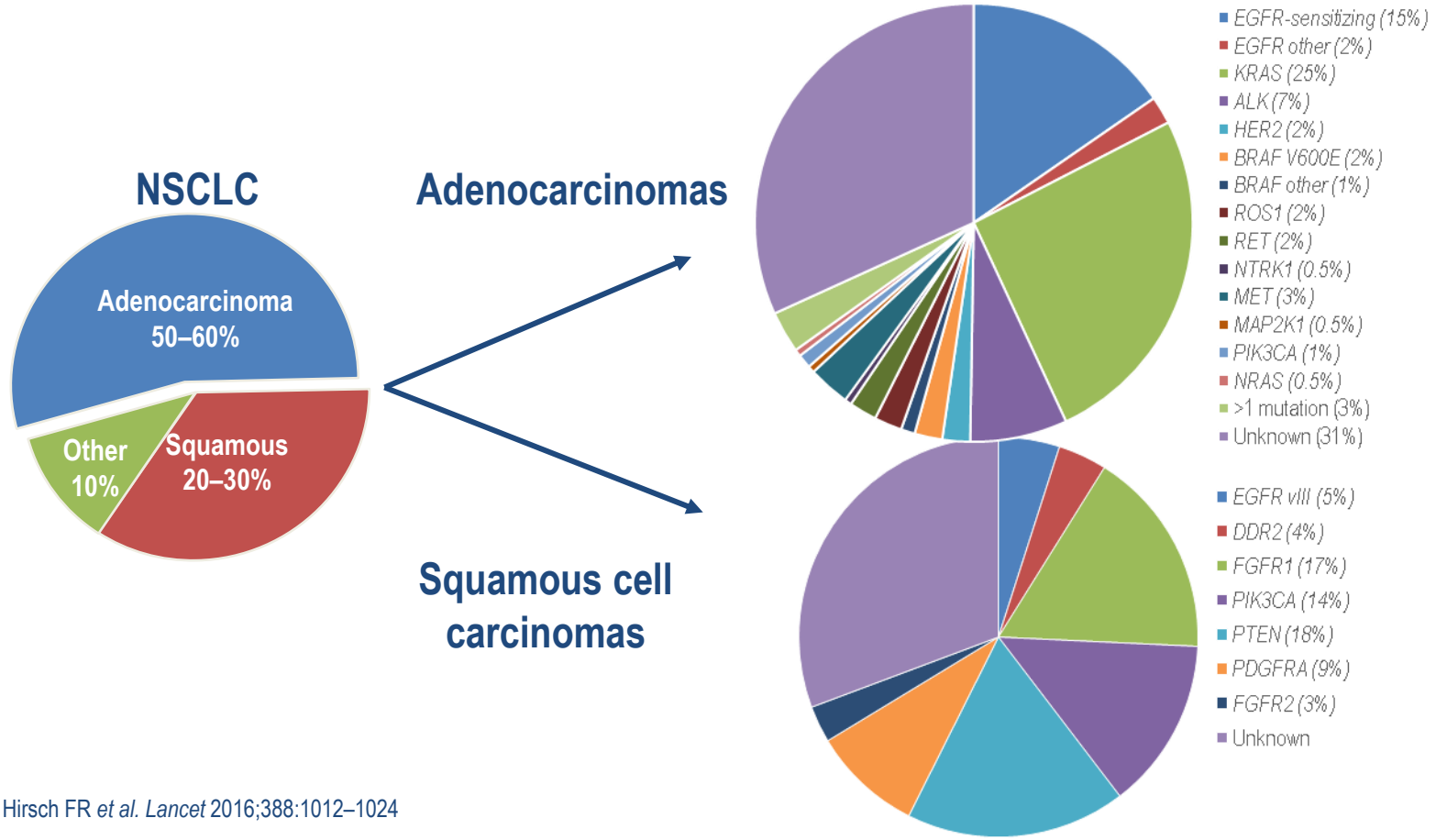
(M.D. Pathology; **Fellowship** Oncopathology**)**

Interpretation of ROS 1 Rearrangement in NSCLC : IHC , FISH, NGS

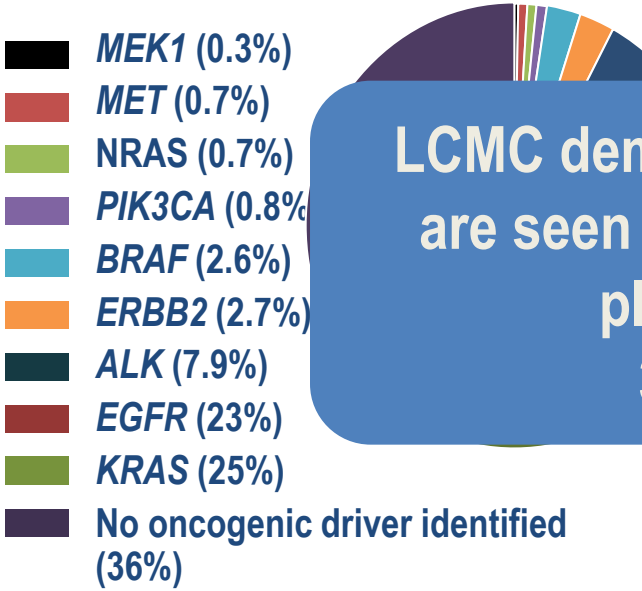
DRIVER MUTATIONS IN NSCLC



Hirsch FR *et al. Lancet* 2016;388:1012–1024

SURVIVAL OF PATIENTS WITH DRIVERS IN LUNG CANCER MUTATIONAL CONSORTIUM: TARGETED VS NO TARGETED THERAPY

Analyzed 733 patients for 10 genes (Full genotyping)



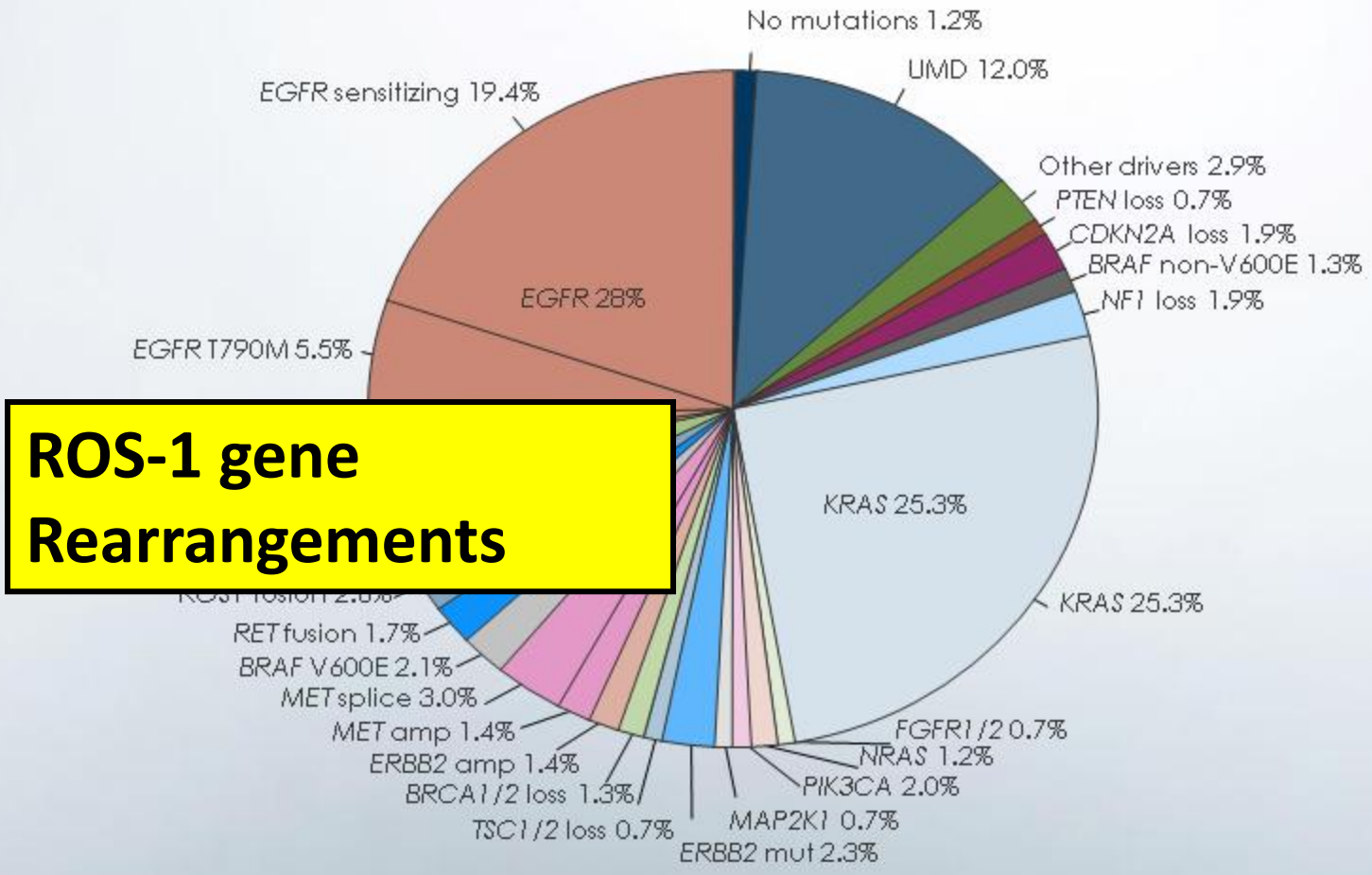
Survival probability in patients with/without targeted therapy[2]

LCMC demonstrates that the best outcomes are seen in patients with identified drivers placed on targeted therapy: 3.5-year median survival

1. Sholl L *et al. J Thorac Oncol* 2015;10:768–777
2. Kris MG *et al. JAMA* 2014;311:1998–2006

LCMC, Lung Cancer Mutational Consortium

Potential application in uncommon mutation: Beyond EGFR and ALK



ROS-1 gene Rearrangements

Jordan EJ, et al. Cancer Discov. 2017;7:596-609.

ROS-1

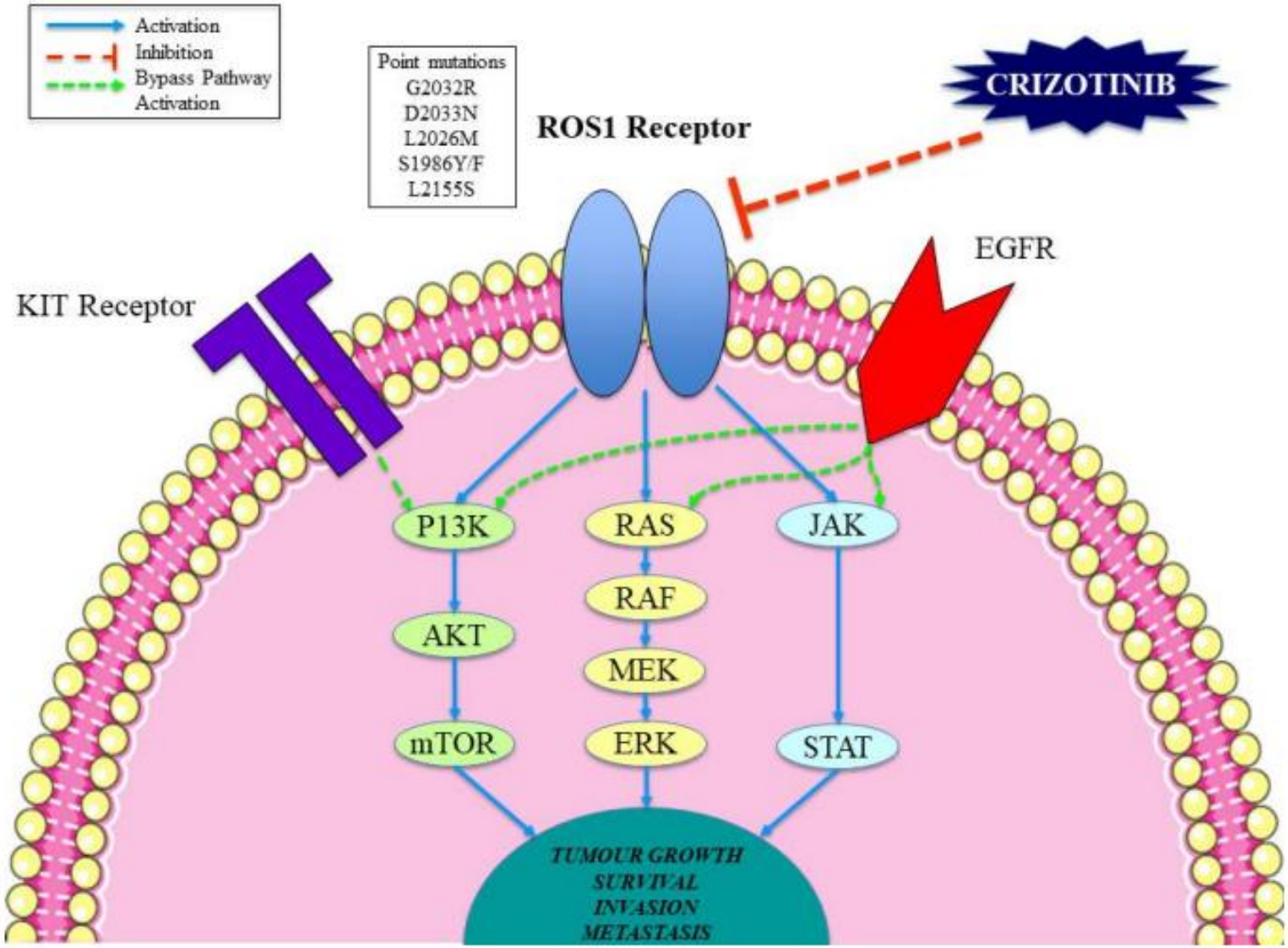
- The proto-oncogene role of ROS-1 was first identified in brain tumors two decades back.

- **Role in lung cancer** was first reported in 2007 by Rikova et al, who identified two other protein fusion transcripts: CD74 and SLC34A2

- Improved sequencing techniques have enabled the discovery of increasing numbers of fusion partners

Main ROS-1-fusion partners in ROS-1-positive NSCLC

Gene	Description	Frequency
CD74	Cluster of differentiation 74 (several subtypes: C6R34, C6R32 C7R32, C3R34)	38–54%
EZR	Ezrin	13–24%
SDC4	Syndecan 4	9–13%
SLC34A2	Solute carrier family-34 member-2 gene	5–10%
TPM3	Tropomyosin-3 gene	3–15%
FIG or GOPC	Fused in glioblastoma (associated with cancers other than NSCLC) or golgi-associated PDZ and coiled-coil motif-containing	2–3%
ADGRG6	Adhesion G protein-coupled receptor G6	1%
ANKS1B	Ankyrin repeat and sterile alpha motif domain containing 1B	1%
CCDC6 or CCKC6	Coiled-coil domain containing 6	1%
CEP72	Centrosomic protein 72	1%
CLTC	Clathrin heavy chain	1%
FAM135B	Family with sequence similarity 135 member B	1%
FBXF17	F-box and leucine-rich repeat protein 17	1%
FRK	Src family tyrosine kinase	1%
KDELR2, ELP-1 or ERD2.2	Endoplasmic reticulum protein retention receptor 2	1%
SKT	Human homologue of murine *Skt* (Sickle tail)	1%
LIMA (or EPLIN)	LIM (Lotus-Intel-Microsoft) domain and actin-binding 1	1%
LRIG3	Leucine-rich repeats and immunoglobulin-like domain 3	1%
MLL3	Mixed lineage leukemia	1%
MPRIP	Myosin phosphatase Rho-interacting protein	1%
MSN	Moesin	1%
MYH9	Myosin, heavy polypeptide 9, non-muscle	1%
MYOC 5	Myosin-gene family myosin VC	1%
RBPMS	RNA-binding protein with multiple splicing	1%
SLC2A4RG	solute carrier family-2 member-4	1%
SLC6A17	Solute carrier family-6 member-17	1%
SLMAP	Sarcolemma-associated protein	1%
SNN	Stannin	1%
SQSTM1	Sequestosome 1	1%
TDP52L1	Tumor protein D52-like 1	1%
TMEM106B	Transmembrane protein 106B	1%
TRG or TFG	TRK (transketolase-related gene)-fused gene	1%
WNK1	Lysine deficient protein kinase 1	1%
ZZCCHC8 or ZCCH	Zinc finger CCHC-type containing 8	1%


Activation
Inhibition
Bypass Pathway Activation
Point mutations
G2032R
D2033N
L2026M
S1986Y/F
L2155S
ROS1 Receptor
CRIZOTINIB
EGFR
KIT Receptor
P13K
RAS
JAK
RAF
AKT
MEK
mTOR
ERK
STAT
TUMOUR GROWTH
SURVIVAL
INVASION
METASTASIS

ROS-1 rearrangements are frequently associated with:

- Young patients
- Women and never smokers
- Predominantly lipedic, acinar, or solid adenocarcinomas (TTF-1 positive)
- Advanced stage (stage III–IV)
- Higher frequency of brain metastases

Incidence and Implications

- ROS1 gene rearrangements occur in **1 to 2.6 %** of NSCLC
- The effectiveness of targeted therapies with TKI in NSCLC depends on the **accurate determination of the genomic status of the tumor**
- **Detecting ROS1 gene rearrangements** offers patients the opportunity to receive highly effective targeted therapies

Lung Cancer: Targets and Therapy

Dovepress

open access to scientific and medical research

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ORIGINAL RESEARCH

Incidence of *ROS1*-Rearranged Non-Small-Cell Lung Carcinoma in India and Efficacy of Crizotinib in Lung Adenocarcinoma Patients

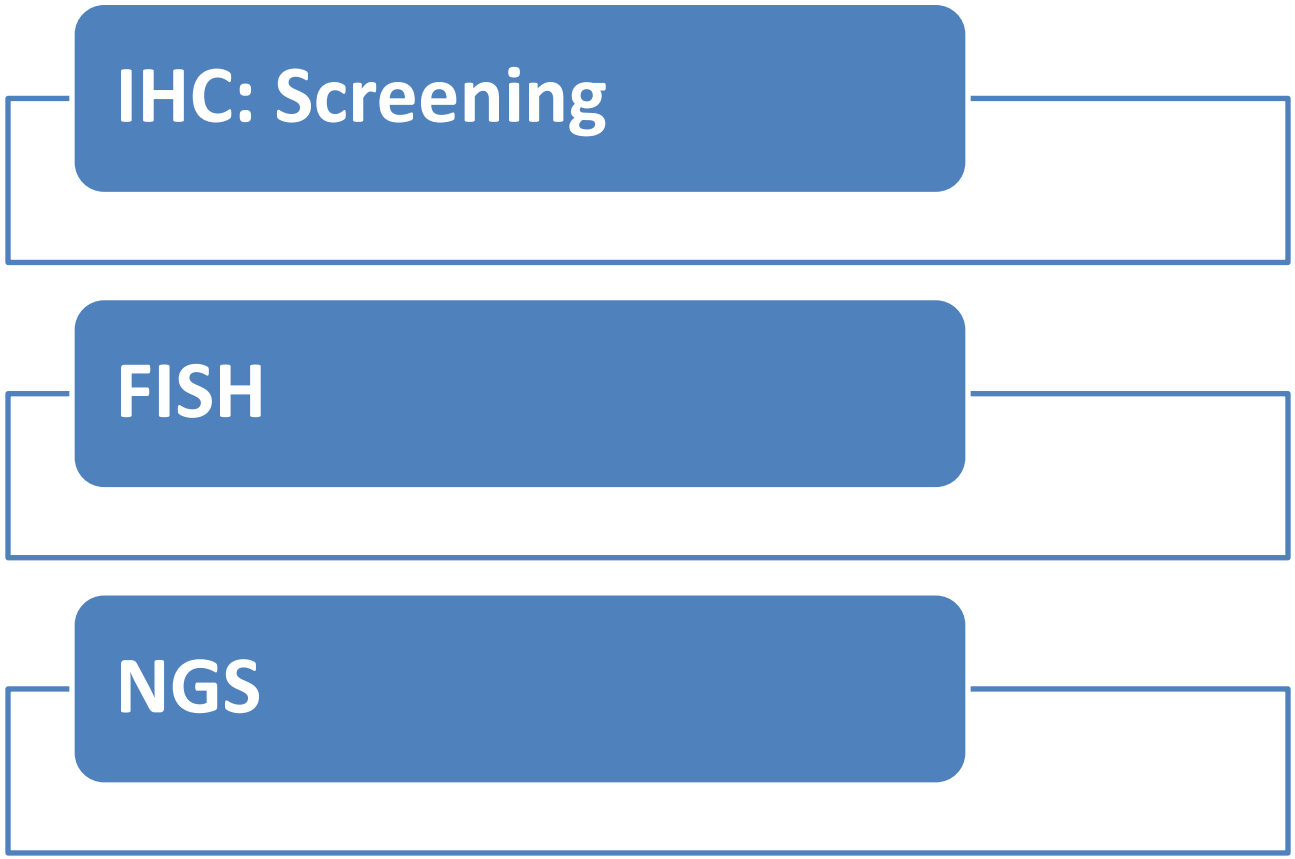
This article was published in the following Dove Press journal:
Lung Cancer: Targets and Therapy

A Mehta et al Lung Cancer: Targets and Therapy 2020:11 19–25

A total of 709 stage IV NSCLC adenocarcinoma patients were included

ROS1-gene rearrangement was present at a relatively higher frequency of **2.8%** (20/709) in north Indian patients

Testing Modalities for ROS-1



IHC

- **Clone D4D6**

  (Cell Signaling Technology, Danvers, MA, USA)

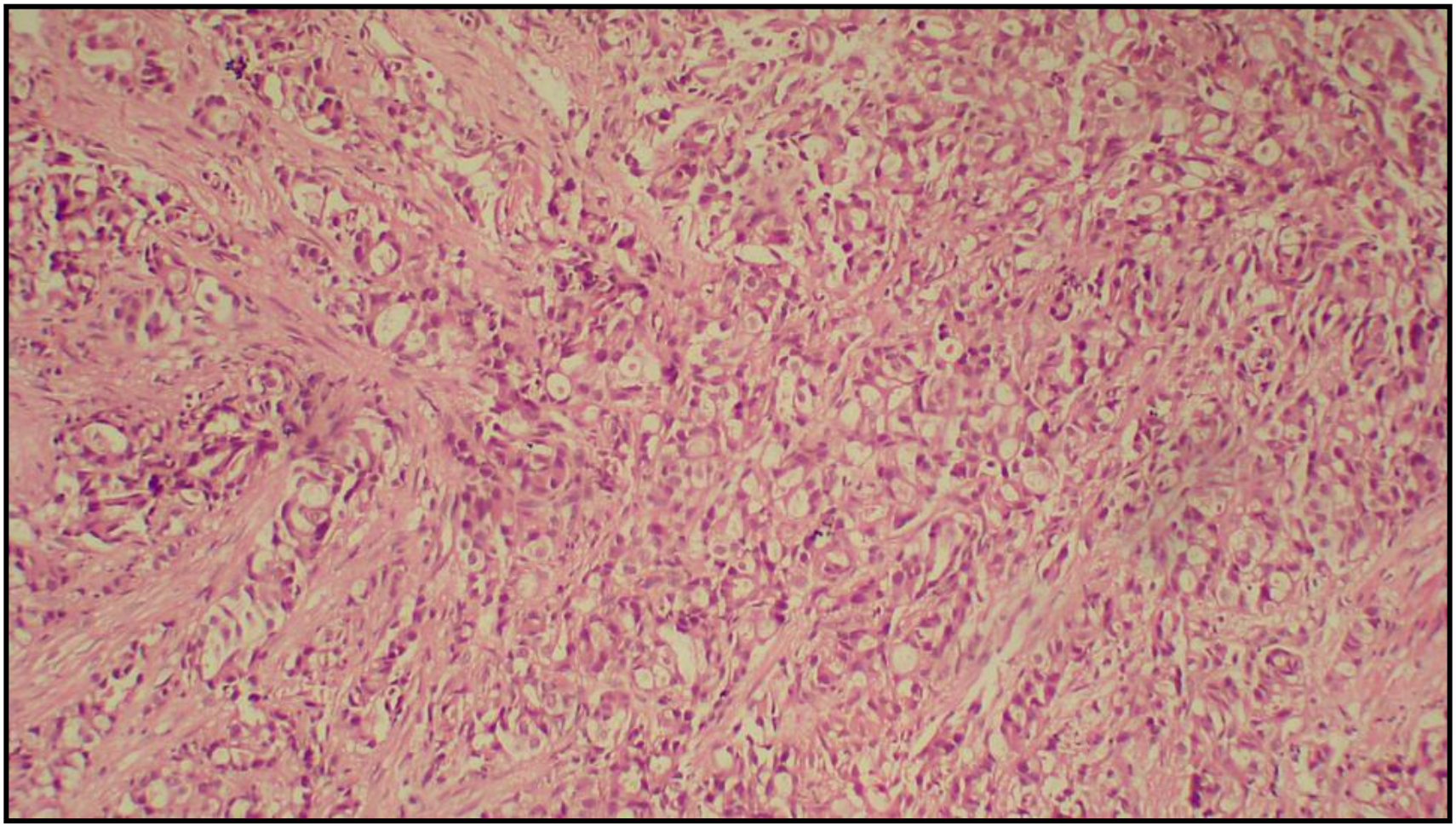
- **Clone SP384**

  (Roche, Ventana, AZ, USA);

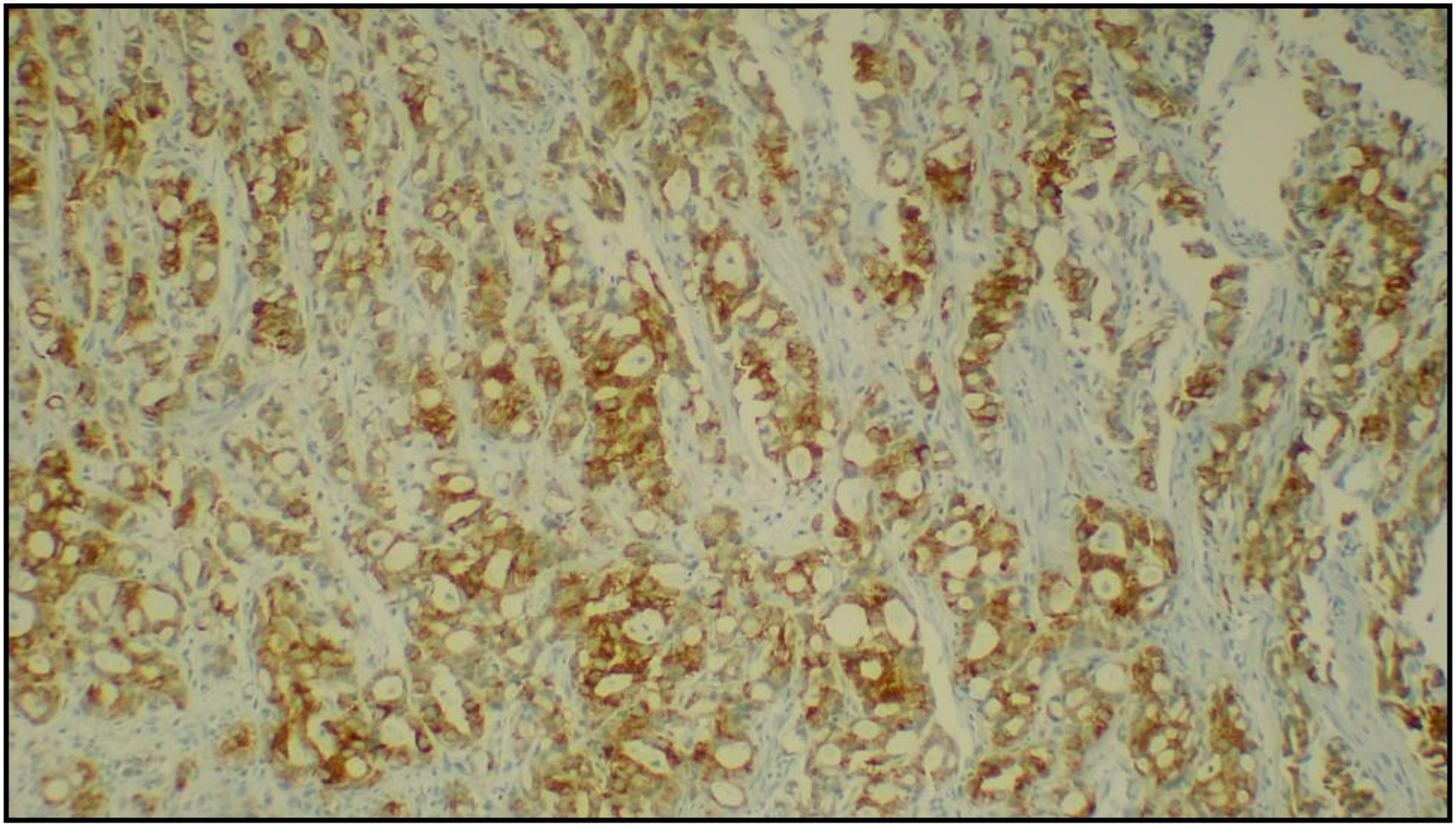
IHC

- Guidelines recommend the use of **IHC as a screening method**
- Clones have high sensitivity (90–100%), compared to FISH and NGS
- ROS-1 specificity is variable, ranging from 70% to 90%, and depends on the clone used and the positivity threshold applied
- All positive IHC cases **must be confirmed with Orthogonal test (FISH/NGS)** before starting on targeted therapy.

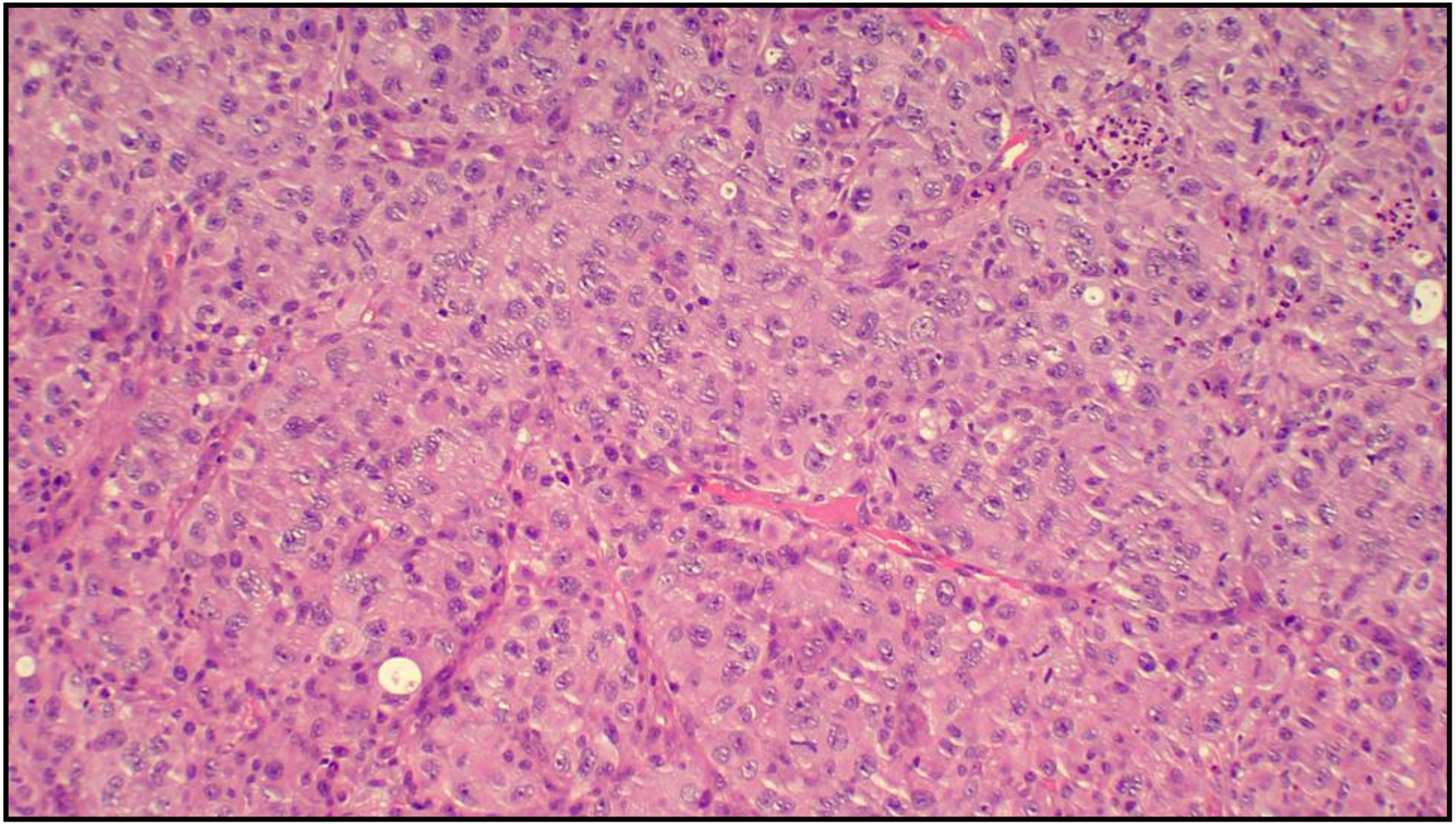
Case 1: NSCLC-ADC with acinar pattern of growth



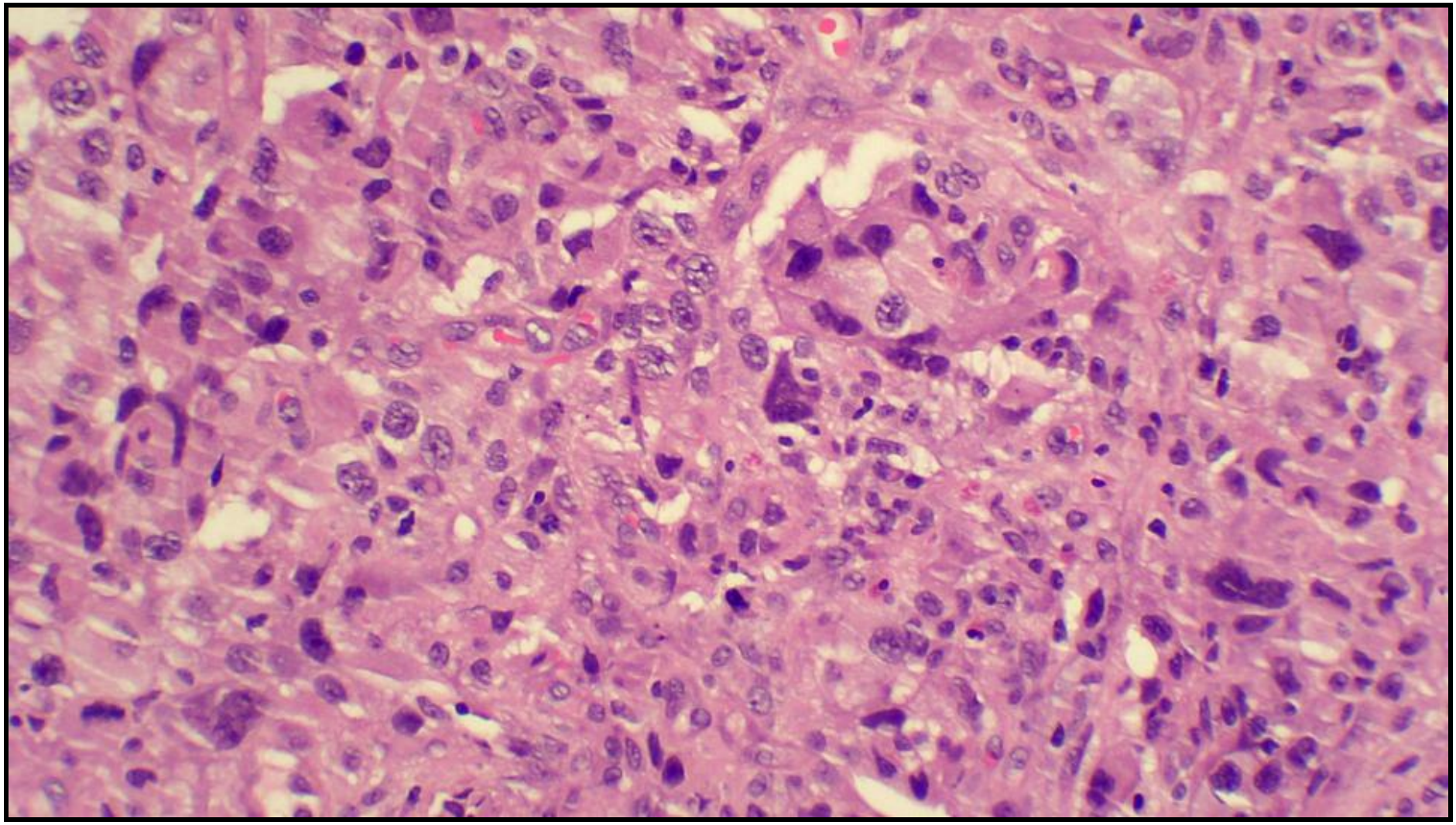
ROS-1: Strong and Diffuse expression



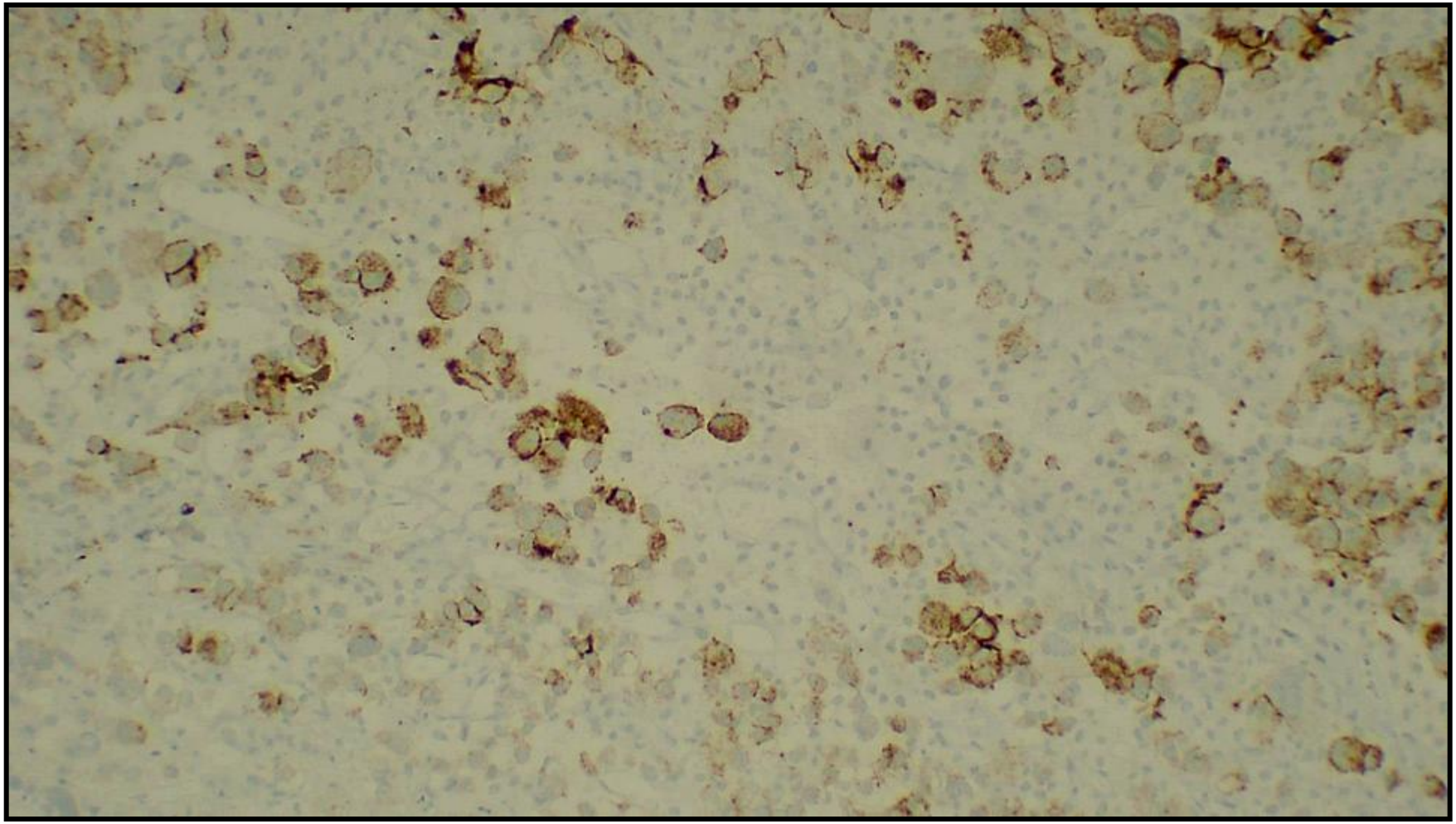
Case 2: NSCLC-ADC with solid pattern of growth



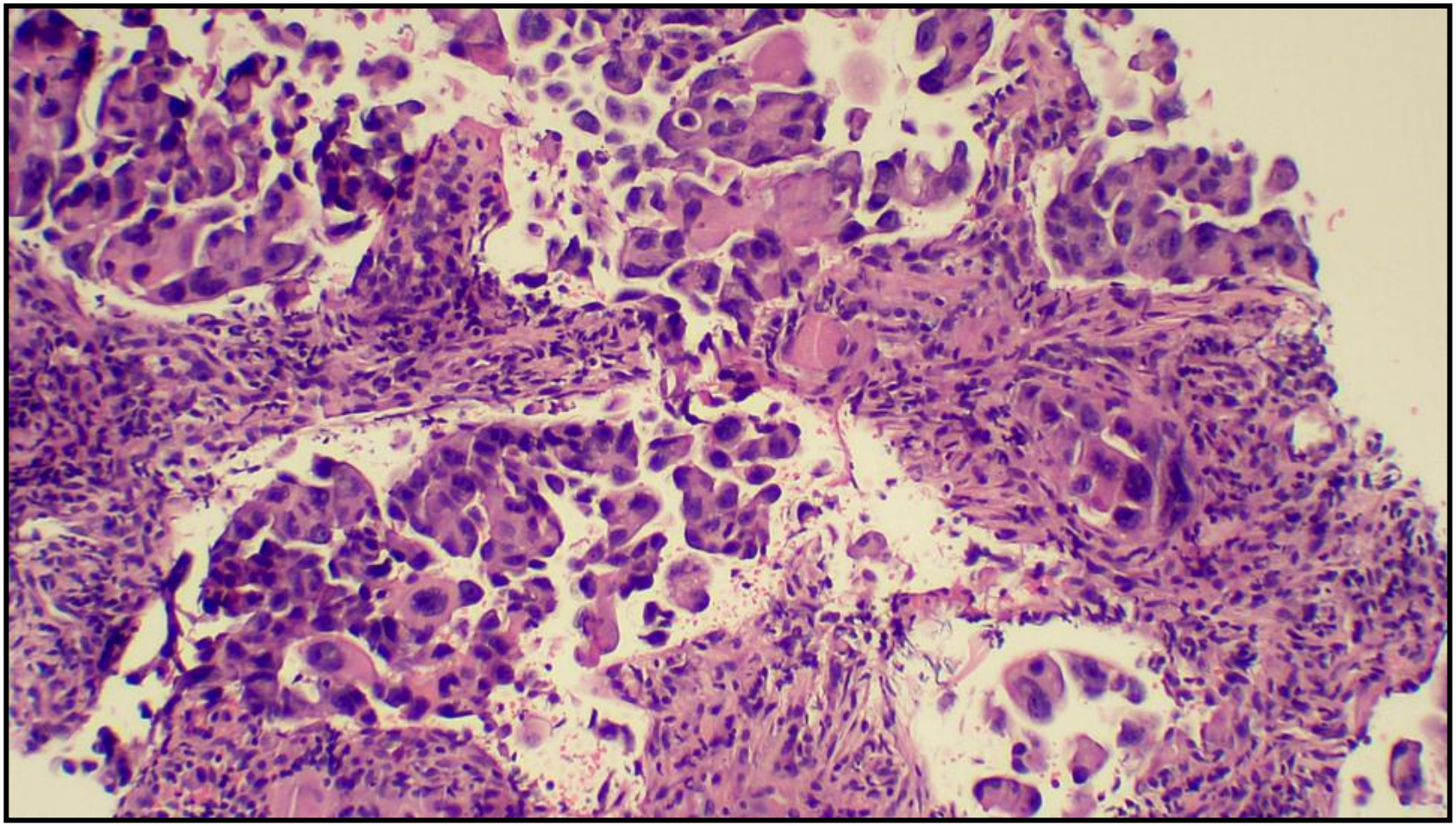
Extreme Pleomorphism and Bizzare cells



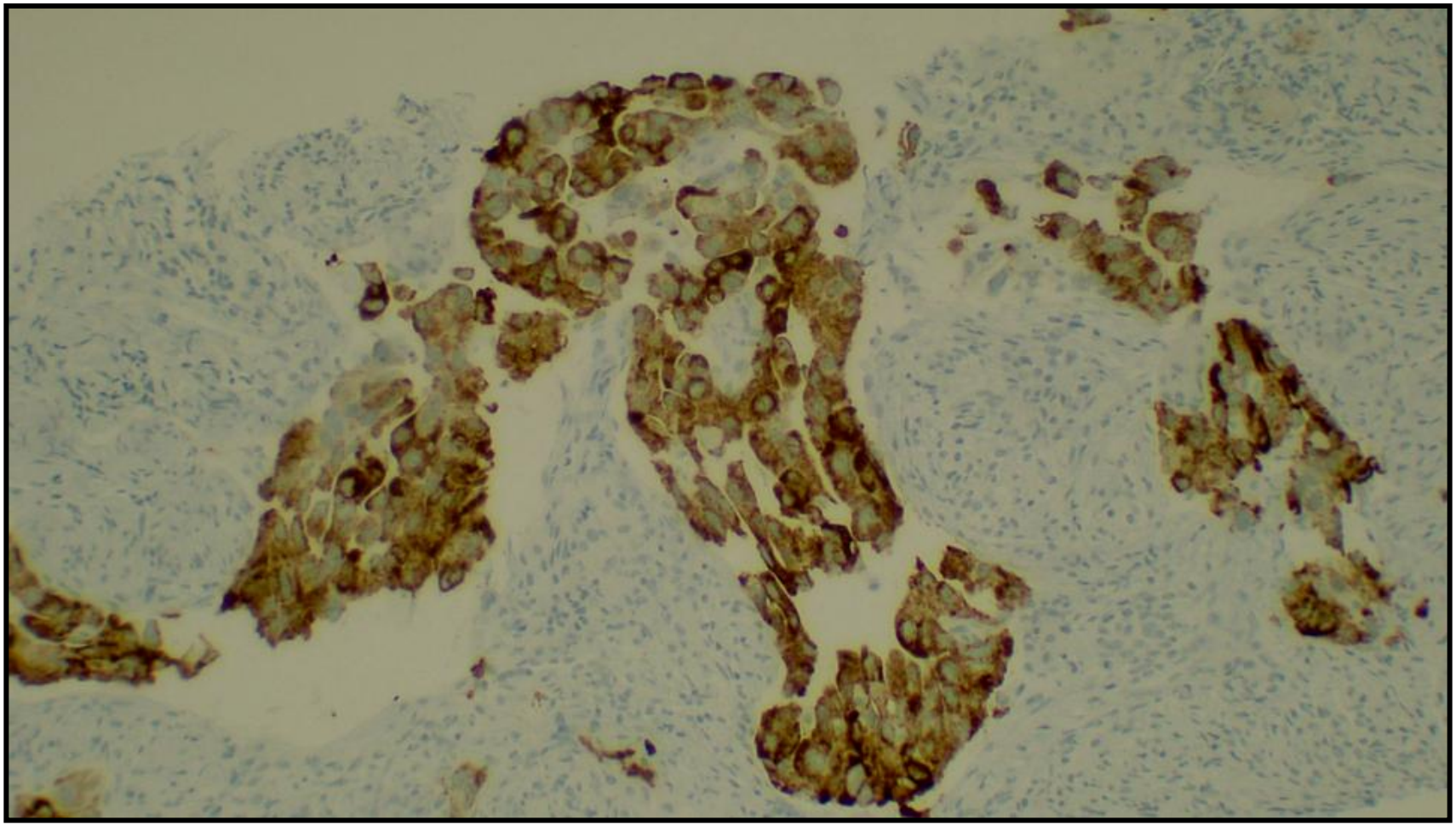
Heterogeneous ROS-1 expression



Case 3: NSCLC-ADC with micropapillary pattern of growth



ROS-1: Strong and Diffuse expression



IHC: Advantages

- Effective screening tool and requires just one section (4 micron)
- Cell Blocks also can be used
- Avoiding unnecessary FISH test
- **Short TAT (Few Hours):** Clinical situations require expedition of results
- Standardization/ Validation is easier
- More laboratories can do it

Disadvantage: IHC criteria

- Different interpretation criteria were suggested with different cut-off points
- Eg, positivity defined with moderate/strong intensity (2+/3+) or with H-score >100 or >150
- **Currently, there is no standard cut-off criteria accepted.**
- Thus, it is recommended that each laboratory validates its own interpretative range

3 Practical Challenging Scenario in ROS-1 IHC
important clinical consequences

Challenging Scenario i HC

1. **True positive IHC**
   orthogonal

- Li
- Risk
  appr

Precise knowledge of the coverage of the assay and its real-world performance can help rule out an NGS false negative result

It now recommended that IHC-positivity and questionable ROS-1 Orthogonal test results be confirmed with another technique

ail to

ng a DNA-based NGS

Challenging Scenario in ROS-1 IHC

2. False-negative IHC result

- Patients with a negative IH… d again for this bioma…
- One…
- …s and/or mucinous cells.
- …gative other driver mutations

One should go for a multigene assay (NGS Testing)

Challenging Scenario in ROS-1 IHC

3. **Significant ROS1 expression ca**

ROS1-amplified t

ROS

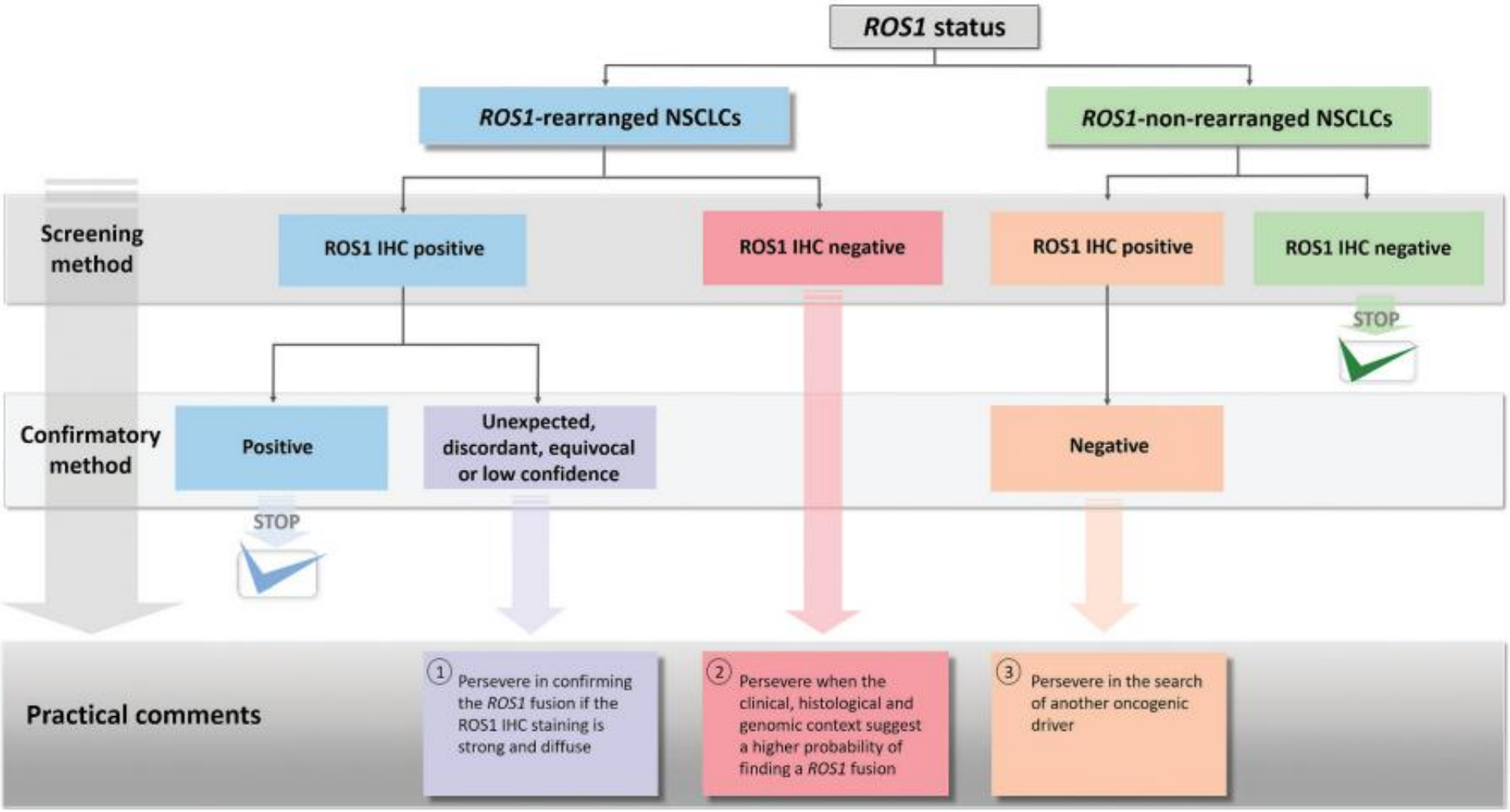
ther oncogenic

ions

utations, BRAF mutations, ALK fusions and abnormalities

If Confirmatory method by BA-FISH Negative for ROS-1 Rearrangement, should go for a multigene assay (NGS Testing)

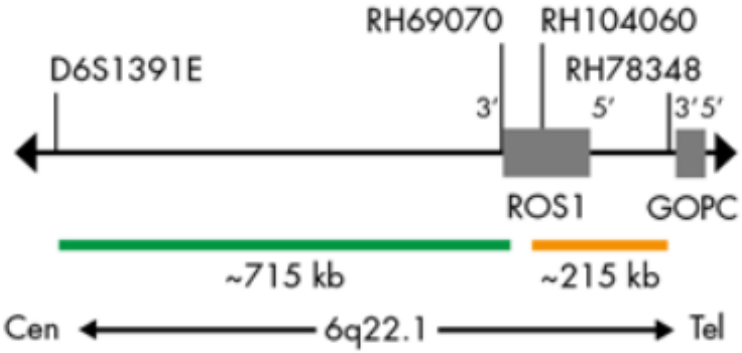
Workflow of ROS1 IHC interpretation



BA-FISH

- Gold standard to diagnose ROS-1 rearrangements
- Played a vital role in the initial clinical trials of Crizotinib
- Using a dual probe break-apart design with 2 different fluorochromes labelled on either side of the fusion break-point (3’ and 5’)

Ideogram of chromosome 6 indicating the hybridization locations.



SPEC ROS1 Probe map (not to scale).

Criteria and Pre-requisite for positive Test

>15% of the cells show separation of both 3’ and 5' probes

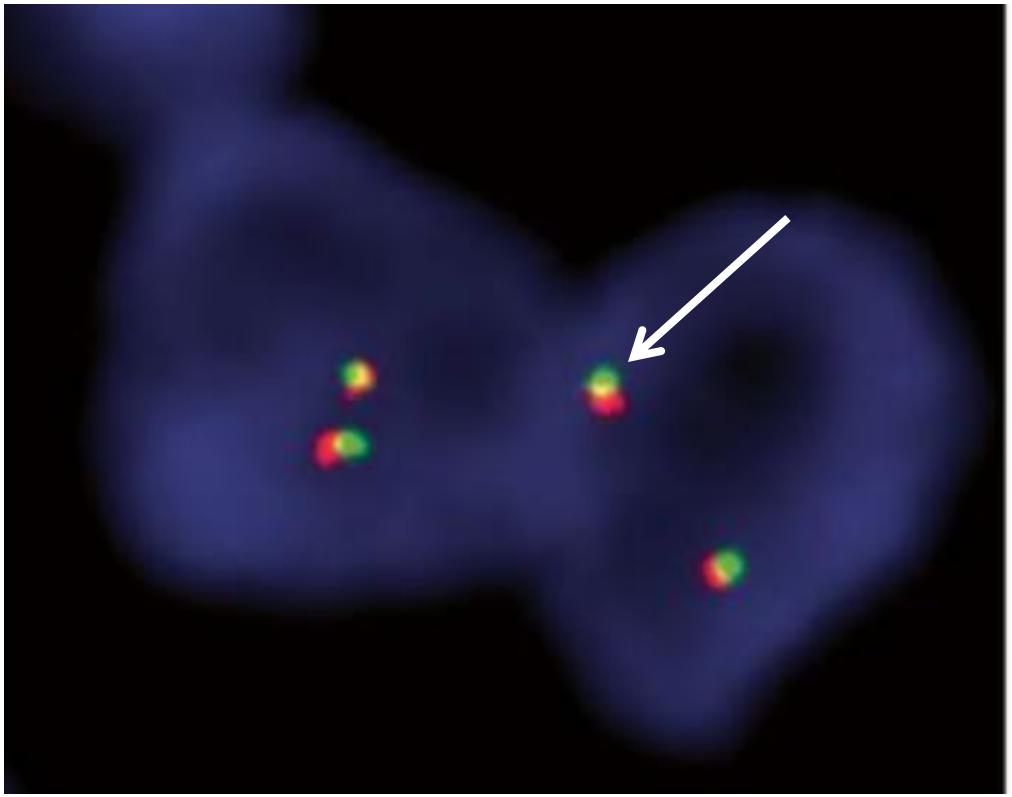
or

>15% of the cells show isolated 3’ signal (centromeric)

- More than **50 viable tumor cells** must be present to validate a positive finding

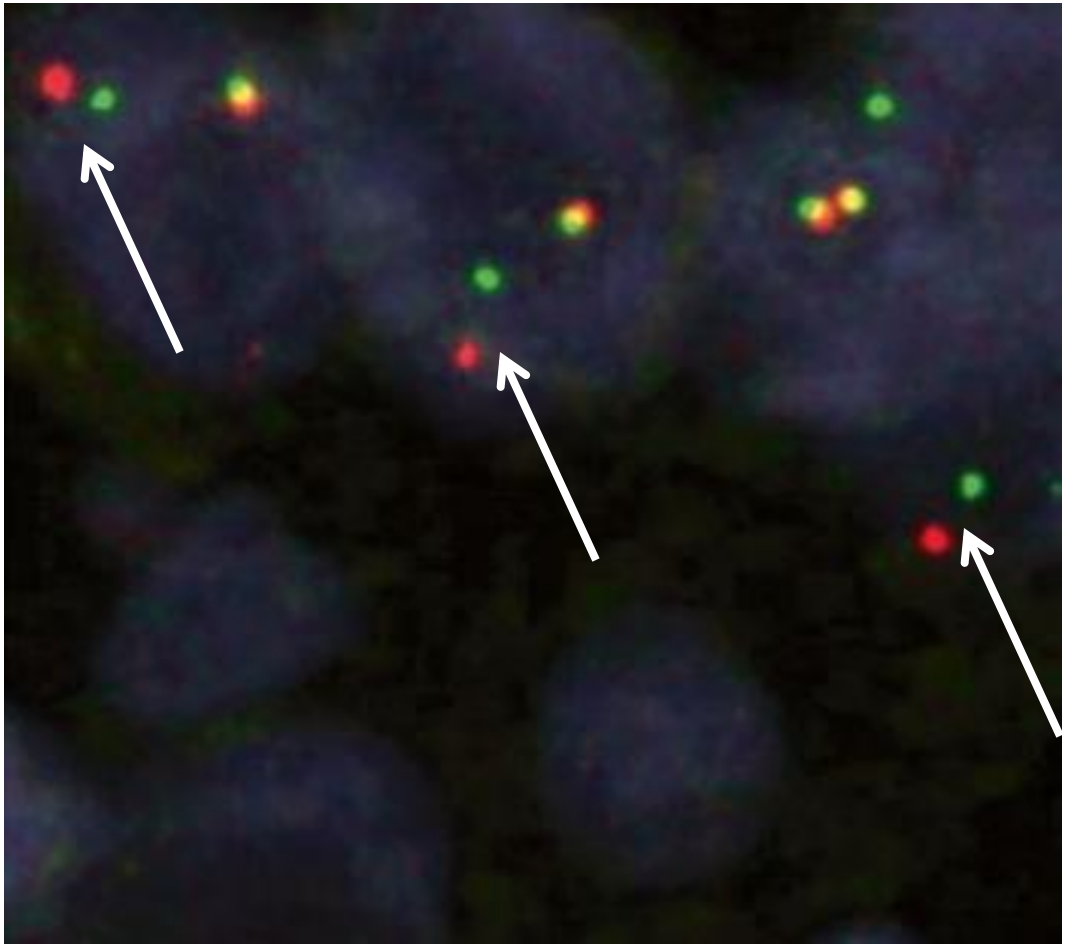
- In uncertain cases (range 10%–15%), a correlation with another diagnostic test is recommended (IHC or NGS)

When ROS1 rearrangement is Absent,
their overlapping produces a “fused” yellow signal

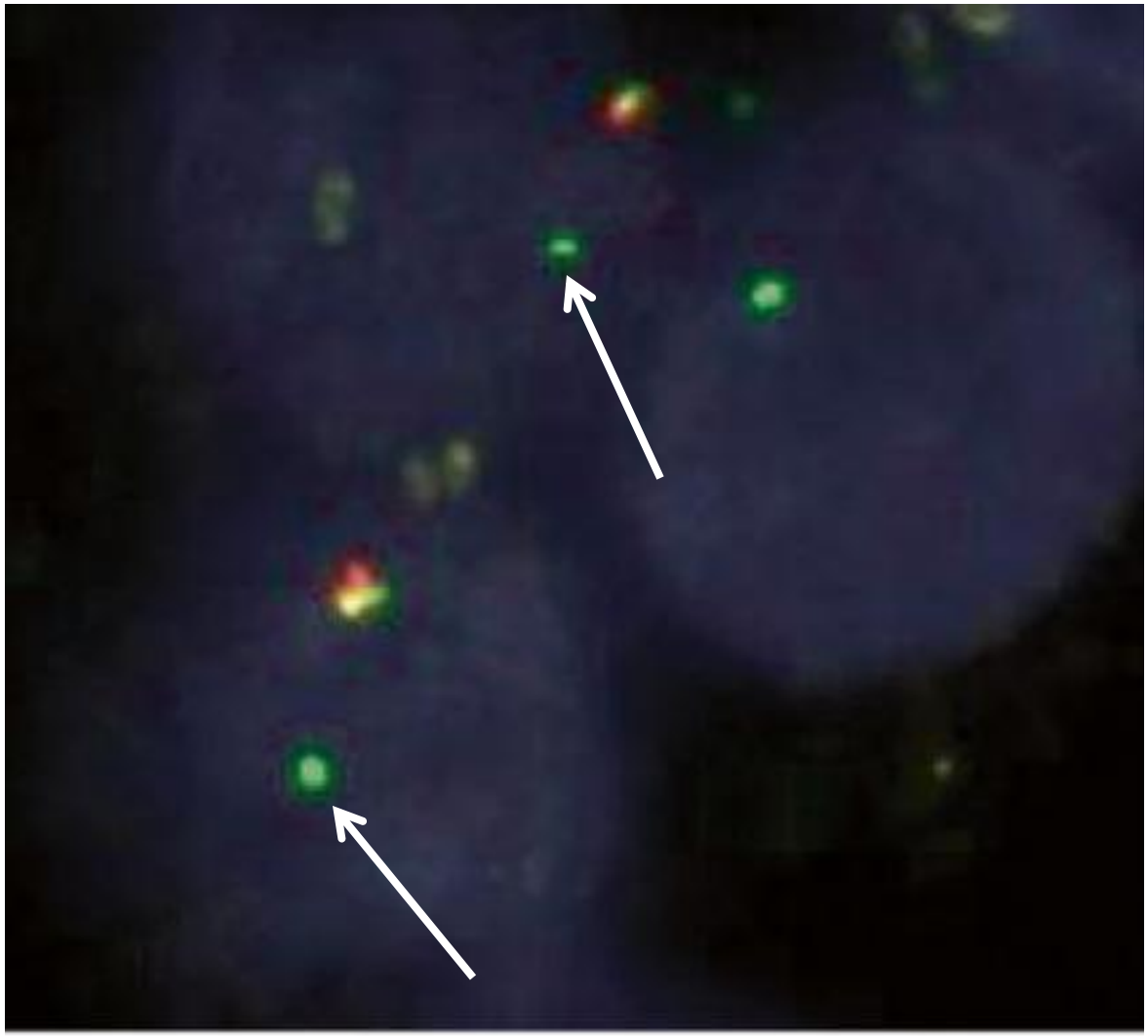


When ROS1 rearrangement is Present

"classic Pattern" with one fusion signal (native ROS1) and two separated 3' and 5' signals



“**Atypical” pattern** with native ROS1 fusion signal and an isolated 3’ signal (usually green) without the corresponding 5’ signal



False Negative BA-FISH

- Certain fusion partners, primarily **GOPC–ROS-1 or EZR–ROS-1**,are known to cause False negative BA-FISH
- Inability of certain FISH probes to detect rearrangements that result from small genomic deletions
- There can be complex staining pattern in which many atypical fusion doublets are seen but the percentage of cells with the typical split signals was below cutoff (15%).

ORIGINAL ARTICLE

Journal of Thoracic Oncology 2018;13:1474-1482

IASLC

Comparison of Molecular Testing Modalities for Detection of *ROS1* Rearrangements in a Cohort of Positive Patient Samples

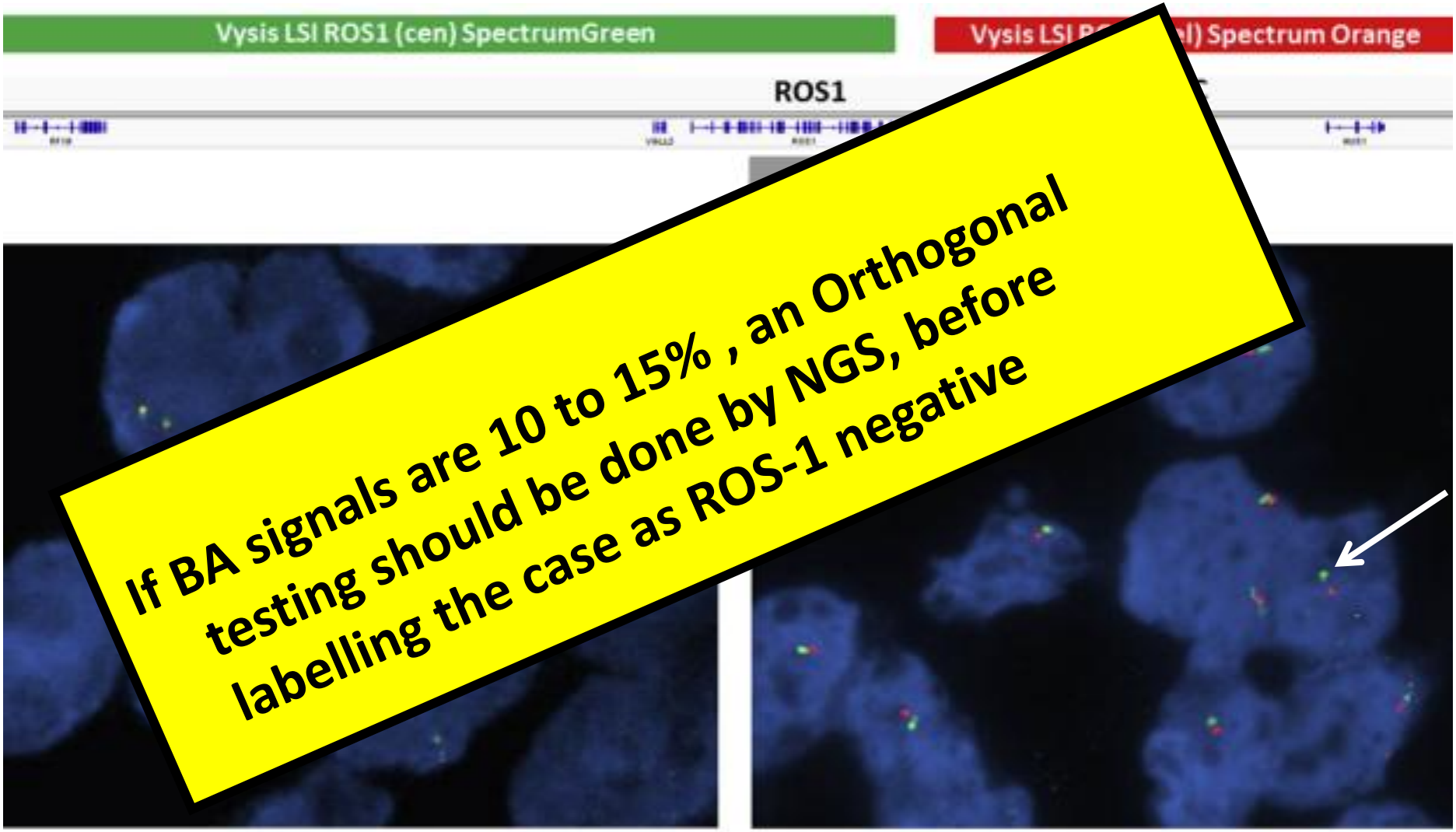
Kurtis D. Davies, PhD,[a] Anh T. Le, BA,[b] Jamie Sheren, PhD,[a] Hala Nijmeh, PhD,[a] Katherine Gowan, BS,[c] Kenneth L. Jones, PhD,[c] Marileila Varella-Garcia, PhD,[a,b] Dara L. Aisner, MD, PhD,[a] Robert C. Doebele, MD, PhD[b,*]

[a]*Department of Pathology, University of Colorado - Anschutz Medical Campus, Aurora, Colorado*
[b]*Department of Medicine - Division of Medical Oncology, University of Colorado - Anschutz Medical Campus, Aurora, Colorado*
[c]*Department of Pediatrics - Section of Hematology, Oncology, and Bone Marrow Transplant, University of Colorado - Anschutz Medical Campus, Aurora, Colorado*

Received 20 March 2018; revised 25 May 2018; accepted 31 May 2018
Available online - 20 June 2018

FISH results were False negative in 2 of 20 tested samples (10%)

Ist False Negative Case



2nd False Negative Case: a complex staining pattern in which many atypical fusion doublets but the percentage of cells with the typical split signals was below cutoff (15%).

False Positive BA-FISH

- Unproductive rearrangements (Non-functional ROS-1 Fusion)
- Aberrant probe hybridization

NGS

- NGS technology consists of massive parallel nucleic acids sequencing and allows simultaneous molecular characterization of multiple genes

- NGS approaches range from targeted panels that include hotspot regions of variable number of genes to whole exome or whole genome sequencing

- Both DNA and RNA can be used as input material for assays

- Allows the detection of SNV, insertion/deletion, CNV and genomic rearrangements

Advantage of NGS for ROS-1 Rearrangement

- Potential to detect several fusions **(known and novel)** and to identify the specific partner of translocation

- Targeted multiplexed panels able to analyze hot-spot regions of **all approved molecular biomarker** (such as EGFR, KRAS, BRAF, ALK, ROS1, HER2, RET, NTRK-1)

- Saving time and histological material in respect to sequential single-target test.

Main ROS-1-fusion partners in ROS-1-positive NSCLC

Gene	Description	Frequency
CD74	Cluster of differentiation 74 (several subtypes: C6R34, C6R32 C7R32, C3R34)	38–54%
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LRIG3	Leucine-rich repeats and immunoglobulin-like domain 3	1%
MLL3	Mixed lineage leukemia	1%
MPRIP	Myosin phosphatase Rho-interacting protein	1%
MSN	Moesin	1%
MYH9	Myosin, heavy polypeptide 9, non-muscle	1%
MYOC 5	Myosin-gene family myosin VC	1%
RBPMS	RNA-binding protein with multiple splicing	1%
SLC2A4RG	solute carrier family-2 member-4	1%
SLC6A17	Solute carrier family-6 member-17	1%
SLMAP	Sarcolemma-associated protein	1%
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TDP52L1	Tumor protein D52-like 1	1%
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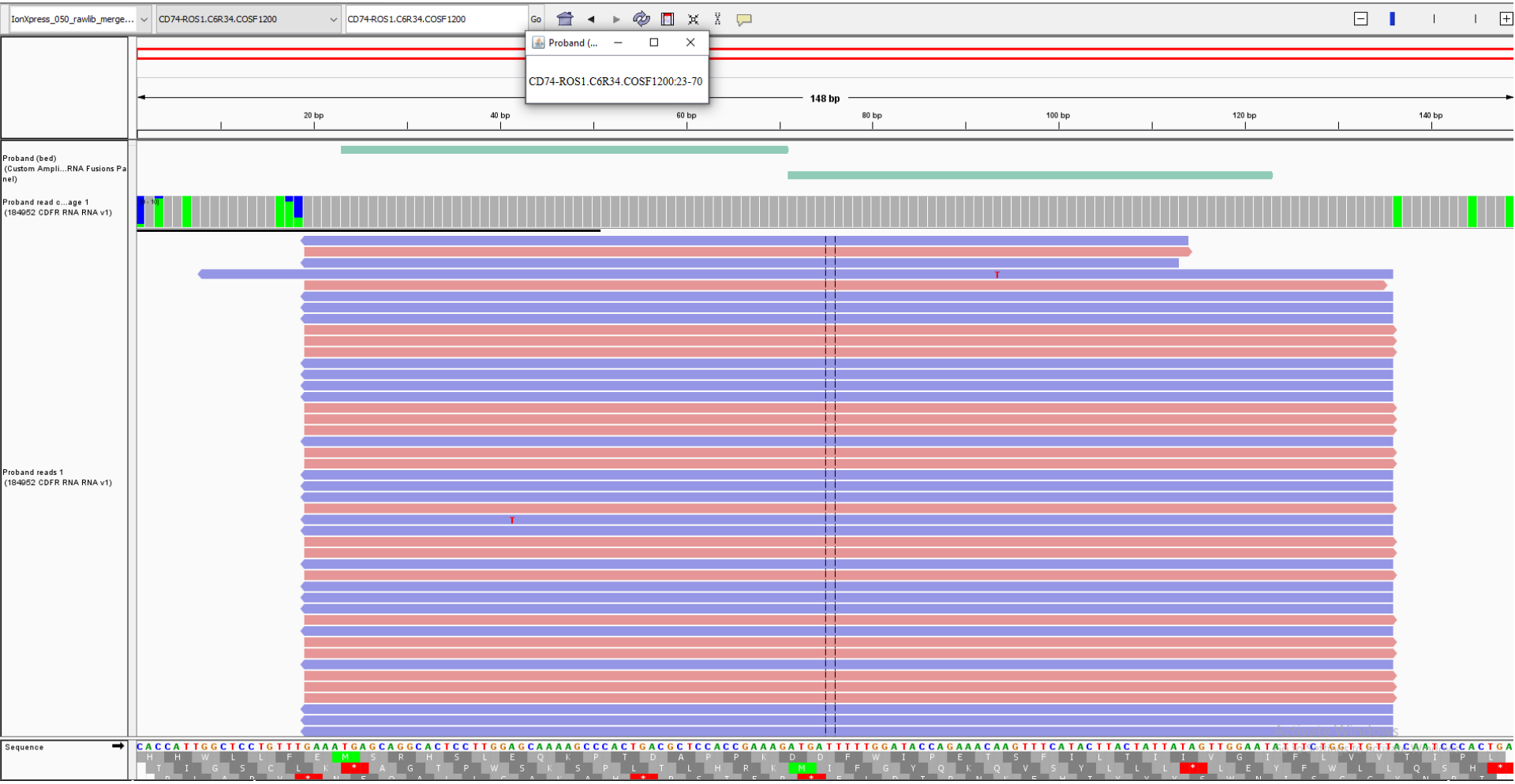
TRG or TFG	TRK (transketolase-related gene)-fused gene	1%
WNK1	Lysine deficient protein kinase 1	1%
ZZCCHC8 or ZCCH	Zinc finger CCHC-type containing 8	1%

DNA based Sequencing

- Because most (but not all) genomic breakpoints that lead to gene fusions occur in introns
- Therefore, assays are designed to detect rearrangements/fusions must sequence introns
- However, introns are known to frequently contain repetitive sequences that are difficult to assess by NGS
- So there will be possibility that genomic breakpoints may occur in intronic regions that cannot be properly sequenced **leading to False Negative results**

RNA-based NGS

- Advantage over DNA-based NGS
- Sequencing can be focused on coding sequences instead of introns, hence reduced false negative cases
- However, drawback of this approach is the high reliance on RNA quality, which can be poor in clinical samples, particularly those that are FFPE processed


IonXpress_050_rawlib_merge...
CD74-ROS1.C6R34.COSF1200
CD74-ROS1.C6R34.COSF1200
Go
Proband (...
CD74-ROS1.C6R34.COSF1200:23-70
148 bp
20 bp
40 bp
60 bp
80 bp
100 bp
120 bp
140 bp
Proband (bed)
(Custom Ampli...RNA Fusions Panel)
Proband read c...age 1
(184952 CDFR RNA RNA v1)
Proband reads 1
(184952 CDFR RNA RNA v1)
Sequence

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Received 20 March 2018; revised 25 May 2018; accepted 31 May 2018
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The DNA-based NGS assay was **False negative** in 4 of 18 tested samples **(22.2%)**

The RNA-based NGS assay was **False negative** in 3 of 19 tested samples **(15.8 %)**

In DNA-based sequencing

- On re-evaluation the coverage of ROS1 introns in this assay, it became apparent that in certain regions coverage was less than complete.

- The presence of repetitive DNA sequence, in intron 31 precluded bait coverage of all desired regions

Journal of Thoracic Oncology 2018;13:1474-1482

In RNA-based sequencing

- Calculation of post-sequencing metric is QC
- Failure of this metric to achieve a defined cutoff is indicative of poor-quality RNA, and precludes interpretation of negative results
- On re-evaluation, all three cases of failed ROS1 fusion detection were associated with failure to achieve this cut-off, thus these **results were interpreted as uninformative and not true false-negatives**.

Journal of Thoracic Oncology 2018;13:1474-1482

To Conclude

- ROS1-positive NSCLCs have been identified as a distinct molecular class
- The effectiveness of targeted therapies depends on the accurate determination of the genomic status of the tumor
- Incumbent upon the Pathologist to make the testing reliable by optimizing:

  Pre-analytical, Analytical and Post analytical steps
- Multidisciplinary communication is essential for the:
  - quality information within the required time frame (TAT)
  - at judicious cost

Thank You