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Immunohistochemical detection of Retinoblastoma protein phosphorylation in human tumor samples

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Abstract

The retinoblastoma protein (pRb) is an important tumor suppressor and cell cycle repressor. pRb is a phospho-protein whose function is regulated primarily at the level of phosphorylation, and therefore, detecting pRb's phosphorylation status in human tissue samples can be clinically informative. Unfortunately, detection of phosphorylated pRb residues can be technically challenging, as these residues can often be weak antigens. In this chapter, we describe an enhanced sensitivity immunohistochemistry protocol for the staining of phosphorylated serine 249 in pRb, in human lung tumor samples.

Keywords

Retinoblastoma protein; tumor microarrays; cell cycle control; phosphorylation; lung cancer; immunohistochemistry

I. Introduction

The retinoblastoma protein (pRb) is an important tumor suppressor that acts as a cell cycle repressor, specifically controlling the G1-S transition of the cell cycle (1, 2, 3, 4, 5). pRb is a phospho-protein whose function is regulated primarily at the level of phosphorylation, its hyper-phosphorylation being associated with a functionally repressed state (6, 7, 8). Studying pRb's phosphorylation state is relevant both to the understanding of oncogenic mechanisms (as most human cancers exhibit hyper-phosphorylated pRb), and also in a clinical setting, as pRb hyperphosphorylation can be a surrogate of pRb tumoral activity and therefore may have prognostic value.

Immunohistochemical staining for total pRb expression as well as for phosphorylation of several of its residues in human tumor samples can be clinically informative. In particular, phosphorylation of pRb in specific residues can be assessed by using commercially available phosphor-specific pRb antibodies in immunohistochemical staining of human tumor samples. The study of pRb phosphorylation in human tissues has been focused primarily on the phosphorylations that are known to be particularly disruptive of pRb's function as a cell cycle repressor. These phosphorylations usually occur in serine or threonine residues in

pRb's pocket domain and in its C-terminal (9). These phosphorylations are known to disrupt pRb's cell cycle repressive capacity by blocking pRb's capacity to bind and block the activities of the proliferation-related E2F transcription factors (9). However, the paradigm of pRb function that is currently emerging is that of a multi-functional protein whose functions go beyond cell cycle control and that encompass other aspects of cellular physiology. In this context, non-canonical pRb phosphorylations (i.e., phosphorylations in other pRb domains that do not necessarily disrupt E2F interactions and cell cycle control) are emerging as phosphorylations of potential interest, both due to their potential clinical implications, as well as for their implications in the biochemical pathways related to pRb's anti-oncogenic function.

In this chapter, we explain the immunohistochemical staining of a pRb non-canonical phosphorylation in serine 249, in human lung tumor samples that were arranged as a tumor microarray. Phosphorylated residues can sometimes be weak antigens and therefore may present problems to their detection. Therefore, their detection may sometimes call for protocols with enhanced sensitivity. Here we describe specifically a protocol that uses the BioGenex Super Sensitive Link-Label IHC kit. The sensitivity of this kit relies on a secondary antibody to which multiple biotin residues have been conjugated in such a manner that the signal is amplified after streptavidin-horseradish peroxidase forms a complex with each biotin. This method is particularly suitable for antigens that give a weak signal using other immunohistochemical staining methods.

2. Materials

This protocol was optimized for formalin-fixed, paraffin-embedded (FFPE) tissues that were part of a commercially available lung cancer tumor microarray. However, you can also get your tissue slides prepared by your institutional histology services facility (*see Note 1*). It is therefore assumed that you have your tissues slides already available before starting this procedure.

2.1 Tissue processing and staining reagents.

1. Xylene (*see Note 2*)
2. Ethanol wash series. You need absolute (100%) ethanol, and then prepare 95%, 90%, 85%, 80% and 75% ethanol dilutions in distilled water (dH₂O).
3. Citrate antigen retrieval solution: Dissolve 1.92 g of trisodium citrate dehydrate and 0.74 g of EDTA in 800 ml of H₂O, adjust pH to 6.2 with 1 N HCL, add 0.5 ml of Tween-20, and complete to final volume of 1 L. This solution can be stored at 4°C for up to 6 months. *See Note 3*.
4. 1X PBS: dissolve 8 g NaCL, 0.2 g KCL, 1.44 g Na₂HPO₄·2 H₂O and 0.24 g of KH₂PO₄ in 800 mL of water. Adjust the pH to 7.2 with HCL, and add distilled water to complete the volume to 1 L. Keep at 4°C.
5. 3% Hydrogen peroxide solution (*see Note 4*)

6. PAP Pen, this is used for drawing hydrophobic barriers around tissue slides.
7. Primary antibody: we optimized this protocol using a rabbit polyclonal antibody recognizing pRb phosphorylated in serine 249 purchased from Sigma-Aldrich (Anti-Phospho-Rb (S249), Cat. No. SAB 1305396-400 ml). We have tried both 1:50 and 1:100 dilutions with good results. Make your dilutions in 1X PBS. For 1:50, add 20 µl of antibody to 980 µl of PBS, and for a 1:100 dilution add 10 µl of antibody to 990 µl of 1X PBS.
8. Super Sensitive Link-Label IHC (BioGenex Cat. No. LP000-ULE). This is a kit that includes a biotin-labelled anti-rabbit secondary antibody. The kit also includes a streptavidin-conjugated horse radish peroxidase (HRP) (*see Note 5*).
9. Blocking solution: we use a blocking solution that is compatible with the Super Sensitive Link-Label IHC. This is a protein block sold by BioGenex (Cat. No. HK112-9K).
10. Diaminobenzidine (DAB). This is a substrate for the HRP, it produces a dark brown precipitate when oxidized by the HRP. We use the DAB solution provided by BioGenex (two components kit, Cat. No. HK542-XAKE).

2.2 Additional laboratory equipment and solutions

1. Conventional oven
2. Humid chamber (*see Note 6*)
3. Standard cover glasses
4. Mounting medium (*see Note 7*)
5. Standard slide racks and jars
6. Microscope with camera, and suitable image capture software
7. Pasteur or plastic pipettes
8. Distilled water
9. Lint-free tissue paper (e.g., Kimwipes or any other brand, provided it is lint-free)
10. Hot water bath

3. Methods

1. Place the slides in a slide rack, and place them in an oven at 57°C for 30 minutes. This will melt the paraffin and will facilitate the de-paraffinization process.
2. Remove paraffin from tissues by submerging slides in their rack in xylene (or xylene substitute) for 30 minutes. Remember that this step should be performed inside a fume hood.

3. Hydrate the slides by subjecting them to ethanol washes as follows: 100% ethanol for 6 minutes, 95% ethanol for 3 minutes, 80% ethanol for 3 minutes, and 75% ethanol for 3 minutes. Complete hydration by submerging the slide in distilled H₂O for 1 minute.
4. Place the slides flat in the humid chamber. From this point onwards, avoid slides getting dry. In the subsequent steps, ensure that the tissue sections are completely soaked in the indicated solutions.
5. Rinse tissue sections on the slides with 1X PBS. You can add approximately 1 ml of PBS on top of the slide (using a Pasteur pipette), paying special attention that you are covering the entire slide surface and that all tissue sections are soaked in PBS. Do 2 washes of 5 minutes each.
6. Cover tissue sections with a layer of 3% hydrogen peroxide block to inactivate endogenous peroxidase activity. Incubate for 15 minutes.
7. Rinse tissue sections with 1X PBS, do 2 rinses of 5 minutes each.
8. Return the slides to a slide rack, and place the rack in a jar filled with the citrate antigen retrieval solution.
9. Perform the antigen retrieval step by submerging the jar (you need to cover the jar first) in a water bath that has been pre-heated to 95-99°C. Leave the jar in the water bath for 40 minutes.
10. Remove the jar from the water bath and let it cool down to room temperature for 20-30 minutes.
11. Remove the slide rack from the jar, and submerge in distilled water for 2 minutes.
12. Remove slides from the rack, and place them flat in the humid chamber. Rinse tissue sections once with 1X PBS for 5 minutes.
13. Carefully dry the slides with a Kimwipe, only in the areas surrounding the tissues (avoid damaging the tissue sections!). After drying the glass surrounding the tissue sections, use the PAP pen to make a hydrophobic circle surrounding the tissue section. Again, be careful not to damage the tissues.
14. Perform the blocking step by adding enough volume of the blocking solution to cover the tissue section. Incubate at room temperature for 1 hour inside the humid chamber.
15. Carefully remove the excess of blocking solution and dry the slides with a Kimwipe. Avoid damaging the tissue sections in this step. You can achieve this by tilting the slide sideways and removing the solution from the edge of the slide. Do not rinse after removing the blocking solution.
16. Incubate in the primary antibody by adding a drop of antibody solution to each tissue section. At this step, the slides should be in the humid chamber. Incubate overnight (12-18 hours) at 4-8°C (**See Note 8**). Remember to perform negative control sections in which the

primary antibody is omitted. These are done by incubating tissues in 1X PBS instead of antibody solution.

17. Remove the primary antibody and rinse the slides a total of 3 washes, 5 minutes each (see **Note 9**). Remove excess PBS with a Kimwipe, but remember that tissue sections must never get dry.

18. Incubate in secondary antibody by covering the tissue sections with enough solution. Incubation should be in the humid chamber at room temperature for 30 minutes.

19. Remove the secondary antibody solution and wash the slides 3 times with 1X PBS, 5 minutes each wash. After the last wash, remove excess liquid with a Kimwipe.

20. Add the streptavidin-HRP mix to each tissue section, and incubate in the humid chamber for 30 minutes at room temperature.

21. Remove the streptavidin-HRP mix and wash the slides with 1X PBS 3 times, 5 minutes each wash. Remove excess PBS after last wash.

22. Prepare the DAB solution following manufacturer's directions, and add a drop of solution to each tissue section. Incubate for a maximum of 2 minutes. When we use the antibody dilutions of 1:50 or 1:100, we have noticed that the optimal signal developing time is around 1 minute, but you must empirically determine the optimal incubation time. See **Note 10**.

23. Stop the DAB reaction by placing the slides in their rack and submerging them in a jar with distilled water. Leave the slides in their rack under running tap water for 5 minutes, being careful that the water does not directly touch the tissue sections.

24. De-hydrate the slides with ethanol baths as follows: 85%, 90%, 95% and absolute (100%) ethanol, each bath should be 2 minutes.

25. Incubate the slides in xylene for 2 minutes.

26. Drain the xylene from the slides and dry them in an oven at 37°C for 30 minutes.

27. Add 2-3 drops of mounting media on top of each tissue section and carefully place a coverslip on top of the section, avoiding the formation of air bubbles inside the preparation. Wipe excess mounting media from the edges using a kimwipe. See **Note 11**.

28. Analyze slides under a light microscope. A representative staining with the Rb anti-phosphor (S249) can be seen in Fig. 1. A clearly defined nuclear staining can be discerned when we use the 1:100 primary antibody dilution. It is recommended that you enlist a pathologist to help you with the scoring. We usually use a two-dimensional scoring system that measures intensity of staining (0, +1, +2, +3) as well as the % of stained cells in a visual field.

4. Notes

1. Be sure that thickness of tissue sections is 4-10 μm . It is also important that the tissue is intact without breaks or folds, as these usually result in staining artifacts.
2. Steps using solvents like xylene must be performed inside a fume hood, and xylene waste must be discarded according to biohazards regulations. Regular xylene can be used, but we use ThermoFisher Shandon Xylene Substitute (Cat. No. 9990505) as it is safer than regular xylene. It has a similar evaporation rate to regular xylene, but it is less sensitizing to skin, and is safer than xylene regarding airborne exposure. Still, we recommend, if possible to handle this reagent within a fume hood.
3. We recommend that you include EDTA in the antigen retrieval buffer when using phosphor-specific antibodies. This inhibits endogenous phosphatase activity therefore enhancing the staining.
4. We use BioGenex Hydrogen Peroxide block (Cat. No. HK111-50K), but standard hydrogen peroxide diluted to 3% will work well. This solution blocks endogenous peroxidase activity, a step that is crucial when using secondary antibodies conjugated to horse-radish peroxidase (HRP). Omitting this step will result in high background resulting from the activity of endogenous peroxidases on the DAB substrate. Liver, kidney and red blood cells are particularly high in endogenous peroxidase activity, but you should nevertheless include this step when processing other tissues as well.
5. The kit's secondary antibody has multiple biotin residues, so the advantage of this kit is its improved sensitivity to weak antigens (such as a phosphor-specific antigen), due to signal amplification. Strictly follow the manufacturer's instructions when preparing the solution of this antibody.
6. All incubations should be carried out in a humidified chamber to avoid drying of the tissues. Drying of the tissue may lead to non-specific binding of the antibody and high background. You don't need to buy a commercially available humid chamber, you can build your own from non-expensive components. Any plastic container with a lid that produces a tight seal will be adequate. You can use an inverted pipette tip case cover as a platform to place your slides. For humidity, paper towels soaked in PBS will be sufficient.
7. You can prepare your own mounting medium, but most commercially available mediums will work well. The important thing to keep in mind is that the mounting medium of choice will be determined by the nature and solubility of the precipitate that will be formed by the action of the enzyme conjugated to the secondary antibody. In this protocol, we use the diaminobenzidine (DAB) substrate for the horse-radish peroxidase enzyme conjugated to the secondary antibody. Be sure that your mounting medium is compatible with this system.
8. Prolonged incubations at low temperatures can dry your slides. For this incubation, be sure that the humid chamber has plenty of PBS. Do not allow the antibody solution to over flood and pass to the adjacent sections. Dilution of the primary antibody is one of the parameters that you can modify should you need to do some troubleshooting to improve the

signal. If you are troubled by high background, try diluting more the primary antibody or blocking for longer time.

9. Thoroughly wash the slides to avoid background, also the slides should have never been left to dry.

10 Set-up a timer, try not to go beyond 2 minutes. This step will produce a dark brown precipitate as the HRP acts on the DAB substrate. Too long a development time can produce very high background. It may be advisable to monitor the darkening of the staining by visualizing the dark precipitate under a light microscope. You may want to add an additional hematoxylin & eosin counterstaining step after this step.

11. Properly sealed slides can last indefinitely stored at room temperature inside a slide box.

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Figure 1.

Lung tumor tissue sections immunohistochemically stained using an anti Rb phospho-Ser249 antibody. A, negative control in which the primary antibody was omitted. B, primary antibody at a 1:50 dilution. C, primary antibody at a 1:100 dilution. Notice the high intensity of the staining when using the antibody diluted at 1:50 (B). The intense staining makes it difficult to appreciate the expected pRb nuclear localization. For that reason, we determined 1:100 as the optimal dilution for this antibody (C), as this dilution allows for a better definition of stained nuclei.