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Useful Immunohistochemical Markers of Tumor Differentiation

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Abstract

Immunohistochemistry (IHC) has been somewhat underutilized in the practice of toxicological pathology but can be a valuable tool for the evaluation of rodent neoplasms, both in a diagnostic and an investigational role. Determining an exact tumor type using standard hematoxylin and eosin (H&E) staining of formalin-fixed tissues can be challenging, especially with metastatic and/or poorly differentiated tumors. Successful IHC is dependent on many factors, including species and tissue type, type and duration of fixation, quality fresh or frozen sectioning, and antibody specificity. The initial approach of most tumor diagnosis IHC applications is distinguishing epithelial from mesenchymal differentiation using vimentin and cytokeratin markers, although false-negative and/or false-positive results may occur. Experimentally, IHC can be employed to investigate the earliest changes in transformed tissues, identifying cellular changes not normally visible with H&E. Individual markers for proliferation, apoptosis, and specific tumor proteins can be used to help distinguish hyperplasia from neoplasia and determine specific tumor origin/type. IHC provides a relatively rapid and simple method to better determine the origin of neoplastic tissue or investigate the behavior or progression of a given neoplasm. Several experimental and diagnostic examples will be presented to illustrate the utility of IHC as a supplement to standard staining techniques.

Keywords

immunohistochemistry; carcinogenesis; rodent pathology

Introduction

There is widespread use of immunohistochemistry (IHC) in both clinical and veterinary diagnostic laboratories. These facilities have years of experience in the development and application of diagnostic IHC. There are many antibodies from which to choose; however, the most abundant are those raised against human antigens. IHC in toxicologic pathology has historically been limited. This has largely been a consequence of the dual constraints of time and cost on large, high-volume rodent studies, combined with the ability of pathologists to utilize hematoxylin and eosin (H&E) staining to diagnose the vast majority of lesions.

At the National Toxicology Program (NTP), a division of the National Institute for Environment Health Sciences (NIEHS), short-term (subchronic) and long-term (chronic) carcinogenicity rodent studies are high throughput, using H&E staining. Traditionally, IHC

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has played a very small role in this work, but more and more, IHC is being requested at various stages of the process, both as an aid in diagnosis and as a tool to evaluate basic mechanisms of carcinogenesis. Non-NTP intramural NIEHS scientists, however, have long used IHC as one of many tools in both carcinogenicity studies and other forms of basic research.

Basic Immunohistochemistry

This section is not meant to be a comprehensive tutorial on all the variations and intricacies of IHC but, instead, is a basic description of the process as it occurs at the NIEHS using the most common protocols and tissue fixation methods. The following general procedure would likely vary with different tissue preservation (e.g., formalin-fixed vs. frozen) and/or with antibody sensitivity and characterization (e.g., biotin conjugated vs. nonconjugated, type of retrieval if any, application time, pH, and temperature) (Key 2006).

We begin with formalin-fixed, paraffin-embedded (FFPE) tissues, processed into unstained slides. These are treated with hydrogen peroxide to quench endogenous peroxidase, which may be seen in erythrocytes and granulocytes and can be a common cause of background staining (Key 2006; Ramos-Vera 2005).

The next step has had a great impact on recent advances in IHC. Formalin fixation, especially over extended periods, causes the blockage of antigen epitopes (antigenic determinants) on the tissue surface via cross-linking, and historically this has severely limited the utility of IHC. Heat-induced epitope retrieval (HIER) acts to remove the formalin blockage, freeing the targeted epitopes to allow for successful binding (Ramos-Vera 2005). The amount of heat and the method by which it is applied varies by tissue type, by conditions under which the tissue was fixed, and by antibody specifications. The longer the time the tissue has been stored in formalin, the more intense a retrieval method may be required. However, there is an upper limit above which overheating the tissue renders IHC results unreliable. Although tissues preserved in formalin for an extended period of time may no longer have identifiable epitopes even with HIER, archival paraffin-embedded tissues may retain sufficient antigen preservation for IHC studies.

After HIER, the primary antibody is applied, and ideally the Fab region (area other than the tail of the “Y”-shaped protein) of the antibody attaches to the recently uncloaked epitope of the antigen on the target tissue. The primary antibody is usually raised in an animal species different from the species of the tissue to be examined. The next step is to apply the secondary antibody, which reacts against the exposed Fc region (the tail of the “Y”) of the primary antibody. This secondary antibody is often referred to as the “linking” antibody, and in this procedural example, an avidin-biotin complex (known as the label) is applied, and it binds to the Fc region of the secondary antibody. Next, a chromagen dye is used to visualize the antibody-antigen reaction, and a hematoxylin counterstain is applied as the last step. Now the slides are ready for viewing. Again, there are numerous methods by which IHC is accomplished and this example illustrates one of the most frequently used detection methods, the Avidin-Biotin Complex detection system (Key 2006).

The use of positive and negative controls is paramount and should be routine in IHC. Five years ago, a guest editor for this journal posed the question of immunohistochemistry results: “Is brown good enough?” (Ward 2004). The answer to that question is unequivocally no. Merely showing an image without any reference to positive or negative controls provides limited information—is the brown stain background? Nonspecific? Does it have any relevance to the question being considered in the study? The importance of positive and negative controls cannot be overstated. Negative controls confirm that the staining being reported is due to the antibody binding, and positive controls confirm that the

antibody is working and that the suspected target of the antibody is the actual target. Internal controls, normal tissues on the stained slide known to be either positive or negative for the marker in question, are excellent confirmatory evidence. While actual images of those controls need not necessarily be presented, discussion of the choice of positive and negative controls should be included in the Materials and Methods section of the manuscript. All of the results reported in the present document, as well as the images included, were compared to positive and negative controls.

General Procedure—NIEHS/NTP

At the NIEHS, IHC studies typically follow a common procedure. All projects begin with a request to the IHC group by a researcher. This researcher may be a pathologist in the NTP, or he or she could be any of the numerous scientists in the Division of Intramural Research (DIR). If the antibody requested is routine and has been performed reliably on that tissue in that species, the IHC laboratory manager will assign the project to any of a number of IHC biologists, and the stain will proceed routinely, with a general screen by the IHC manager and/or pathologist at the end of the process prior to release of the slides to the requestor.

Many requests, however, are not routine. In these cases, after the project has been assigned to an IHC biologist, an extensive literature review is conducted. This is done to determine applicability of the particular antibody or antibodies to the species and tissue in question and also to begin to determine suitable dilution ranges and detection methods. Once the literature review is completed, an antibody search/acquisition is conducted. There are numerous Web sites and online catalogs for finding and purchasing antibodies, and information sheets on these antibodies can provide further information as to the suitability, dilution range, antigen retrieval, and other techniques to assist in the development process. A particular vendor's antibody may be more suitable to the tissue/species than another, so often multiple antibodies are purchased for use.

The development process is the longest of the procedural steps. It may involve numerous attempts of trial and error. Choosing the proper negative and positive controls is very important, and most of the development is performed on these tissues rather than the more valuable case samples. Choosing the proper retrieval method, if HIER is to be done at all, elimination of background staining, and selecting an appropriate dilution for the primary and secondary antibody can take days to weeks. Some protocols allow for the use of an automated stainer, which can save time and resources during the subsequent optimization stage. Others, however, need a manual application of the various steps in the protocol. Some antibodies seem to work better with the automated system, while others show better results with the manual method.

Once initial development is complete, and the staining characteristics in the positive control tissues are acceptable to the IHC team, the researcher is typically contacted to obtain approval for final optimization and application to the case slides. This consultation is very important to ensure that all interested parties are in agreement prior to application of the IHC technique to what may be very valuable research tissues. Most often the work put into the development stage allows for a smooth application to case tissues, and slides can be stained relatively quickly, especially if an automated system is used. Whenever possible, each slide stained will have a paired negative control to ensure the validity of the stain and eliminate false-positive results. Lastly, all stained slides are reviewed by the IHC laboratory manager for general quality, and the completed project is released to either the researcher or a pathologist for evaluation.

Diagnostics

Immunohistochemical techniques may be used effectively to classify neoplasms of uncertain origin during histopathological evaluation of NTP carcinogenicity studies. For example, the classification of a lesion as “Neoplasm NOS (Not Otherwise Specified),” while uncommon, does insert a level of uncertainty into the NTP tables. The potential exists for such a classification to allow a compound to be incorrectly labeled a suspect carcinogen or incorrectly cleared of carcinogenic potential, depending on the resultant incidence data. A more specific “Carcinoma NOS” or “Sarcoma” diagnosis might not be specific enough to adequately address discrepancies in the incidence tables, and moreover the H&E classification of a poorly differentiated or anaplastic neoplasm into an epithelial or mesenchymal lineage may not be appropriate without confirmatory IHC.

Another potential use for IHC is to increase the accuracy of historical incidence tables. Previously during this meeting at the NTP Satellite Symposium, we learned of a histiocytic sarcoma that was incorrectly diagnosed as a meningioma; the correct diagnosis was made only after F4/80 IHC confirmed a histiocytic lineage for the neoplasm. While it is not practical to use IHC to confirm all previously diagnosed neoplasms, those of uncertain cellular origin or those on which the consensus of the Pathology Working Group was divided may warrant additional investigation.

The identification of a tissue of origin is of utmost importance in human or veterinary diagnostic medicine, especially when dealing with tumor metastases in distant organs. This correct identification can markedly impact the treatment and prognosis of the patient. While such direct life-or-death impacts are not relevant in toxicologic pathology, the identification of a correct tissue of origin may be important in identifying target tissues and determining accurate tumor incidences. Although the discovery of a large primary mass can provide good evidence for the histogenesis of metastatic lesions, there are times when the primary may be smaller than the metastases, or the histopathologic appearance of the metastases may differ from that of the primary. In addition, neoplastic masses may be found in a body cavity with no obvious attachment to a known organ and/or no identifiable information available in the Individual Animal Necropsy Report. While standard H&E staining will often provide a successful resolution to either of these circumstances, it may not always be the case, and IHC could therefore be employed to provide the answer or support previous suspicions.

The importance of using a broad panel of antibodies in any IHC investigation cannot be overemphasized. The situations mentioned above may give the impression that a single antibody will be definitive. This is certainly not the case. The responsible investigator will apply a range of antibodies to any unknown mass, including both those expected to be positive and those expected to be negative (Bahrami, Truong, and Ro 2008). This practice will prevent merely confirming one's bias toward a particular diagnosis and will provide a more informative and accurate evaluation.

Broad Differentiation

The first step in an IHC diagnostic investigation of a neoplasm is to allow for differentiation into any of the three major cell lineages: epithelial, mesenchymal, or hematopoietic. Certain IHC stains are well suited to this. Once the broad classification is made, additional stains can be used to further classify the neoplasm.

Cytokeratins (CK) are intermediate filaments found in epithelial cells of all types and are therefore specific markers for an epithelial cell lineage. They have been classified into subtypes numbered 1 through 20, and their expression is frequently organ- and tissue-specific. In addition to the type of epithelium, the subtypes of cytokeratins that epithelial

cells express depend on the stage in the sequence of terminal differentiation and the stage of development. Any initial screen should include “pancytokeratin,” which is a blend of CK common to most if not all epithelial tissues and is characterized by the antibody AE1/AE3 (Figure 1A). Once the initial differentiation is made, more specific individual cytokeratins such as CK7 and CK20 can be used to better characterize an epithelial tumor. These are both simple keratins with restricted tissue/neoplastic distributions. See Table 1 for the differentiation of human epithelial neoplasms using CK7 and CK20 (Bahrami, Truong, and Ro 2008; Dabbs 2006).

Vimentin is an intermediate filament that is present in most mesenchymal cells (Figure 1B). It is found in almost all sarcomas and melanomas but is variable in lymphomas and even some carcinomas (Bahrami, Truong, and Ro 2008). Therefore, it has high sensitivity and poor specificity. It may be coexpressed with CK in a wide range of carcinomas and other tumors (Table 2). This illustrates the need for a broad panel, as a positive or negative result with a vimentin stain needs to be evaluated in the context of other IHC stains.

A good broad marker for hematopoietic neoplasms is CD45, especially for (but not restricted to) those of a lymphoid lineage. As with vimentin and other screening markers, there may be variable staining results in individual anaplastic and early-stage neoplasms; this reinforces the need for a broad screening panel. Once lymphoid lineage has been determined, additional stains such as CD3 (T-cell), CD79a (B-cell), and/or PAX-5 (B-cell) can be used to better distinguish lymphoid cell types, and subsets of neoplastic T-cells can be further characterized using CD4 or CD8 markers (Figure 1C). Histiocytic sarcoma, a systemic neoplasm of histiocytes/macrophages, can be diagnosed using lysozyme, vimentin, CD68, ED1/ED2 (Figure 1D), or F4/80. Mononuclear cell leukemia (MCL), a common neoplastic finding in aged Fischer 344 rats, can be confirmed using OX-8 (Frith, Ward, and Chandra 1993). A paucity of reliable markers for MCL diagnosis may be due to the relative ease with which it is diagnosed using standard H&E staining and a rather specific host signalment. The characterization of other tumor types using various IHC markers is illustrated in Table 3.

Examples

Four examples are described below, this work having been done by the NIEHS/NTP-IHC group. These included the initial workup of a possible case of erythroleukemia in a rat, gastrointestinal stromal tumors (GISTs) versus leiomyomas in mice, uterine granular cell tumors in mice, and myoid thymoma.

Example 1. Erythroleukemia?—A female F344 rat in the untreated control group of a two-year carcinogenicity study exhibited an enlarged spleen with a neoplastic cell population in the blood vessels of numerous other organs. An initial diagnosis of mononuclear cell leukemia (MCL) was made from the H&E-stained section, but there was some uncertainty, so an IHC review of the splenic sections was requested. Among the differential diagnoses were MCL, increased extra-medullary hematopoiesis (EMH), and erythroleukemia. A plan was developed using diagnosed examples of MCL and increased EMH as comparison slides, restricting the cases selected to untreated female controls from this study. One could therefore presume similar fixation and storage conditions, tissue handling, and necropsy techniques between the case tissues and those with the confirmed diagnoses. The incidence tables from this study were used to identify animals with diagnosed MCL and EMH in the spleen, and a pathologist not involved in the initial histopathological or IHC evaluations confirmed the diagnoses using the original H&E slides. As seen in Figures 2A and B, there was disruption of normal splenic architecture in the case sample, replaced by sheets of nucleated and anucleate erythrocytes as well as large

polygonal cells with amphophilic cytoplasm and irregular large nuclei. The confirmed diagnoses of MCL also exhibited loss of normal splenic architecture, with a majority population of variably sized cells with basophilic to amphophilic cytoplasm and irregular single nuclei (Figures 2C and D). For the confirmed EMH samples, normal splenic architecture was retained, with multiple areas of red pulp containing a mixture of nucleated erythrocytes, megakaryocytes, myeloid precursor cells, and numerous macrophages distended with granular iron pigment (Figures 2E and F). Paraffin blocks from all the samples in question were retrieved, and unstained slides of spleen were obtained for IHC evaluation.

A series of IHC stains was applied to the case slides and two each of the confirmed MCL and EMH slides. Those chosen included hemoglobin (Hgb), PAX-5 (B lymphocyte marker), CD3 (T-cell marker), and ED2 (rat macrophage marker). Hgb staining in the case sample of spleen was prominent and widespread, involving the vast majority of the neoplastic cell population (Figures 3A and B). In contrast, Hgb staining of the confirmed cases of MCL was sporadic, involving scattered individual cells and clumps of cells, with most of the section showing negative or light nonspecific staining (Figures 3C and D). The EMH slides exhibited positive staining in multiple areas, corresponding to the H&E-described foci of hematopoietic cells, separated by negative zones that represented the normal white pulp of the spleen (Figures 3E and F). PAX-5 staining was negative for the neoplastic cells in the case slide; rare cells that stained positively were interpreted as residual normal B lymphocytes (Figures 4A and B). The spleens with confirmed MCL exhibited rare aggregates of patchy positive PAX-5 staining cells (Figures 4C and D). Spleens with EMH showed positive staining of the follicles and marginal zones of the white pulp as expected with normal splenic architecture (Figures 4E and F). CD3 staining showed negative results in the case samples and MCL spleens and expected periarteriolar lymphoid sheath (PALS) staining of the white pulp of the EMH spleens. ED2 staining was negative in the study case and MCL spleens, and there was patchy positive stain in the macrophages of the red pulp of the EMH spleen (partially obscured by granular iron pigment). These comparative findings between the case sample, confirmed MCL, and confirmed EMH in the spleen suggested that the neoplastic cell population of the study case was neither MCL nor severely increased EMH. Additional IHC (CD45, OX-8) and electron microscopy were suggested to further characterize the leukemic infiltrate.

Example 2. GISTs—Gastrointestinal stromal tumors (GISTs) are a recently described type of mesenchymal neoplasm often diagnosed as smooth muscle tumors (leiomyoma or leiomyosarcoma). GISTs are the most common intestinal mesenchymal neoplasm in humans, and there is ongoing research to develop appropriate animal models of this disease (Sommer et al. 2003). The application of several IHC markers to distinguish muscle tumors from GISTs is especially helpful due to the marked differences in IHC profile (Fletcher et al. 2002):

- Muscle tumors: desmin positive, smooth muscle actin positive, KIT negative, Ras negative
- GISTs: desmin very rare positive, 60%–70% smooth muscle actin negative, KIT positive, Ras positive

Desmin is a muscle-specific intermediate filament found in both smooth and striated muscle, while smooth muscle actin is a cytoskeletal contractile protein specific to smooth muscle cells. KIT (CD117) is a tyrosine kinase receptor whose mutation has been associated with carcinogenesis. Ras is a proto-oncogene involved in signal transduction.

A transgenic mouse model under investigation develops neoplasms histologically comparable to GISTs from the stomach to the colon. The H&E appearance is that of a mesenchymal/spindled tumor. IHC performed on these tumors has demonstrated vimentin, CD34, Ras, and KIT positivity in the tumor cells. Both desmin and smooth muscle actin were negative in the neoplastic tissue but positive in the adjacent intestinal musculature. Ras and KIT positivity was shown to be overexpressed from the smallest areas of hyperplasia to overt and invasive GISTs.

Example 3. Uterine Cervical Granular Cell Tumors—A recent NTP study (Veit et al. 2008) examined four mouse uterine cervical granular cell tumors (GCTs) using IHC and electron microscopy to characterize the histogenesis of this type of neoplasm. GCTs contained large polygonal cells filled with PAS positive eosinophilic granules; granules were shown to be secondary lysosomes by electron microscopy. IHC demonstrated smooth muscle actin and desmin positivity for these tumors and negative staining for neural markers such as S-100 and neuron specific enolase (NSE), suggesting a myogenic rather than neural origin. Close examination of these slides often revealed normal cells/tissues away from the negative-stained neoplasm that clearly stained positively for NSE or F4/80. These internal controls confirmed the negative staining as real and not a result of improper fixation or other artifact.

Example 4. Myoid Thymoma—The final example shown was myoid thymoma, with IHC for pan-cytokeratin positive for the specific epithelial component and desmin staining positive for the interspersed muscular component. This is an uncommon variant of thymoma. Without desmin staining, the muscular portion of this neoplasm might not be recognized.

Investigative/Experimental

The diagnosis and/or confirmation of various neoplasms represents only a portion of the carcinogenesis IHC performed at NIEHS; a majority of cancer-related IHC work is to provide support for NIEHS's intramural investigators. This is often basic science, assisting in the investigation of novel tumor markers, carcinogenic mechanisms, rodent models, and other aspects of carcinogenesis. The IHC group is a core facility and thus provides service to all branches of the institute. Some of these efforts focus on historical tissues stored at the NTP Archives, whereas others involve ongoing research projects. While carcinogenesis is a significant portion of the research efforts, noncarcinogenic mechanisms also are extensively studied, and the IHC group supports these investigations as well.

Examples

Four examples are cited below, this work having been done by both the NIEHS/NTP-IHC group and in a separate study at Colorado State University (CSU). These included the investigation of hepatic preneoplasia in F344 rats, FAM 84a and β -catenin in mouse liver, and Bmi-1 in human xenografts in immunocompromised mice.

Example 1. Foci of Altered Hepatocytes—In the first example, performed at CSU, IHC was used to determine a potential carcinogenic mechanism. Female F344 rats were exposed to several different dosages of PCB 126 and arsenic, with variable durations of exposure (Dean et al. 2002; and previously unpublished data). Rats exposed for thirty weeks had foci of altered hepatocytes (FAH), which were classified by H&E staining as eosinophilic or clear cell foci. For this investigation, morphometric analysis was performed, and the unclear borders between foci and normal hepatic tissue visible with H&E staining were insufficient for adequate tracing and quantification. IHC using glutathione-s-

transferase pi (GSTpi) was performed on the liver sections, enabling the visualization of sharp lines of demarcation between the normal liver and FAH, including the identification of positively stained individual cells. Additional IHC for transforming growth factor alpha (TGF α) and transforming growth factor beta receptor II (TGF β RII) showed that some of these GSTpi positive FAH also expressed increased TGF α and decreased TGF β RII staining compared to the surrounding normal hepatic parenchyma. This suggested that a subset of these GSTpi positive FAH possessed a competitive growth advantage over surrounding hepatic tissue due to increased expression of the growth-stimulatory TGF α and decreased expression of the growth-inhibitory TGF β RII. Therefore, these particular FAH would be more likely to grow and progress to overt neoplasia.

Example 2. Hepatocellular Neoplasia and FAM 84a—The protein “Family with sequence similarity 84 member A (FAM 84a)” is upregulated in colon cancer and may play a role in cell migration (Kobayashi et al. 2006). An NIEHS investigator was exploring its influence in hepatocellular neoplasms. The IHC group was provided with liver slides from mice exposed to a variety of hepatocarcinogens; these sections contained previously diagnosed and confirmed hepatocellular carcinomas (HCC), adenomas, and FAH. FAM 84a IHC demonstrated patchy strong staining in the HCC and adenomas, with patchy less intense staining in adjacent FAH. The surrounding nonneoplastic hepatic parenchyma was negative. This suggests that FAM 84a may play a role in chemically induced liver FAH and neoplasia.

Example 3. Hepatocellular Neoplasia and β -Catenin—Beta-catenin (β -catenin) is a subunit of E-cadherin and functions in epithelial cell adhesion. β -catenin also acts as a transcription factor as part of the Wnt signaling pathway and can play a role in cancer progression. Hepatocellular carcinomas from archived liver samples were provided for β -catenin IHC. As seen in Figure 5, there is focal loss of the typical membrane staining pattern (present in normal liver and most of the HCC tissue), with a shift of staining to a cytoplasmic/nuclear pattern. This pattern shift suggests the migration of β -catenin to the nuclear compartment of the cell, where it can influence transcription and result in a more malignant cell phenotype (Thompson and Monga 2007).

Example 4. Neoplasia and Bmi-1—Bmi-1 is a transcriptional regulator protein that normally targets the tumor suppressor proteins p16 and p19. Its overexpression is implicated in carcinogenesis, and it can be found amplified in various neoplasms (Datta et al. 2007; Sasaki et al. 2006). Positive nuclear staining typical of Bmi-1 was demonstrated in an implanted tumor cell line in SCID mice, indicating that Bmi-1 overexpression may play an important role in the carcinogenicity of this cell line.

There are numerous other antibodies used by the NIEHS/NTP-IHC group routinely in carcinogenesis investigations. Table 4 lists the most common and/or most recently used markers, roughly categorized by group/function. This is not meant to be a complete list of all potential immunohistochemical markers, but merely those performed by our group.

Challenges

A large portion of the samples received for processing and staining by the NIEHS/NTP-IHC group are retrieved from the NTP Archives and may have been stored in formalin for long periods. This often results in either tissues falsely negative for the marker of interest despite positive tissue controls, or unreliable results with nonspecific staining (e.g., nuclear vs. cytoplasmic vs. membranous pattern). Tissues preserved in paraffin blocks allow for easier epitope retrieval, although initial fixation times often vary between studies and may vary within a study. The preferred timeline for processing of formalin-fixed tissues is twenty-four

hours in 10% neutral buffered formalin, followed by transfer to 70% histology-grade ethanol and storage at 4°C until processing and embedding in paraffin. It is often very difficult to obtain reliable IHC results from tissues that have been stored in formalin for long periods of time, although this may vary by the tissue type and by the antibody being used. Vimentin is a marker often used to check for loss of signal due to long-term fixation (Dabbs 2006), although this is not foolproof. The recommendation to utilize a broad panel of IHC markers rather than individual markers is especially applicable in these cases. The HIER techniques discussed earlier can be modified to unmask epitopes in tissues stored in formalin for extended periods, although there is the ever-increasing risk of overretrieving the tissue, resulting in overwhelming background stain.

In addition to overapplication of HIER, other common causes of nonspecific antibody binding (background staining) are improper dilution, using antibody raised in the same species as the tissues being investigated, and general errors in technique. This can be described as poor signal-to-noise ratio, with the best IHC techniques having excellent signal and no background noise. Methods to combat this issue vary from using a wide range of dilutions during development to use of commercial “mouse-on-mouse” background reduction kits, to co-incubation of primary antibodies with thiol-reactive compounds such as reduced glutathione (GSH) (Rogers, Cormier, and Fox 2006).

IHC is subject to the balance between the precision of diagnosis and the costs in terms of time and resources necessary to achieve that precision. Toxicologic pathology IHC often lacks the sense of urgency inherent to surgical pathology IHC—there is no patient on the operating table awaiting a prognosis or determination of intraoperative palliative versus curative treatment. Over the long term, however, devoting the time and resources to utilize IHC in toxicologic pathology investigations may pay future dividends in the proper identification of hazardous and/or carcinogenic compounds either at the preclinical stage of development or after widespread clinical and/or environmental exposures, potentially preventing large-scale clinical illness. For NTP studies, the ideal time to consider using IHC techniques is either during the initial histopathological evaluation at the study laboratory or during pathology peer review (PPR). This ensures that any PPR will have all of the pertinent data in hand prior to final evaluation of the substance in question.

The final challenge to be commented on is the application of human IHC markers to laboratory animals. Most if not all of the antibodies discussed in this document have proven utility in human tissues, but there may be wide variation once applied to veterinary tissues. What works well in the dog may not work in the rat, and even that which works in the rat may not work in the mouse. Any given antibody may vary not only between species but also between tissue types, fixation methods, and even commercial distributors and lot numbers. This variability is yet another reason for the extended development time and an increased cost required to properly investigate all but the most routine antibodies, and underscores the importance of having a dedicated team of researchers, pathologists, biologists, and technicians.

About the Laboratory

The Immunohistochemistry Group is part of the Cellular and Molecular Pathology Branch (CMPB) of the NTP and NIEHS. It is a core laboratory, serving both NTP and NIEHS investigators and collaborators. IHC is the most widely used specialized technique provided by CMPB, although immunofluorescence and electron microscopy are also routinely utilized. The CMPB-IHC Web site is <http://www.niehs.nih.gov/research/atniehs/labs/lep/path-support/immuno/index.cfm>. At this site, one can research antibody protocols and specifications, learn what routine antibodies

are offered, identify sources of reagents, and view photomicrographs of many of the various markers.

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Figure 1.

IHC presentation of positive control tissues for selected markers. All are at 10X magnification, except for Figure 1B at 20X magnification. (A) Pancytokeratin staining of the epithelial tissues of the nonglandular stomach. (B) Vimentin staining of the glomeruli and adjacent perirenal vasculature. (C) CD79a staining of the spleen. Note presence of B lymphocytes in the marginal zone and follicles of the white pulp. (D) ED2 staining of the macrophages in the red pulp of this rat spleen.

Figure 2.

Comparative H&E staining of potential erythroleukemia in a negative control rat spleen, compared to confirmed MCL and EMH in the spleen of two other control rats. Figures A, C, and E are at 10X magnification; Figures B, D, and F are at 40X. (A, B) Case slide—Disruption of normal architecture, large numbers of nucleated erythrocytes and large polygonal cells. (C, D) MCL—More severe disruption of splenic architecture, large numbers of mononuclear neoplastic cells. (E, F) EMH—Normal splenic architecture, with multifocal aggregates of hematopoietic cells and accumulated yellow iron pigment.

Figure 3.

Comparative Hgb staining of potential erythroleukemia in a negative control rat spleen, compared to confirmed MCL and EMH in the spleen of two other control rats. Figures A, C, and E are at 10X magnification; Figures B, D, and F are at 40X. (A, B) Case slide—Most neoplastic cells are positive for Hgb. (C, D) MCL—Hgb positive cells are uncommon, other than some nonspecific light staining. (E, F) EMH—Aggregates of Hgb positive cells match up with the multifocal hematopoietic tissue seen with H&E staining in Figure 2.

Figure 4.

Comparative PAX-5 staining of potential erythroleukemia in a negative control rat spleen, compared to known MCL and EMH in the spleen of two other control rats. Figures A, C, and E are at 10X magnification; Figures B, D, and F are at 40X. (A, B) Case slide—All neoplastic cells are negative for PAX-5. (C, D) MCL—PAX-5 positive cells are present as infrequent loose aggregates. (E, F) EMH—Multifocal dense accumulations of PAX-5 positive cells match up with known B lymphocyte areas in the white pulp of the spleen with normal architecture.

Figure 5.
β-catenin staining of hepatocellular carcinoma, 10X magnification. Note the focal transformation of positive stain from the relatively normal membranous pattern to a potentially more malignant cytoplasmic and nuclear pattern.

Table 1 Differentiation of human epithelial neoplasms based on CK7/CK20 staining. S100-, HMB45-, CD45-.^a

CK7+/CK20+	CK7-/CK20+
Transitional cell carcinoma	Colorectal adenocarcinoma
Urothelial carcinoma	Merkel cell carcinoma
Pancreatic carcinoma	Gastric adenocarcinoma (subset)
CK7+/CK20-	CK7-/CK20-
Endometrial adenocarcinoma	Prostate adenocarcinoma
Mesothelioma (2 of 3)	Renal cell carcinoma
Cholangiocarcinoma	Squamous cell carcinoma
	Hepatocellular carcinoma
	Mesothelioma (1 of 3)

^aMust be S100/HMB45/CD45 negative to rule out melanoma (S100, HMB45) and hematopoietic (CD45) tumors.

Table 2

Neoplasms with cytokeratin/vimentin coexpression.

Some carcinomas

- Renal, endometrial, thyroid, some ovarian

Other carcinomas don't reliably express vimentin

- Colonic, small intestinal, prostate, transitional cell

Synovial cell sarcoma

Mesothelioma

Carcinosarcoma

Table 3

IHC determination of other tumor types.

Peripheral Nerve Sheath Tumors (PNST)	Muscle Tumors
• Examples: Schwannoma, Neurofibroma • Neural markers: S100, Leu-7, Protein Gene Product 9.5	• Vimentin & desmin for broad characterization • Smooth muscle actin for smooth muscle • Myogenin for skeletal muscle, with PTAH to confirm cross-striations
CNS Tumors	Neuroendocrine Tumors
• In rodents, GFAP reliably stains reactive astrocytes but not neoplastic astrocytes as would be seen in an astrocytoma, although there remains some disagreement here.	• Synaptophysin • Chromogranin

Table 4

Routine and/or common antibodies utilized by the NIEHS/NTP-IHC group.

Tumor Suppressors, Apoptosis	Cell Cycle Proteins
• p53 • APC • TEN • Caspase-3	• Cyclin D1 • Ki-67, PCNA, BrdU • p15, p19, p21
Transcription Regulators	Leukocyte Markers
• NF-kappa B • HDAC 1/2 (histone deacetylase) • NELF-E • WT-1	• CD3 • PAX-5, CD79a • F4/80, ED2 (CD163)
Other	Angiogenesis
• Chromogranin A, Synaptophysin • S100, Neuron-specific enolase (NSE) • Desmin, Smooth muscle actin • MMP-9 (matrix metalloproteinase) • ER alpha, beta • Uroplakin III	• VEGF • CD31/PECAM • Factor VIII