Pitfalls Caveats in Chromogenic Immunostaining

Subjects: Others

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In daily diagnostic pathology services, chromogenic immunostaining using formalin-fixed, paraffin-embedded (FFPE) sections is indispensable to help in the histopathological diagnosis fundamentally based on hematoxylin and eosin (H&E) staining. Differential diagnoses are determined first, and appropriate immunohistochemical markers for the diagnoses are then selected. The expression of lymphocyte surface markers, hormones and tumor markers contributes to the functional classification of tumors. The degree of malignancy can be analyzed by immunostaining for p53 and Ki-67 (MIB-1), a cell proliferation marker, in a variety of malignancies including carcinomas of the oral cavity, colon, and breast.

Keywords: chromogenic immunostaining ; epitope retrieval ; fixation ; histopathological diagnosis ; sensitivity ; specificity ; trouble-shooting

1. Overview

Chromogenic immunohistochemistry (immunostaining using an enzyme-labeled probe) is an essential histochemical technique for analyzing pathogenesis and making a histopathological diagnosis in routine pathology services. In neoplastic lesions, immunohistochemistry allows the study of specific clinical and biological features such as histogenesis, behavioral characteristics, therapeutic targets, and prognostic biomarkers. The needs for appropriate and reproducible methods of immunostaining are prompted by technical development and refinement, commercial availability of a variety of antibodies, advanced applicability of immunohistochemical markers, accelerated analysis of clinicopathological correlations, progress in molecular targeted therapy, and the expectation of advanced histopathological diagnosis. However, immunostaining does have various pitfalls and caveats. Pathologists should learn from previous mistakes and failures and from results indicating false positivity and false negativity. The present review article describes various devices, technical hints, and trouble-shooting guides to keep in mind when performing immunostaining.

2. Techniques of Immunohistochemistry

Techniques of immunohistochemistry (IHC) or immunostaining have already been established [1][2][3][4][5][6][7][8]. Particularly for diagnostic IHC, chromogenic IHC (immunostaining using enzyme-labeled probes) is commonly utilized. Measures and tools to prevent technical artifacts and appropriate trouble-shooting tips are needed. The present review article provides an overview of technical aspects, knowhow, pitfalls, and trouble-shooting guides for routinely performing chromogenic immunostaining, especially for experienced personnel with immunohistochemical expertise. It should be emphasized that all data presented herein were obtained by the author and/or the author’s colleagues.

3. The Need for Chromogenic Immunostaining in Diagnostic Pathology

IHC is critical both to informing the biology of lesions and to formulating the histopathological diagnosis. The patient’s prognosis and response to therapeutic approaches may thus be predicted.

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IHC markers can be classified into four types, as with other biomarkers: diagnostic, prognostic, predictive, and therapeutic. A diagnostic marker defines the nature (histogenesis/origin) of the lesion. A prognostic marker suggests clinical/biological characteristics that provide information on the likely course of the disease and inform a probable

outcome of the patient. A predictive marker predicts the response of the lesion to targeted therapy, and a therapeutic marker represents a structure that can be used as a target of therapy.

We now have a variety of cell-specific markers available to clarify the nature of neoplasms and non-neoplastic lesions. Cytokeratins are useful to show the epithelial nature of the cells (Figure 1) [12]. For determining molecular targeted therapy of breast cancer, for example, such markers as estrogen receptor (ER), progesterone receptor (PgR), human epidermal growth factor receptor-2 (HER2), and Ki-67 are consistently immunostained [13].

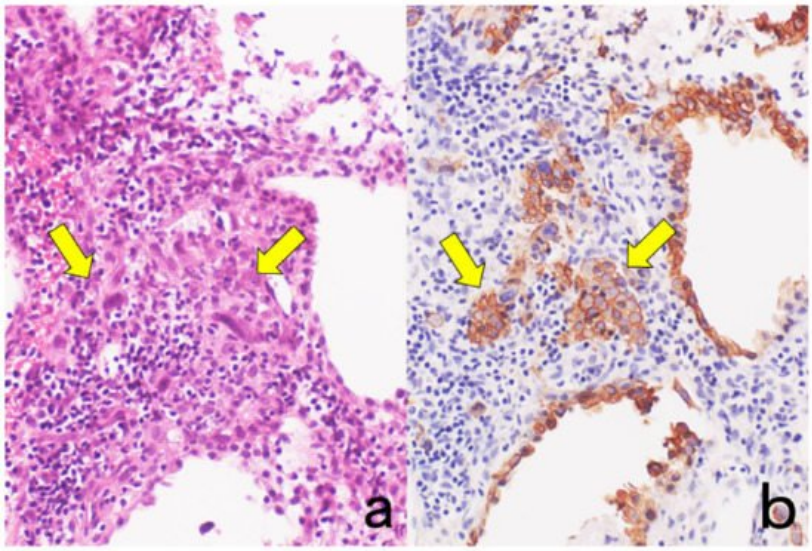

Figure 1. Cytokeratin for identifying intermediate trophoblasts in the placenta. (**a**): H&E, (**b**): cytokeratin immunostaining. Intermediate trophoblasts (arrows) are observed in the stroma of the placental tissue sampled by curettage. Cytokeratin immunoreactivity with a monoclonal antibody CAM5.2 clearly illustrates their distribution.

Appropriate applications of chromogenic immunostaining to diagnostic pathology are dependent upon the following three points: (a) how to stain, (b) how to select IHC markers, and (c) how to evaluate the immunostaining findings. The present review article focuses on chromogenic immunostaining for diagnostic pathology that routinely uses FFPE sections.

4. Selection of Methodology for Chromogenic Immunostaining

A variety of primary antibodies and immunostaining kits are commercially available, and automated instruments have become popular. Now, chromogenic immunostaining is adequately sensitive and technically reliable. As a secondary reagent with high sensitivity, horseradish peroxidase (HRP)-labeled polymers such as Envision Flex (Agilent Technologies/Dako, Santa Clara, CA, USA), Simple Stain Max (Nichirei, Tokyo, Japan), and Novolink (Leica Biosystems/Novocastra, Nussloch, Germany) are widely used [14]. When necessary, catalyzed signal amplification (CSA)-II using fluorescein isothiocyanate (FITC)-labeled tyramide (Agilent/Dako) can be applied in which the anti-FITC antibody mediates amplification of the chromogenic signal (Figure 2) [15]. We have now abandoned biotin-labeled techniques such as the avidin biotinylated peroxidase complex (ABC) method and labeled streptavidin biotinylated peroxidase (LSAB) method for the following reasons: (a) endogenous biotin activity in mitochondria is retrieved by pretreatment with heat-induced epitope retrieval (HIER) [16], (b) three steps are required for immunostaining, and (c) the sensitivity of detection is inadequate when compared with the polymer technique. The original version of CSA using biotinylated tyramide should thus be avoided [15].

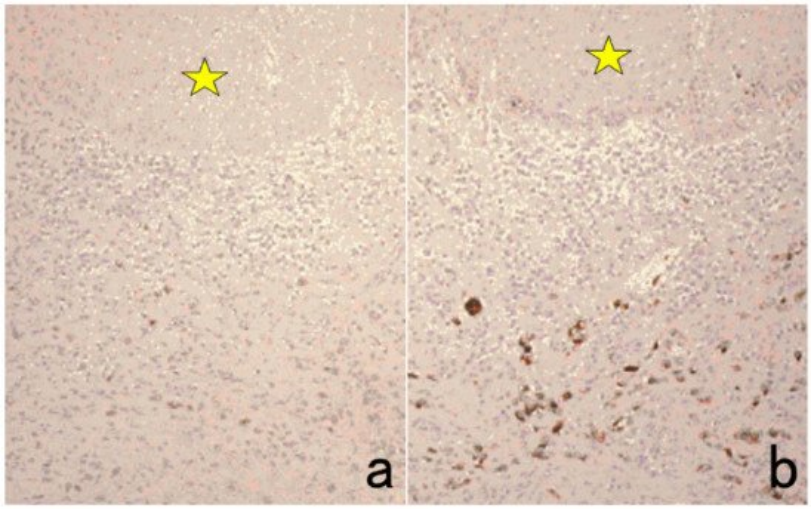

Figure 2. p53 immunostaining without HIER in esophageal squamous cell carcinoma. (**a**): Polymer method (EnVision) and (**b**): catalyzed signal amplification (CSA)-II method. Submucosally invading cancer cells express p53 in the nuclei. The immunoreactivity is significantly enhanced by the CSA-II method using FITC tyramide as an amplifier. Stars indicate non-cancerous esophageal squamous mucosa.

Antigenic detection with ultra-high sensitivity techniques such as CSA-II is not necessarily needed because of the augmentation of background staining and the difficulty in maintaining the highly diluted antibodies in refrigerators or freezers. The method used should be selected based on ease of handling, stability, and reproducibility. The antibodies must be diluted with 0.01 M phosphate-buffered saline (PBS), pH 7.4, containing 1% bovine serum albumin (BSA) to avoid inactivation of antibody reactivity. Repeated freezing and thawing damages the diluted antibodies.

When there is a 100 µL aliquot of an antibody at a working dilution of 1:1000, a recommended management suggestion for the antibody is as follows. Half of the volume is kept in an undiluted form, and the remaining 50 µL is diluted at 1:10 with BSA-PBS to allow 10 plastic tubes containing the remaining 50 µL of 1:10-diluted antibody to be stored in a freezer after appropriate labeling. It is critically important to avoid the repeated freezing and thawing of diluted antibodies to ensure reliable and reproducible chromogenic immunostaining. To eliminate the risk associated with repeated freezing and thawing cycles, the addition of 25–50% glycerol in the diluent as a cryopreservative is a convenient and practical method of choice [17].

FFPE sections are the common target of chromogenic immunostaining in diagnostic pathology, so the antibodies selected must be applicable to the FFPE sections. Diaminobenzidine (DAB) solution, containing 20 mg DAB and 3 mg hydrogen peroxide in 100 mL of 50 mM Tris-HCl buffer, pH 7.6, is routinely utilized for the final chromogenic reaction in brown. The nuclei are briefly counterstained with Mayer’s hematoxylin in deep blue (usually by dipping for 10 s).

5. Conclusions

Nowadays, it is clearly expected that chromogenic immunostaining will produce beautiful and specific images. Biomedical engineers (i.e., medical technicians) should know how to select appropriate markers and how to judge the results. Pathologists and investigators must understand the pitfalls and caveats of immunostaining procedures. To apply immunostaining to diagnostic pathology services, it is critically important for pathologists to know how to stain, how to select markers, and how to judge the results. The importance of close teamwork and cooperation between the biomedical engineers and pathologists cannot be understated.

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