Chapter 2

Selection and Validation of Antibodies for Signal Transduction Immunohistochemistry

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Abstract

The in situ expression levels and subcellular localization of molecules involved in signal transduction using specific antibodies can be useful for prognosis and diagnosis of human diseases such as cancer. In addition, it has the potential to be helpful in monitoring biologic response to targeted therapies. The increasing availability of such antibodies makes these studies feasible. However, compared to typical immunohistochemical stains in which stabile molecules such as cytokeratins are targeted, additional validation may be required for signal transduction immunohistochemistry.

Key words: Antibody validation, Phosphoprotein, Immunohistochemistry

1. Introduction

The study of signal transduction pathways is nearly synonymous with the study of phosphoproteins (1). Phosphoproteins are involved in regulating nearly all cellular functions. Phosphorylation states can determine key properties of proteins including enzyme activity, protein–protein physical interactions, protein–nucleic acid physical interactions, and subcellular localization. The first antibody against phosphoproteins was discovered almost 30 years ago (2). Ten years later, researchers successfully developed antibodies specific for phosphorylated tyrosine and threonine (3, 4), but these antibodies were mostly useful in Western blotting (WB) analyses where different phosphoproteins could be determined by their molecular weight. Nevertheless, researchers applied these antibodies in immunohistochemical studies of human cancer tissues and discovered a significant increase in phosphorylated proteins in these tissues (5).

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The current commercial availability of phosphorylation–state-specific antibodies makes the in situ study of signal transduction molecules possible and is opening many opportunities in diagnostic pathology and targeted therapeutic monitoring. However, an extra degree of validation may be required for the selection of the antibody suitable for signal transduction immunohistochemistry (IHC), because the detection of the phosphoproteins is highly dependent on the antibody sensitivity and specificity, as well as tissue integrity.

2. Materials

2.1. Cell Controls for Validation of Anti-pAKT Antibody

1. HT-29 cell line (ATCC, Manassas, VA).
2. McCoy's medium (Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA).
3. Hydrogen peroxide.
4. Wortmannin (Cell Signaling Technology, Danvers, MA).
5. 10% Buffered formalin.

2.2. Automated IHC

1. HistoGel system (Richard-Allan Scientific, Kalamazoo, MI).
2. Immunostainer Discovery (Ventana Medical Systems, Tucson, AZ).
3. Cell Conditioning 1 (CC1, pH = 8, Ventana Medical Systems).
4. Reaction buffer (Ventana Medical Systems).
5. Background Sniper (Biocare Madical, Concord, CA).
6. Endogenous Biotin Blocking Kit (Ventana Medical Systems).
7. Anti-pAKT (S473) (736E11) antibody (Cell Signaling Technology).
8. OmniMap anti-rabbit HRP (Ventana Medical Systems).
9. ChromoMap kit (Ventana Medical Systems).
10. Hematoxylin.
11. 95% Dehydrant, 100% dehydrant, xylenes (Richard-Allan Scientific).
12. Cytoseal XYL (Richard-Allan Scientific).

3. Methods

3.1. Search Primary Antibodies: Vendors and Primary Literature

There are some free online search tools available that allow the user to search multiple companies at once. For example, www.antibodybeyond.com, www.linscottsdirectory.com, www.biocompare.com, or www.antibodydirectory.com are good places to start. With these tools, the antibody search can often be narrowed by antigen, species, type (monoclonal, polyclonal), or application. Importantly, one of the crucial requirements is that the chosen specific antibody must be completely described by the commercial vendor. The species, type (monoclonal vs. polyclonal), subclass, the structure of the immunizing antigen, and specificity must be known (6). This can often eliminate potential problems of using less-specific antibodies or antibodies not suitable for IHC. Furthermore, if peer-reviewed literature exists, this can give a realistic review of the performance of the antibody in a particular setting. Such literature can also provide the basis for optimizing immunoreactivy of the antibody (7).

3.1.1. Monoclonal and Polyclonal Antibodies

Based on the type of production, antibodies are divided into monoclonal and polyclonal. In general, monoclonal antibodies are produced by a single B-cell clone using hybridoma techniques. This provides excellent specificity; the antibody binds to a single epitope and it is less likely to cross-react with other proteins. The first monoclonal sequence-specific phosphoprotein antibody was successfully produced in the early 1990s (8). The phosphopeptide immunization approach was later applied but with the production of polyclonal instead of monoclonal antibodies (9).

Monoclonal antibodies have very high homogeneity in comparison to polyclonal antibodies that are prone to "batch-to-batch" variability. Usually, if experimental conditions are kept constant, results will be highly reproducible between experiments. Thus, one of the disadvantages of using polyclonal antibodies, especially for quantitative analysis of signal transduction proteins, is that every new lot must be reevaluated. Polyclonal antibodies contain a mix of antibodies recognizing multiple epitopes on any one antigen, and thus may cause higher nonspecific background staining and be less specific than monoclonal antibodies. On the other hand, polyclonal antibodies may demonstrate high affinity allowing higher dilutions when compared to monoclonal antibodies. Finally, polyclonal antibodies are also more tolerant to changes in the antigen induced by sample processing. Although it seems that monoclonal antibodies are more suitable for signal transduction IHC, a few examples of particularly well-suited polyclonal antibodies are exceptions to this generalization.

3.2. Validation of the Antibody

In the clinical laboratory, we are "at the mercy" of manufacturers. Regulations such as those governing analyte-specific reagents are meant to ensure that reagents are correctly manufactured and labeled as to content. For most antibodies in diagnostic use, we have some idea, based on the results of positive and negative control tissues, that antibodies are specific. Since most applications in routine IHC are lineage or cell-of-origin assignments, normal tissues with stable targets are often suitable. However, signal transduction proteins are very labile by nature; therefore, we believe that an extra degree of diligence may be required (see Note 1). Although the exact method for this type of antibody validation is not established, several independent methods can be used to validate the antibody specificity, including immunostaining of different stimulated and unstimulated cultured cells (see Note 2), immunostaining of tissue with peptide preincubation controls, and genetic (knockout) controls (see Notes 3 and 4). Archival primary human tissues are difficult to use as controls since there may be substantial variability in tissue processing, most importantly delays in fixation, that will affect phosphoprotein levels.

3.2.1. Automated Immunohistochemistry of pAKT

1. 2×10^7 cells are treated with 5 mM hydrogen peroxide for 15 min and 1 μM wortmannin for 1 h, harvested, and fixed in 50 ml of 10% buffered formalin overnight at 4°C with gentle shaking.
2. Paraffin-embedded cell blocks are prepared using the "HistoGel" system for IHC. All samples are processed overnight using the conventional histological techniques and embedded in paraffin, using an automatic apparatus (Tissue-TEK VIP, Miles Scientific). The melted wax temperature does not exceed 60°C.
3. IHC is performed using an automated immunostainer. After deparaffinization and heat-induced epitope retrieval (HIER) using standard Cell Conditioning 1, slides are incubated in Background Sniper for 30 min. Subsequently, Avidin and Biotin block is applied for 20 min.
4. Samples are incubated with 1:50 dilution of the anti-pAKT (S473) (736E11) for 2 h at room temperature (see Notes 5 and 6).
5. OmniMap anti-rabbit HRP is added and incubated for 30 min. Staining is then visualized by using ChromoMap DAB kit (see Notes 7 and 8).
6. Finally, cells are counterstained with hematoxylin for 1 min. After rinsing, the slides are submerged for 2 min twice in 95% dehydrant, 100% dehydrant, and in xylenes and slides are mounted in Cytoseal XYL.

If the antibody is suitable for WB, paired WB and IHC can be performed (Fig. 1a). In this case, WB should result in detection of

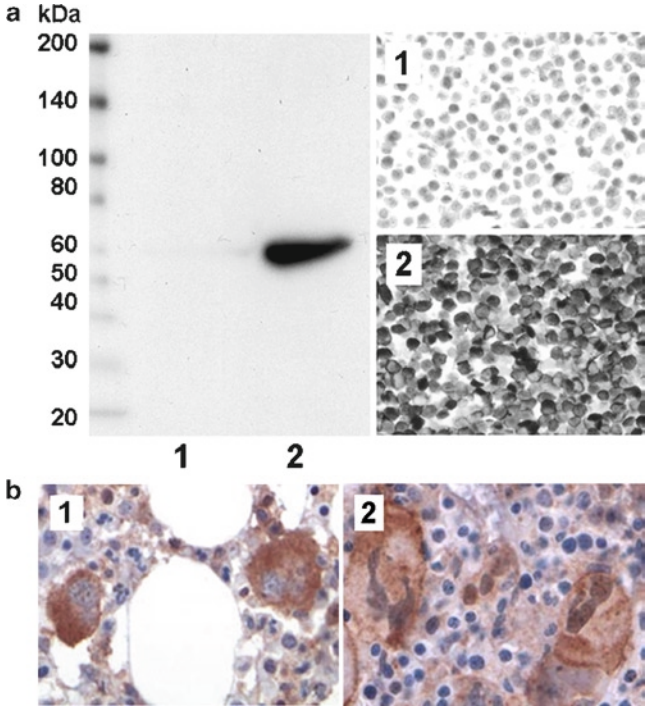

Fig. 1. (**a**) Western blot analysis of pAKT (S473) of HT-29 culture cells treated with (*1*) AKT inhibitor, wortmannin and (*2*) hydrogen peroxide. Negative (*1*) and positive (*2*) IHC stainings correspond to lanes of WB. (**b**) pSTAT5 (Y694/9) IHC staining of (*1*) normal bone marrow showing cytoplasmic localization of pSTAT5 in megakaryocytes and of (*2*) refractory anemia with ringed sideroblasts associated with thrombocytosis sample with nuclear positive pSTAT5 megakaryocytes.

a single band (or multiple bands if family members share the same motifs) of appropriate molecular weight. Alternatively, methods such as ELISA, intracellular flow cytometry (10), reverse phase protein microarray (11), or mass spectrometry (12, 13) can prove the status of signal transduction protein levels in the cells that can be prepared as controls for IHC. Using these known positive and negative controls, the specificity of the antibody can be validated followed with further confirmation in particular tissues.

4. Notes

1. Since little data of signal transduction protein patterns in human tissues exist, one would need to use judgment, informed by knowledge of active biologic processes in various cell types (Fig. 1b), as to whether the antibody remains specific and is sensitive enough to be used in studied tissues. This can be even more complicated, because some signal transduction proteins can oscillate between different cell

compartments in a very short time after their activation (14). Antibodies against different sites of the same protein or against total protein that produce the same staining pattern may be an important strategy for establishing specificity.

2. For semiquantitative analysis of signal transduction proteins using IHC, a further method of validation is needed. Cell lines manipulated to produce different levels of intended targets (Fig. 2a) or cell lines known to express high, medium, and low levels of (Fig. 2b) a particular signal transduction

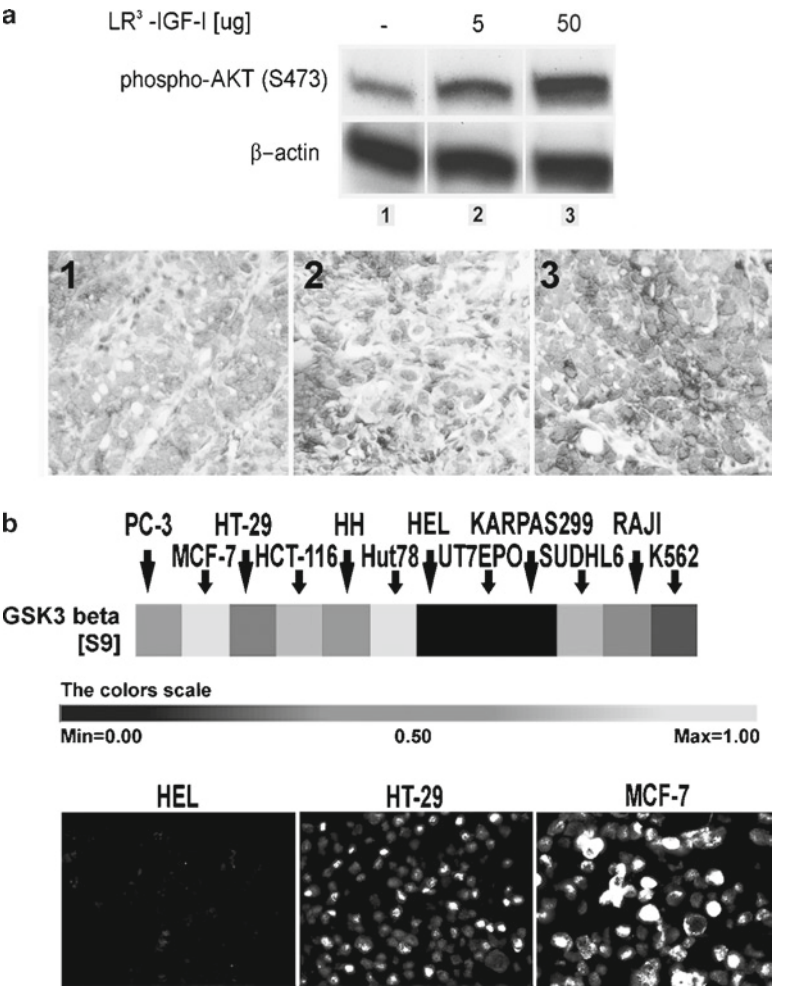

Fig. 2. (**a**) Western blot and IHC analysis of pAKT (S473) in HT-29 mouse xenografts treated with LR3-IGF1. Numbers correspond to lanes of WB. (**b**) The expression of pGSK3 beta (S9) was determined by using Kinetworks phosphoprotein screen and Quantum dot based immunofluorescence staining in the panel of various human cell lines. Phosphoproteins are normalized to the cell line with the highest expression of corresponding phosphoprotein, and relative intensities are shown on the heat map on a 0–1 scale. Three human cell lines with low, medium, and high expression of pGSK3beta (S9) represent different intensities of Quantum dot based staining.

protein may be useful to confirm the specificity and sensitivity. Such information is available for some phosphoproteins (12, 15, 16), but more published data are needed. Based on the same principles, validated antibody can be used for the truly quantitative analysis, using immunofluorescence assay (Fig. 2b).

3. Besides validation methods mentioned above, antigen adsorption, where the antibody is mixed with the appropriate purified antigen before application to the tissue section or cells, is a powerful way to look for nonspecific reactivity of the antibody. Using this approach, we can confirm, especially with monoclonal antibodies, that the cloning process was performed efficiently and only one antigen epitope is recognized. However, this does not provide information on whether other tissue proteins may cross-react with the tested antibody.
4. The last method, especially for validation of antiphosphoprotein antibody, is using (as negative control) tissue sections or cells pretreated by alkaline phosphatase. Although this method excludes only cross-reaction with nonphosphorylated proteins, it is still more useful than using omission controls (staining without primary antibody) that can be only used as a control for evaluation of the specificity of the secondary antibody.
5. The rate of binding between antigen and antibody is dependent on the affinity constant. This constant can be affected by many factors such as temperature, pH, and buffer type. Further, changing the antibody concentrations can also control the amount of antibody–antigen complex formation. Often, the manufacturing company has guidelines for starting dilutions. Typically, two to tenfold dilutions above and below the manufacturer's recommended dilution provide a good starting point. Depending on the type of antibody and type of tissue, a range of 1–5 μg/ml should be used for an initial titration. Moreover, it is important to realize that antibody dilutions may vary between different tissues.
6. If an antibody has not been tested yet, a systematic approach using a wide range of dilutions, as well as different antigen-retrieval methods, is needed. A checkerboard design combining all combinations of chosen retrieval conditions and primary antibody concentrations will allow one to quickly identify promising conditions that one can focus on during a more detailed study. For example, a broad checkerboard experiment may identify that low pH heat-induced epitope retrieval stains positive and negative control tissue appropriately. Focusing secondary experiments on more detailed antibody concentrations, epitope retrieval, incubation times, and temperatures can then be systematically tested. Incubation

for most routine IHC protocols is 30–90 min at room temperature. However, for detection of signal transduction proteins, it is better to use longer incubation times with higher dilutions in order to eliminate nonspecific staining.

7. Primary antibodies can be directly labeled, but this staining is rarely used, usually due to low sensitivity. Secondary antibodies, labeled with the first step in a detection (such as biotin) system, are widely available from commercial sources and are generally of good quality. Because high quality secondary antibodies are widely available, selection is only based on the type of the primary antibody. The most important criterion is that secondary antibody must be directed against the species in which the primary antibody was raised. Furthermore, if the primary antibody is monoclonal, the secondary antibody should match the class (isotype) of the primary antibody. For example, if the primary antibody is rabbit IgG, an anti-rabbit IgG should be used.
8. Selecting an optimal secondary antibody and optimizing the immunoreactivity can improve immunostaining and reduce false-positive or -negative staining. However, especially for poorly expressed signal transduction proteins, special polymer detection systems (such as EnVision (DakoCytomation, Carpinteria, CA), ImmPRESS (Vector Laboratories, Burlingame, CA), or MACH4 (Biocare Medicals, Concord CA)) that amplify signal may be more useful.

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