Immunocytochemical Detection of ALK and ROS1 Rearrangements in Lung Cancer Cytological Samples

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Abstract

The detection of molecular alterations as *ROS1* and *ALK* rearrangements are performed at the diagnosis of advanced stage lung adenocarcinoma. These alterations allow the treatments with tyrosine kinase inhibitors. Cytological samples are very useful as up to 40% patients are diagnosed with this type of sample. Here we describe the immunocytochemistry technique usable to reveal the overexpression of ALK or ROS1 tyrosine kinase receptors secondary to *ALK* and *ROS1* rearrangements respectively.

1. Introduction

The emergence of tyrosine kinase inhibitors (TKI) has change the diagnostic and therapeutic management of non-small cell lung cancer. When advanced stage is diagnosed, *EGFR* mutational status and *ALK* and *ROS1* rearrangement are now routinely performed in the anatomy and pathology laboratory ***(1–3)***. As up to 40% of lung cancers are diagnosed on cytological sample ***(4)*** (e.i pleural or pericardial effusion, bronchoalveolar lavage, bronchial brush of the nodule, endobronchial ultrasound-guided transbronchial needle aspirates (EBUS-TBNA)…), these types of sample are very useful for the evaluation of *ALK* and *ROS1* rearrangement. Immunocytochemistry (ICC) revealing ALK and ROS1 proteins could be used to detects an overexpression of these tyrosine kinase receptors secondary to *ALK* or *ROS1* rearrangement ***(5)***.

The last guideline updated for molecular testing from the College of American Pathologists, the International Association for the Study of Lung Cancer, and the Association for Molecular Pathology ***(1)*** approves the use of cytological sample with adequate cellularity and highlights the performance of smear preparations.

ROS1 rearrangement is rare, accounting for 2-3% of non-small cell lung cancer ***(6–8)***. ROS1 ICC may be used as a screening test in advanced-stage lung adenocarcinoma. A positive result should be confirmed by a molecular or cytogenetic testing ***(1)***. *ALK* rearrangement is found in 3 to 7% of non-small cell lung cancer ***(9, 10)***. Numerous publications have established the performance on ALK

immunohistochemistry (IHC) and it is now an acceptable alternative to *Fluorescent in situ* hybridization (FISH), a high positivity is enough to start the treatment with TKI ***(1, 11–13)***.

This chapter provides the materials and the protocol that will allow investigators to successfully identify *ALK* or *ROS1* rearranged cells by immunocytochemistry[1,2].

2. Materials

The following materials are for ICC protocol that we use to label rearranged *ALK* or *ROS1* cells using monoclonal antibodies against ALK or ROS1.

2.1 Equipment

1. Cell culture hood (if working on cell culture)

2. Laboratory fume hood

3. Cytospin™ centrifuge

4. Pipettes (P10, P20, P200, P1000) and tips

5. Freezer (−20°C)

6. Kova® slides

7. Cytospin™ slide[3]

8. Paraformaldéhyde (PFA) powder

9. NaOH 1M

10. Phosphate-buffered saline 1X and 10X (PBS) stored at room temperature

11. H_2O_2 10V

12. Methanol

13. SensiTEK HRP (ScyTek) provides Super Block (to block the non-specific fixation sites) the biotinylated secondary antibody (mouse, rat, Guinea Pig or rabbit), and the streptavidin/HRP label[4]

[1] This technique works on cytological materiel, either from cell culture or from patient's cytological sample (e.g pleural effusion).

[2] This technique can be used with other antibodies (e.g. anti-cytokeratins).

[3] You can use cytospin slide or classic slide. If you use classic slide, we recommend circling the cell spot obtained after cytocentrifugation with an immunostaining guard pen to help visualize the cytocentrifugation spot.

14. DAB Quanto (Thermo Fisher Scientific) contains the 3,3′-diaminobenzidine (DAB) chromogen and substrate solutions [5].

15. Primary antibodies: anti-ALK antibody [6,7], Anti-ROS1 antibody [8].

16. Positive control: A cell line such as HCC78 for *ROS1* rearrangement or H2228 for *ALK* rearrangement is usable as positive control in each experiment you perform to validate the experiment. Alternatively, you can use as positive control a patient's cytological sample with an already known rearrangement.

17. Negative Control: a negative control should be performed in each experiment to confirm the absence of non-specific staining. We recommend using a total non-immune mouse or rabbit IgG as primary antibody, depending on the specie of your ALK and ROS1 primary antibody. The dilution of the negative control may be adjusted to have the same final concentration as the ALK or ROS1 primary antibody.

18. Sterile water

19. Aquatex® or another aqueous mounting medium

20. Optique microscope

2.2 Solutions

2.2.1 PFA 4%

. Add 40g of PFA in 500mL of sterile water at 60°C

. Agitate for one hour

. Add 1 to 2 mL of NaOH 1M until the lightening of the solution

. Wait until the solution return to room temperature

. Add 500 mL of PBS 2X

. Adjust at pH 7.2

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[4] Several companies offer similar IHC products.

[5] Several companies offer similar products.

[6] Several companies offer similar primary antibodies.

[7] The international recommendation recommends the 5A4 or D5F3 clone ***(14–16)***.

[8] The international recommendation recommends the D4D6 clone ***(17–19)***.

. This buffer can be stored at -20°C until utilization[9]

2.2.2 Peroxidase blocking solution:

Add 10 mL of H_2O_2 to 90 mL of methanol[10]

3. Methods

The same protocol is used to label *ALK* rearranged or *ROS1* rearranged cells.

3.1 Slides preparation

1. Cells can come from patient's cytological sample or from cell culture. If you work with patient's cytological sample containing precipitated proteins, you can prewash the sample in PBS 1X or other isotonic saline solution, this help to decrease background staining. If you work with cultured cells, you should resuspend and wash them twice in PBS 1X before the next step to remove residual culture medium.

2. Evaluate the cell count with a Kova® slide.

3. Adjust the volume (you can concentrate or dilute) to obtain ≈ 1000 cells/µL and prepare a cytospin with 200 µL[11].

4. Cytospin at 450 x g for 3 minutes.

5. If you do not perform the immunocytochemistry the day you prepare the cytospin slides, freeze them at -20°C until their utilization.

3.2. Immunocytochemistry

[9] Once the PFA 4% is defrosted, you must use it in the week.

[10] Peroxidase blocking solution should be prepared just before the immunocytochemistry.

[11] You may confirm the slide cellularity before performing the immunocytochemistry. You can color one slide with Papanicolaou or May Grunwald-Giemsa staining. Cells should be in sufficient quantity, but not too many that you can distinguish one from another. There must be enough neoplastic cells for analysis (more than 50 ref jain)

1. Every step of the method is performed at room temperature. As the revelation method used chemistry technique, there is no need to be in a dark room.

2. If needed, defrost cytospin slides for 10 minutes.

3. Under laboratory fume hood, fix cells in PAF 4% for 10 minutes[12].

4. Remove PAF 4% from the slide by putting the slide vertically.

5. Wash the slide twice in PBS 1X for 5 minutes.

6. Under the laboratory fume hood, prepare the peroxidase blocking solution: add 10 mL of H_2O_2 in 90 mL of methanol[13].

7. Under the Laboratory fume hood, incubate the slide in the peroxidase blocking solution for 30 minutes in a slide staining jar.

8. Wash the slide twice in PBS 1X for 5 minutes.

9. Add 100 µL of Super Block from the SensiTEK HRP kit, and incubate for 10 minutes.

10. Wash the slide in PBS 1X for 5 minutes.

11. Prepare the dilution in PBS 1X of the primary antibody depending on the manufacturer's instructions.

For anti-ALK antibody (5A4, ab17127, Abcam) the dilution is 1/25; for anti-ROS1 antibody (D4D6, #3287, Cell signaling), the dilution is 1/250.

12. Add 100 µL of diluted primary antibody and incubate for 30 minutes[14].

13. Wash the slide in PBS 1X for 5 minutes.

14. Add 100 µL of biotinylated secondary antibody provided in the SensiTEK HRP kit and incubate for 15 minutes.

15. Wash the slide in PBS 1X for 5 minutes.

16. Add 100 µL of streptavidin/HRP provided in the SensiTEK HRP kit and incubate for 20 minutes.

17. Wash the slide in PBS for 5 minutes.

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[12] You can overlay the cells spot with PAF 4% when placing your cytospin horizontally

[13] As the detection kit use exogenous peroxidase activity and as cells can contain endogenous peroxidase, we recommend to inhibit endogenous peroxidase by incubating cells with H202. This process reduces the non-specific background staining due to endogenous peroxidase.

[14] The time can be modified depending on the manufacturer instruction if you use another primary antibody.

18. Add 16 µL of diluted DAB Quanto chromogen (30 µL of chromogen in 1mL of commercial buffer present in the kit) and incubate for 5 minutes.

19. Wash the slide in PBS 1X for 5 minutes.

20. Wash the slide in sterile water for 1 minute.

21. Add Mayer's hemalun solution for nucleus conterstaining for few seconds.

22. Wash the slide in sterile water.

23. Add a cover slip with Aquatex® or another aqueous mounting agent.

3.3 Data analysis (Figure 1)

1. Slides could be observed under optique microscope as soon as the mounting agent is dry.

2. The positive control should have a brown extranuclear staining.

3. Cells incubated with mouse or rabbit IgG (negative control) should be white or beige but not as brown as the slide with ALK or ROS1 primary antibody.

4. For ROS1, a positive staining even weak and in fewer neoplastic cells should lead to a confirmation by a second method (FISH or NGS)[15] ***(17, 20)***.

5. For ALK, the range of intensity staining can be graded (0 to 3+), the 3+ do not need a confirmation with another technique and should lead to the treatment with TKI. The weaker intensities 1+ and 2+ should be confirmed by FISH ***(1)***.

6. Slides can be conserved at room temperature in a closed slide boxe

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Figure 1 legend:

A: HCC78 cell line showing a positive staining against anti-ROS1 antibody (positive control).
B: Adenocarcinoma cells from EBUS-TBNA showing a negative staining against anti rabbit IgG (negative control for ROS1 antibody).

C: Adenocarcinoma cells from pleural effusion showing a positive staining using anti-ALK antibody.
D: Adenocarcinoma cells from EBUS-TBNA showing a negative staining against anti mouse IgG (negative control for ALK antibody).

B, C and D are from 3 different patients. Obj x40. Scale bar represents 10 µm.