Review Article
Immunohistochemistry in diagnostic surgical pathology: contributions of protein life-cycle, use of evidence-based methods and data normalization on interpretation of immunohistochemical stains

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Abstract: Immunohistochemical (IHC) staining of formalin-fixed and paraffin-embedded tissues (FFPE) is widely used in diagnostic surgical pathology. All anatomical and surgical pathologists use IHC to confirm cancer cell type and possible origin of metastatic cancer of unknown primary site. What kinds of improvements in IHC are needed to boost and strengthen the use of IHC in future diagnostic pathology practice? The aim of this perspective is to suggest that continuing reliance on immunohistochemistry in cancer diagnosis, search and validation of biomarkers for predictive and prognostic studies and utility in cancer treatment selection means that minimum IHC data sets including “normalization methods” for IHC scoring, use of relative protein expression levels, use of protein functional pathways and modifications and protein cell type specificity may be needed when markers are proposed for use in diagnostic pathology. Furthermore evidence based methods (EBM), minimum criteria for diagnostic accuracy (STARD), will help in selecting antibodies for use in diagnostic pathology. In the near future, quantitative methods of proteomics, quantitative real-time polymerase chain reaction (qRT-PCR) and the use of high-throughput genomics for diagnosis and predictive decisions may become preferred tools in medicine.

Key words: Immunoperoxidase, protein lifecycle, surgical pathology, proteomics, evidence based methods, normalization

Introduction
Immunohistochemical methods in diagnostic pathology have a long history [1, 2]. Immunohistochemical staining methods include use of fluorophore-labeled (immunofluorescence) and enzyme-labeled (immunoperoxidase) antibodies to identify proteins and other molecules in cells. In diagnostic surgical pathology, immunoperoxidase methods (usually single antigen-antibody and less commonly double antibody-antigen combinations) (Figure 1) are widely used to extract additional information that is not available by hematoxylin and eosin staining and light microscopy or by transmission electron-microscopy. The advantage is that the molecules are identified in-situ in the cell. Immunohistochemistry is now used in surgical pathology to determine cancer cell types, cancer subtype classifications and possible cell-of-origin in metastatic cancer of unknown or undetermined primary site. In all instances, accepted and standardized morphologic criteria are used in addition to immunohistochemical staining of the tissue. The morphologic criteria for cancer diagnosis do not encompass the proposed biologic hallmarks of cancer [3].

This perspective is to review and promote the inclusion of some information to improve the interpretation of immunohistochemical data such as protein life-span and signaling, evidence-based methods and quantitative data and normalization.
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Protein structure, modifications, life-span and implications for Immunohistochemistry

Protein synthesis in the cell is highly regulated [4]. The proteins undergo many modifications before full maturation and functional activation. Life-span modifications in normal, stressed and cancer cells include sumoylation and ubiquitination and subsequent degradation in the proteosome and probably rescued by de-ubiquitination, by chaperones and chaperonins [5-7] and the effects of microRNA [8]. A widely known functional modification is phosphorylation that occurs on serine and threonine amino-acids, and these changes may affect life-span [9]. There are numerous protein databases that are freely available that permit inquiry of protein structure, cellular and tissue distribution, developmental and evolutionary history, functional status, mutations and other relevant information [10]. Furthermore, since synthetic peptides are frequently used for generating antibodies (mono-and polyclonal), the functional significance and contribution of the peptide segment and structural information in relation to the function of the whole molecule should be taken into account when interpreting the immunohistochemical staining result. Phospho-specific antibodies are now available for immunohistochemical use to determine the functional status of the protein and their use may further improve the results of immunohistochemical staining [11]. The productive use of phospho-specific antibodies will rest heavily on further elucidation of the cellular phospho-proteome [12] and optimization of phospho-specific polyclonal and monoclonal antibodies and tissue processing [13]. The p53 Example (Figure 2 a-c): One of the most investigated proteins in cell biology and pathology is p53. As an example, p53 is altered in many human cancers (>18,000 mutations) and involved in cell death and survival, DNA damage response [14, 15] and affects the transcription of a large gene/protein set in the cell [16]. p53 undergoes many modifications as wild-type or mutant protein and influences its cytoplasmic or nuclear location [17-20], the function and life-span of p53 and cellular interactions with its known and unknown targets and their function [21]. There are now competing and continually improving methods of proteomics to quantify and determine presence of protein(s) in cells [22-25]. Proteomics is useful in searching for and defining biomarkers using high-throughput methods such as the whole cell proteome.

Figure 1. a. Human prostate core biopsy with double immunohistochemical staining for high molecular weight cytokeratin (K903) and AMCAR (alpha-methyl-CoA-racemase). The dark brown stain (K903) highlights the basal epithelial cells and the light brown cytoplasmic stain AMCAR in prostate cancer cells including dysplastic cells in high-grade prostatic intraepithelial neoplasia (HGPIN). The differential localization and distribution are useful in confirming areas of invasive carcinoma (x40) in addition to conventional criteria for malignancy. b. Bcl-2 (anti-apoptotic protein) and Ki-67 in human lung carcinoma (courtesy Epitomics,Inc). This also highlights differential localization of the two proteins; Bcl-2 to cytoplasm and Ki-67 nuclear and also suggests that Ki-67 staining cells are different from Bcl-2 staining cells and the transcription cycle of the proteins.
Diagnostic and predictive biomarkers

Biomarkers are currently proposed for various aspects of cancer such as early detection to selection of cancer patients for treatment. The biomarkers can be detected by immunohistochemical methods, quantitative proteomic methods and methods such as quantitative real-time polymerase chain reaction (qRT-PCR) [26]. The promotion of molecular and individualized medicine is based on the improvement and miniaturization of methods of proteomics and genomics in the search for biomarkers of disease onset, progression and treatment response [27]. A recent commentary also emphasizes the need to base the use of markers in diagnostic or predictive immunohistochemical staining on known biological pathways and underlying biology of the cancer or disease process [28]. Furthermore, there is
growing interest in including defined biomarkers in clinical trials [29].

**Evidence-based methods (EBM) and standards for reporting of diagnostic accuracy (STARD)**

Another question is whether the rules of Evidence-based medicine (EBM) that are adopted in other sections of laboratory medicine (clinical chemistry) can be applied to immunohistochemical interpretation before adoption for routine use [30, 31]. The rules of EBM applied to laboratory values include agreement statistics (raw, kappa, expected and odds ratio), confidence intervals, sensitivity, specificity, positive and negative predictive values, likelihood ratios, pre- and post-test probabilities; all of these datasets are useful for estimating diagnostic accuracy and are used in other diagnostic settings [32]. The STARD criteria include 24 item check list. Including EBM and STARD criteria may reduce bias in their use in diagnosis or treatment selection. The proposal for minimum datasets in immunohistochemical publications (MISFISHIE) is encouraging, although quantitative analysis and protein structure data are not now included. Many publications provide immunohistochemical analysis as percentage of cases and control that are positive and negative for the antigen/protein under study. A recent study on immunohistochemical markers for mesothelioma listed the markers and percentage positive in the cases used but no sensitivity or specificity information [33]. Few studies provide sensitivity and specificity analysis; one recent study on lymphatic markers provided these analyses [34]. A recent study on use of markers for defining the possible primary site of metastatic adenocarcinomas had some markers with variable sensitivities and specificities [35]. The drawbacks of immunohistochemical staining that include inadequate antibody validation and many technical issues with a host of suggestions have been highlighted [36]. Some drawbacks of conventional immunohistochemical staining include lack of multiplexing, limited dynamic range and lack of correlation with functional protein and treatment response [36, 37]. The use of rabbit monoclonal versus mouse monoclonal antibody to estrogen receptor (ER) changed the level of positivity in breast cancer [38] (see **Figure 3**).

**Normalizing IHC scores**

How do we determine protein content in tissues? There are no reliable methods to quantify tissue protein content by immunohistochemical methods. Many authors use different methods to estimate protein / antigen level in the literature [39] including intensity levels (0-3) or percent of cells that stain or a combination of the two scoring methods and attempts at cutoff values. Many suggestions relating to quantitative methods in immunohistochemistry relate to its impact in high-throughput methods such as tissue microarray (TMA) [39, 40]. In a recent study, simulated mRNA levels were related to possible levels of protein detected by immunohistochemistry [41]; these methods will be difficult on an individual case based on protein life-span, modifications and mutations, tissue retrieval and fixation and other limitations. A study of Her-2 in breast cancer cell

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**Figure 3.** Comparison of rabbit and mouse monoclonal antibodies in immunohistochemical detection of Her-2 (courtesy of Epitomics, Inc). The differences in intensities and percentage of cells stained is notable.
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lines and tissue highlights the contribution of the primary antibody dilution on the level of Her-2 protein detected by immunohistochemical methods [40] especially as Her-2 belongs to a protein family with complex interacting networks [42]. Unlike routine diagnostic immunohistochemical methods, high-throughput tissue microarrays, protein and DNA microarrays generate a lot of data. The methods of data analysis and presentation proposed for DNA and protein microarrays- including methods to remove noise in the data such as normalization, false and negative discovery rates [43-47] are designed to improve interpretation, and utility of the information. Can bioinformatics tools to determine such cut-offs [49] and these attempts created different estimates for HER2 that are different from the standardized criteria for HER2 [50]. Image analysis computer programs that can be used easily are needed [51, 52] and [53] and are coming on-stream [54, 55]. A recent overview of quantitative image analysis software for immunostaining lists several commercial sources though costs may be a limitation to adoption of specific software [56]. Furthermore, as the interest in computer-assisted image analysis grows within the surgical pathology community an awareness of the multiple methods of image analysis, noise remov-

Table 1. Summarized Comparison of Immunohistochemical Method and Liquid Chromatography-Mass Spectrometry (LC-MS) in Tissue Proteomics

<table>
<thead>
<tr>
<th>Method of Protein Detection</th>
<th>Advantages</th>
<th>Drawbacks</th>
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<tbody>
<tr>
<td>Immunohistochemistry</td>
<td>Protein location and distribution seen</td>
<td>Limited ability to quantitate protein content</td>
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<tr>
<td></td>
<td>Detectable in small and large tissue biopsies and fixed tissues</td>
<td>Problems with antibody types, limited ability to detect protein modifications</td>
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<td></td>
<td>Validation of other high-throughput studies (DNA microarray)</td>
<td>Limited or lack of Evidence based Criteria</td>
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<td>Other Proteomics (i.e LC-MS)</td>
<td>100's to 1000's of peptides and proteins detected</td>
<td>Single or dual detection ability</td>
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<td></td>
<td>Can peruse databases for protein function and Gene ontology</td>
<td>Variable scoring methods and reproducibility</td>
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<tr>
<td></td>
<td>Robust Bioinformatics</td>
<td>No normalization methods</td>
</tr>
<tr>
<td></td>
<td>High throughput</td>
<td>Limited throughput</td>
</tr>
<tr>
<td></td>
<td>High level quantitation</td>
<td>Limited capacity for clinical biomarker profiling (only with tissue microarrays)</td>
</tr>
<tr>
<td></td>
<td>Can detect modified proteins</td>
<td>Cannot locate identified peptides to cell type(s)</td>
</tr>
<tr>
<td></td>
<td>Great potential for use in detecting clinical biomarkers</td>
<td>Need fresh or frozen tissue samples</td>
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normalization be used in immunohistochemical evaluation and what methods can be used for normalization? One can use endogenous proteins for normalization as is used in northern blots for messenger ribonucleic acid (mRNA) levels [48] and for Western blotting and quantitative real-time polymerase chain reaction (qRT-PCR). A relative protein expression level can then be used. What are the minimum methods to quantify protein/antigen levels in the cell? Some investigators used al, image quality, and their effects on the results should be noted [57, 58]. The DAB-stained slides can be analyzed by spectral imaging [59], color deconvolution [54, 55, 60, 61], Hue-Saturation-Intensity [61], normalized RGB [62] and CMYK [63] and other methods. In a recent study of predictive biomarkers in breast cancer, automated image analysis was necessary to use 42 antibodies in the assessment of marker utility [64]. The growth of many image analysis methods for the popular
DAB-stained tissues need internal normalization as done for RT-PCR and western blotting to truly compare the results.

**Alternative methods for biomarker identification and selection**

The role of IHC data sets and analysis in diagnostic pathology are being challenged by other quantitative methods such as DNA microarray and qRT-PCR in cancer detection, classification and predicting cancer treatment response. Recently proposed molecular classifications of cancers and their use in cancer treatment planning are based on DNA microarray methods that have well-defined methods and analysis [65-68] and in some cases new entities unknown by light microscopic methods have emerged. The DNA microarray methods have been used to separate primary and secondary cancers in lung [69] and separate colonic from ovarian cancer origins [70] and to determine cancer of unknown primary sites [71]. The future of a needle core biopsy of suspicious mass may be (a) routine hematoxylin and eosin, immunohistochemistry including normalization and analysis, EBM and STARD (b) isolation of protein content for 2-dimensional gel electrophoresis and western blotting, protein and antibody arrays and mass spectrometry (c) isolation of total messenger ribonucleic acids (mRNA), cDNA synthesis, microarray expression studies, single nucleotide polymorphism (SNP) and array comparative genomic hybridization (array CGH) and copy number variation (CNV) of genes (Table 1). The continued use and dependence on immunohistochemical staining in diagnostic surgical pathology will need the use of EBM and STARD methods and minimum datasets and integration of protein networks and function, and image analysis with normalization or definable cut-offs.

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**References**

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