Immunohistochemistry Innovations for Diagnosis and Tissue-Based Biomarker Detection

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Abstract

Purpose of Review Immunohistochemistry is an integral technique for tissue-based diagnostics and biomarker detection with broad worldwide adoption. Advances in core chemistries, antibody design, and automation have ushered unprecedented sensitivity, specificity, and reproducibility in immunohistochemistry assays. As a result, clinical immunohistochemistry assays that utilize dual-color approaches and mutation-specific antibodies provide novel tools in clinical diagnostics that until recently were in the realm of investigational research. This review provides an overview of innovations in clinical immunohistochemistry assays with emphasis on those used for patients with hematopoietic neoplasms.

Recent Findings Advances in clinical-grade immunohistochemistry techniques have allowed labs to develop and validate multiplex assays that improve diagnostic utility—such as CD5/PAX5 and TCF4/CD123 dual-color stains—and have the potential to enhance the specificity of biomarker detection. In addition, the increased availability of immunohistochemistry assays that detect mutant proteins (e.g., BRAF V600E and IDH1 R132H) provides a helpful replacement and/or adjunct for molecular testing. These techniques are highly reproducible, entail reasonable technical and interpretation complexity, and are relatively cost-effective, making them valuable novel tools in modern cancer care.

Summary Multiplex and mutation-specific immunohistochemistry assays represent important innovations that provide improved utility in the context of personalized medicine and targeted therapy.

Keywords Dual immunohistochemistry · Multiplex immunohistochemistry · Mutation-specific antibody · BRAF V600E · CD123 · IDH1 · LEF1 · PAX5 · TCF4

Introduction

Immunohistochemistry is an integral technique for tissue-based diagnostics and biomarker detection that is broadly utilized worldwide. It is mostly applied in conjunction with morphologic evaluation as a diagnostic tool, and in the context of cancer diagnostics, it serves as a robust and cost-effective

platform for integral biomarker detection [1••]. Increasingly, immunohistochemistry markers for mutant proteins are becoming available. Prognostication and minimal residual disease detection may also be accomplished by immunohistochemistry [2, 3].

The origins of immunohistochemistry have roots in immunofluorescence and similar tissue-based techniques for visualizing protein antigen expression via antibody-reporter complex binding. The use of fluorochrome-conjugated antibodies was first developed by Coon et al. in 1941 [4]. Other visualization techniques included ferritin, radioactive agents, and heavy metals. Efforts over the ensuing decades saw the replacement of most of these approaches by current immunohistochemistry technologies whose core principle involves localized enzyme-catalyzed production of a chromogenic signal visible in situ by routine light microscopy. Thus, it was not until approximately half a century after the initial efforts of Coon that immunohistochemistry as we know it today become practical for general usage [5].

This article is part of the Topical Collection on *Molecular Testing and Diagnostics*

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In this review, we provide an overview of innovative approaches in diagnostic immunohistochemistry, with particular emphasis on uses in hematopoietic neoplasms. We describe dual-color immunohistochemistry combinations as well as mutation-specific immunohistochemistry assays currently utilized in clinical practice.

Principles of Immunohistochemistry

Immunohistochemistry is based on antibody binding to a tissue-based antigen typically within formalin-fixed paraffin-embedded (FFPE) tissue sections. The antigen is usually a protein or glycoprotein located within one or more subcellular compartments, such as the cell membrane, cytosol, mitochondria, and/or nucleus. The primary antibody may be monoclonal or polyclonal, usually of the IgG class, produced by immunizing a mammalian host such as mouse, rabbit, goat, or horse. Monoclonal antibodies are typically generated using hybridoma technologies whose details are beyond the scope of this review [6]. Immunohistochemistry assays used in clinical practice most commonly involve the use of a species-specific secondary antibody. Through biotin-avidin binding, a peroxidase-containing polymer is brought in proximity to the antigen-antibody complex to effectuate precise generation and deposition of chromogen molecules in the immediate vicinity of the complex. The most commonly used chromogen is 3,3′-diaminobenzidine tetrahydrochloride (DAB), a stable brown pigment. Chromogens of different color, including 3-amino-9-ethylcarbazole (AEC) and Fast Red®, result in a red pigment. Several steps preceding and following the application of the primary antibody have been optimized over the years to improve antibody binding (antigen retrieval), reduce background reactivity, and enhance the clarity of the signal. Antigen retrieval is achieved using heat or enzymatic protein digestion methods. Reducing background reactivity is commonly achieved using blocking agents to neutralize endogenous biotin and peroxidases. Dual-color immunohistochemistry, which entails the use of two (or more) chromogens commonly entails tandem antigen retrieval and primary antibody application steps.

The selection of antibodies for dual-color immunohistochemistry stains should be done with careful consideration of the subcellular localization of the target proteins. Most commonly, one of the proteins is restricted to the cell surface and/or cytosol whereas the second protein is restricted to the nucleus.

A number of pre-analytic, analytic, and post-analytic factors can impact the quality of immunohistochemistry stains. Efforts to minimize the impact of such factors are built into routine laboratory practices, codified in the USA by certification under the Clinical Laboratory Improvement Amendments of 1988 (CLIA) and accreditation by agencies such as the College of American Pathologists (CAP) [1••]. A number of

guidelines have also been published to further improve immunohistochemistry standardization and reproducibility, particularly in the context of biomarker evaluations that guide therapy decisions [7•]. Ongoing quality control and quality improvement should be integral to any clinical immunohistochemistry operation, ensuring testing stability over time, instruments, and laboratory operators [1••].

The use of digital image analysis to assist in the evaluation of immunohistochemistry assays is well-established. Whole-slide imaging technologies coupled with developments in artificial intelligence represent a new frontier for which immunohistochemistry is particularly well-suited. A detailed discussion of these topics is beyond the scope of this review.

Multiplex Immunohistochemistry Assays

CD3 and CD20

CD3 and CD20 are core lineage markers expressed by T-cells and B-cells, respectively. Both markers have been a mainstay of tissue-based diagnostic evaluation, particularly in the evaluation of lymphomas and leukemias. CD3 is a multimeric protein complex comprised of four polypeptide chains—epsilon (ε), gamma (γ), delta (δ), and zeta (ζ), which assemble and function as three pairs of dimers ($\varepsilon\gamma$, $\varepsilon\delta$, $\zeta\zeta$). The CD3 complex associates non-covalently with the T cell receptor (TCR), a critical actuator of T cell function [8]. CD3 is a pan T-lineage-defining marker, expressed on the surface and/or in the cytoplasm of normal and neoplastic T cells throughout all stages of T cell differentiation. Other markers associated with T-lineage differentiation include CD2, CD4, CD5, CD7, and CD8. With rare exceptions, surface and/or cytoplasmic CD3 expression is identified uniformly in mature T cell lymphoma and T lymphoblastic leukemia/lymphoma [9–11].

CD20 is a non-glycosylated tetraspanin of the membrane spanning 4-A family whose two extracellular loops contain the epitopes recognized by most anti-CD20 antibodies [12]. CD20 is regarded as a specific B-lineage marker, alongside CD19, CD79a, PAX-5, and CD22, and it is expressed on the surface of normal and neoplastic B cells throughout nearly all stages of B cell differentiation. CD20 is the target of rituximab [13]. CD20 is almost universally positive in non-Hodgkin lymphoma and in sizeable subsets of Hodgkin lymphoma and B lymphoblastic leukemia/lymphoma [13, 14].

Dual-color immunohistochemistry provides a practical tool for simultaneous evaluation of T cell and B cell populations, since these populations express CD3 and CD20 in a mutually exclusive manner, respectively. This approach permits assessment of the distribution of these populations within tissue compartments, such as in the evaluation of lymph node tissue. CD3/CD20 dual-color immunohistochemistry can provide valuable information in the initial work-up of a tumor comprised of small

round cells, particularly in limited biopsy material. In addition, CD3/CD20 dual-color immunohistochemistry can provide diagnostically useful insight into the spatial distribution of T cells and B cells in lymphomas that do not lend themselves easily for flow cytometry immunophenotyping, such as nodular lymphocyte-predominant Hodgkin lymphoma and angioimmunoblastic T cell lymphoma [15–17].

Some limitations of CD3/CD20 dual-color immunohistochemistry should be pointed out. Lack of both CD3 and CD20 expression may be seen in some types of lymphoma; thus, the stain may not by itself definitively exclude the presence of lymphoid neoplasia. Such a scenario might be seen in plasmablastic lymphoma, anaplastic large cell lymphoma, and/or B lymphoblastic lymphoma/leukemia. On the other hand, rare B cell lymphomas might express CD3, and conversely, rare T cell lymphomas might express CD20 [18, 19].

CD5 and PAX5

The *PAX5* gene encodes a 52-kD protein of the same name (also known as B cell-specific activator protein) that is a member of the paired-box containing (*PAX*) family of transcription factors. The PAX5 protein plays a critical role in B cell development, and its expression spans B cell ontogeny from the pro-B to mature B cell stages, with notable downregulation in plasma cells. PAX5 is generally considered the most sensitive and specific marker for B cell lineage in tissue sections [20].

Encoded by a gene located on chromosome 11q12.2, the CD5 protein is a member of the scavenger receptor cysteine-rich (SRCR) superfamily of membrane-anchored proteins whose expression spans T cell ontogeny from the pro-T to mature T cell stages. CD5 is expressed normally on a small subset of B cells. The encoded protein contains three SRCR domains and is believed to act as a regulator of T cell proliferation. CD5 also enhances B cell survival through autocrine interleukin-10 production [21]. Aberrant CD5 expression is a hallmark of several B cell neoplasms, including chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL) and mantle cell lymphoma [22]. In addition, a number of low-grade and high-grade B cell neoplasms show occasionally aberrant expression of CD5 [23–26].

The combination of CD5 (DAB chromogen) and PAX5 (Fast Red® chromogen) in a single dual-color immunohistochemistry stain thus offers a number of advantages. The stain allows simultaneous detection of T-cells by virtue of CD5 expression in a membranous staining pattern, and detection of B cells by virtue of PAX5 expression in a nuclear pattern. Furthermore, in B cell neoplasms with aberrant CD5 expression, the stain permits the identification of co-localized CD5/PAX5 signals by neoplastic B-cells (Fig. 1). The latter feature makes CD5/PAX5 immunohistochemistry a better tool for evaluation of lymphoid neoplasms than the use of dual-color

CD3/CD20 immunohistochemistry. The stain has been in use at our institution since 2014, without notable downsides. On occasion, dim CD5 expression detectable by flow cytometry immunophenotyping might not be detectable by immunohistochemistry. This discordance is uncommon and relates to the inherent higher sensitivity of fluorescence signal detection in flow cytometry.

CD19 and LEF1

LEF1 belongs to the LEF/TCF family of transcription factors and is involved in lymphocyte development. LEF1 is expressed normally in T cell and B cell precursors. Distinctive overexpression of LEF1 in CLL/SLL was initially detected by gene expression profiling. Subsequent studies using immunohistochemistry showed that LEF1 expression correlates with CLL/SLL with high accuracy (70% sensitivity and 92–100% specificity) [27, 28]. LEF1 expression may be decreased when CLL/SLL tumor cells undergo plasmacytic differentiation [29].

LEF1 is expressed by T cells and a sizeable subset of hematopoietic precursors in the bone marrow (unpublished data), rendering distinction between LEF1 expression by CLL/SLL cells and background elements challenging at times. To circumvent this limitation, CD19/LEF1 dual-color immunohistochemistry permits the evaluation of LEF1 staining specifically within the B cell compartment. The combination of LEF1 (DAB chromogen) and CD19 (Fast Red® chromogen) permits more precise evaluation of LEF1 expression in B cells, a feature that supports CLL/SLL in conjunction with other findings such as CD5/PAX5 coexpression.

CD123 and TCF4

Blastic plasmacytoid dendritic cell neoplasm (BPDCN) is a rare malignant hematologic neoplasm that arises from precursors of plasmacytoid dendritic cells (pDCs) [30]. BPDCN is characterized by the expression of the pDC-associated markers CD123 and TCF4.

CD123, the interleukin-3 (IL-3) receptor α-chain, is the low-affinity subunit of the IL-3 receptor and promotes high-affinity binding to IL-3 when co-expressed with the β-subunit. CD123 is encoded by a 40 kilobase gene that maps to pseudo-autosomal regions of the X and Y chromosomes. CD123, a 43.3 kDa single-pass transmembrane protein, is characteristically expressed at a high level in pDCs and BPDCN. CD123 is expressed in other hematologic malignancies, including lymphoblastic leukemia/lymphoma [31], hairy cell leukemia [32], acute myeloid leukemia [33, 34], and systemic mastocytosis [35]. Due to its lack of specificity, the use of CD123 as a marker for BPDCN has been supplemented by other markers, including CD4, CD56, CD303, and TCL-1, in the absence of lineage-specific markers including CD3, CD19, and myeloperoxidase [36].

Fig. 1 CD5/PAX5 dual-color immunohistochemistry. **a** Normal tonsil showing PAX5 staining of B cells within follicles and CD5 staining of T cells in interfollicular areas (100×). **b** Bone marrow involved by chronic lymphocytic leukemia/small lymphocytic lymphoma comprised of neoplastic cells with CD5 (red) and PAX5 (brown) coexpression. Note occasional CD5+ T cells lacking nuclear brown signal (400×)
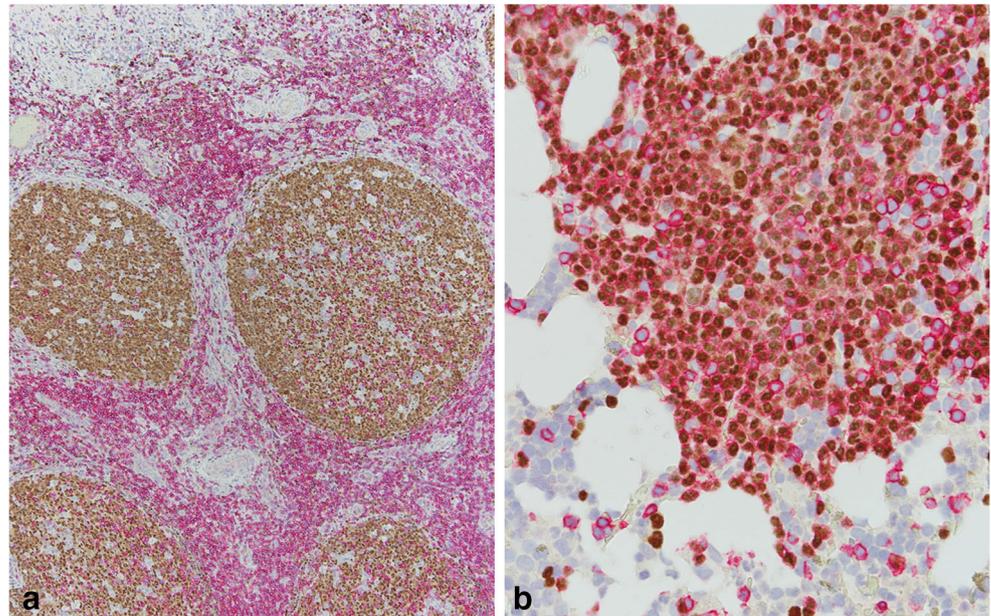
TCF4 has been identified as a key regulator controlling the committed development of plasmacytoid dendritic cells (pDCs) from common dendritic cell progenitors through a regulatory network of secondary transcription factors [37, 38]. The *TCF4* gene encodes a transcription factor that is a member of the E-box Tcf4/Lef family (also known as SL3-3 enhancer factor 2 (SEF2), and immunoglobulin transcription factor 2 (E2-2)) [30]. Ceribelli et al. identified a BPDCN-specific transcriptional network regulated by TCF4, and they showed that it plays a master regulatory role in programming BPDCN cells [39].

Hypothesizing that the sensitivity of CD123 expression and the obligatory dependence on TCF4 are characteristic of pDCs and hence BPDCN, we optimized and validated a TCF4/CD123 dual-color immunohistochemistry stain. This stain has become the mainstay of BPDCN diagnosis at our institution (Fig. 2). In a recent study, we demonstrated that TCF4/CD123 co-expression using dual-color immunohisto-chemistry has an analytic sensitivity of 100% and specificity of 99.8% for BPDCN [40].

CD138 and Cyclin D1

CD138 (also called syndecan-1) is a transmembrane heparan sulfate proteoglycan associated with cell-to-cell and cell-to-matrix adhesion [41]. It is expressed normally in many tissues by epithelial cells and plasma cells. CD138 is also expressed in epithelial malignancies and plasma cell neoplasms.

Cyclin D1 is encoded by the *CCND1* gene on chromosome 11q32 and plays a key role in cell cycle regulation and cellular proliferation [42]. Cyclin D1 is normally expressed in histio-cytes, endothelial cells, basal epithelial cells, adipocytes,

fibroblast, glial cells, spermatocytes, and smooth muscle cells [43]. Constitutive upregulation of cyclin D1 expression is detectable by immunohistochemistry in epithelial malignancies and several hematolymphoid neoplasms, particularly mantle cell lymphoma and plasma cell myeloma with t(11;14).

Plasma cell myeloma with t(11;14) has been generally regarded as a standard-risk group, with an overall survival of 8–10 years [44]. A range of correlation levels between cyclin D1 expression and the presence of t(11;14)(q23;q23) has been reported, with the highest correlation being in cases with strong and homogeneous cyclin D1 expression [44, 45]. Conversely, 80–100% of plasma cell myeloma cases with t(11;14)(q32;q32) are usually positive for cyclin D1 [44–46].
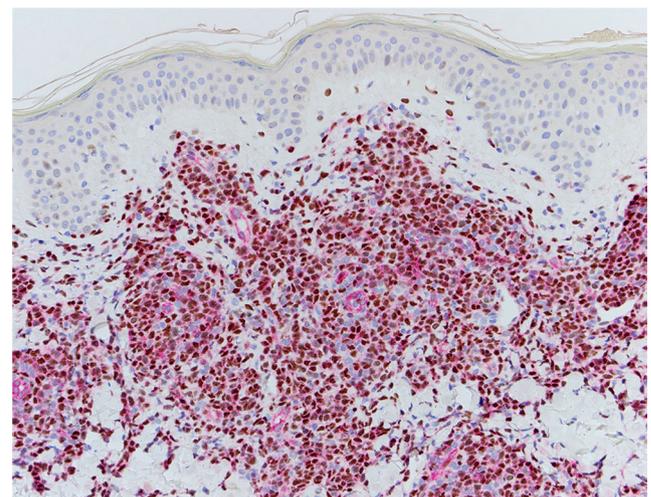
Fig. 2 CD123/TCF4 dual-color immunohistochemistry. Blastic plasmacytoid dendritic cell neoplasm involving skin. Neoplastic cells show CD123 (red) and TCF4 (brown) coexpression. Note CD123 expression by endothelial (200×)

Discordances between the translocation type and cyclin D1 protein expression could be explained on the basis of variant genetic alterations, including the presence of extra copies of chromosome 11 [45].

CD138/cyclin D1 dual-color immunohistochemistry provides a useful tool to assess cyclin D1 expression specifically within plasma cells. Besides facilitating the identification of cyclin D1-positive plasma cell neoplasms, CD138/cyclin D1 assessment can serve as a diagnostic aid in distinguishing B cell lymphoma from plasma cell myeloma with t(11;14) since the latter can occasionally express CD20 [47]. In one report, CD138/cyclin D1 dual-color immunohistochemistry was helpful in a case with synchronous hairy cell leukemia and cyclin D1-positive plasma cell myeloma. Namely, in this case, both the hairy cell leukemia and plasma cell myeloma cells were positive for cyclin D1, and only the latter expressed CD138 [48].

Mutation-Specific Immunohistochemistry

IDH1 R132H

The *IDH1* gene encodes isocitrate dehydrogenase 1, an enzyme that catalyzes the oxidative decarboxylation of isocitrate to alpha-ketoglutarate with concurrent reduction of NADP+ to NADPH [49, 50]. *IDH1* mutations result in a mutant IDH1 protein that catalyzes alpha-ketoglutarate into R-enantiomer of 2-hydroxyglutarate (2-HG) with reversal in the NADPH steady state in favor of NADP+. Increased levels of 2-HG disrupt the function of cytoplasmic and nuclear ketoglutarate-dependent enzymes by competitive receptor binding due to similarity of both substrates. In turn, this results in cell differentiation arrest, possibly caused by epigenetic regulation of DNA and histone hypermethylation. Additionally, 2-HG inhibits prolyl hydroxylases resulting in HIF1-alpha activation and abnormal collagen metabolism. These downstream factors lead to tumor formation and disruption of the blood–brain barrier, respectively. Moreover, in leukemogenesis, 2-HG activates NF-kB through ERK signaling, upregulates HOXA and MAPK genes, and downregulates CDKN2A and ATM, which together promote cellular proliferation and leukemia initiation [51].

The most common mutation in this gene is *IDH1* p.R132H, characterized by substitution of guanine by adenine at codon 395 changing arginine (R) to histidine (H) at position 132. Multiple mutation hotspots other than R132H have been discovered [52]. Apart from *IDH1* (cytoplasmic), mutations in *IDH2* (mitochondrial) at certain codons also cause similar pathogenic effects. Diffuse gliomas are the most common malignancies harboring *IDH1/IDH2* mutations, and the *IDH1/IDH2* mutation status is currently a major criterion in classification of glial neoplasms. Myeloid neoplasms, including acute myeloid leukemia (AML), intrahepatic cholangiocarcinoma and chondrosarcoma also have high rates of *IDH1/IDH2* mutations [53, 54]. IDH1 mutations are more common in gliomas, while IDH2 is more frequently detected in AML [55].

Monoclonal antibodies against IDH1 p.R132H (clone IMab-1 and H09) were developed and have demonstrated high sensitivity (> 90%) and specificity (100%) [56, 57]. In various studies, detection of IDH1 p.R132H by immunohistochemistry showed near 100% accuracy compared to molecular techniques [3, 49, 58]. The staining pattern of IDH1 pR132H is typically cytoplasmic, with or without nuclear staining, and the intensity may vary from weak to strong. In a large study by Kurt et al, IDH1 p.R132H expression was evaluated in hematologic malignancies and showed expression restricted largely to myelomonocytic cells (including leukemic cells) and megakaryocytes; erythroid precursors, lymphoid cells, endothelial cells, and osteoblasts were largely negative [3]. Interpretation of IDH1 p.R132H immunohistochemistry is usually straightforward in glial neoplasms, with most tumors showing clear positive or negative status [49].

While detection of IDH1 p.R132H by immunohistochemistry is currently a mainstay in the evaluation of glial neoplasms, molecular testing using next-generation sequencing platforms are used more commonly for *IDH1/IDH2* mutation profiling for hematologic malignancies. Molecular testing does offer the advantage of detecting mutations other than *IDH1* p.R132H, an advantage that is not shared by IDH1 p.R132H immunohistochemistry [3]. Notwithstanding, some have advocated IDH1 p.R132H immunohistochemistry as an adjunct tool in hematologic malignancies in cases where molecular technique fail (due to sampling error or tissue quality), as this could occasionally provide a conduit for patient clinical trials evaluating IDH inhibitors [59]. In our experience, IDH1 p.R132H immunohistochemistry might provide a more direct estimate of residual disease burden in patients with hematologic malignancies.

BRAF V600E

V-Raf murine sarcoma viral oncogene homolog B1 (*BRAF*) is a gene encoding the B-Raf protein, a serine/threonine signal transduction protein kinase in the Raf family. It plays a role as a MEK/ERK activator within RAS-RAF-MEK-MAPK-ERK (mitogen-activated protein kinases) pathway, an important regulator of cell growth, proliferation and differentiation [60]. Wild-type B-Raf is regulated by RAS-dependent phosphorylation. Upon *BRAF* gene mutation, downstream MEK/ERK activity can be either enhanced or impaired but still capable of leading to constitutive MAPK pathway activation. The most common mutation in this gene is *BRAF* p.V600E, a single nucleotide change leading to amino acid substitution of valine to glutamic acid [61].

The BRAF p.V600E mutation is common across many human cancers [62]. This includes, in particular, melanoma (40–60%) papillary thyroid carcinoma (45%), colonic adenocarcinoma (10–15%), hairy cell leukemia (~ 100%), and Langerhans cell histiocytosis [62–68]. Capper et al. first reported a mutation-specific monoclonal mouse antibody, VE1, as a specific and sensitive marker for detecting BRAF p.V600E by immunohistochemistry in routinely processed FFPE tissue of melanoma and papillary thyroid carcinoma [69]. Positive staining for BRAF p.V600E by immunohistochemistry results in a cytoplasmic signal whose intensity can vary across from weak to strong and from diffuse to focal. Strong and homogeneous/diffuse positivity by immunohistochemistry correlates best with molecular detection of BRAF p.V600E assessment. The significance of detecting a nuclear signal on BRAF p.V600E immunohistochemistry remains unknown [69, 70]. No single optimal cutoff point for BRAF p.V600E immunohistochemistry has been established, and criteria published in the literature vary by tumor type.

In melanoma, immunohistochemistry detects BRAF p.V600E with up to 97–100% sensitivity and specificity [70–72]. A similarly high correlation between immunohistochemistry and molecular testing is identified in papillary thyroid carcinoma [73–77]. The concordance rate in lung adenocarcinoma, epithelioid glioblastoma, and hematologic malignancies, including hairy cell leukemia, Langerhans cell histiocytosis, and Erdheim-Chester disease, was also reported to be high [63, 78–81]. On the other hand, the correlation between immunohistochemistry and molecular testing in colorectal carcinoma is less robust, with some investigators recommending caution in using BRAF p.V600E immunohistochemistry as a surrogate marker for BRAF p.V600E mutation [82]. A number of pre-analytic, analytic, and biological factors, in addition to variability in interpretation criteria, have been suggested as possible explanations for discordance between BRAF p.V600E detection by immunohistochemistry and molecular diagnostics [76, 82, 83].

Conclusion

Immunohistochemistry has evolved over decades to become a crucial component of pathology practice, impacting patient care directly in view of its role in diagnosis and biomarker detection. Modern immunohistochemistry technologies permit high reproducibility and versatility within a clinical environment, enhanced by continuous quality monitoring and ongoing quality improvements to ensure optimal results. These technological advances have pushed immunohistochemistry into new paradigms such as multiplex assays and mutation-specific markers. As digital image analysis, whole-slide imaging, and artificial intelligence algorithms advance further, we predict a commensurate increase in the role of immunohistochemistry and multiplex immunohistochemistry assays as underpinnings of cancer diagnosis and therapy.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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