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Immunohistochemistry- a brief note on technical protocols

Dr. L.Malathy, Dr. Sangeetha Priya.P ,Dr. N. Aravindha Babu MDS, Dr. N.Anitha

Department of Oral Pathology and Microbiology
Sree Balaji Dental College and Hospital
Bharath Institute of Higher Education and Research

Sangeethapriya.omfp@gmail.com

Abstract-

In the microscopic diagnosis of neoplasms, the application of immunologic research methods to histopathology has been resulted in marked improvement. Although histologic analysis of hematoxylin and eosin stained tissue sections remains as gold standard diagnostic method and it is at the core of the practice of head and neck surgical pathology, additionally immunohistochemistry had become a powerful tool to the pathologist. It has a significant advantage in the diagnosis of difficult tumors. The selection of antibodies for the immunohistochemical testing is made on the basis of their tumor specificity and the likelihood that they will react with the tumor under evaluation using the antibody. IHC is a well-established technique to facilitate the diagnosis of infectious and neoplastic processes in animals. Immunohistochemical studies have traditionally focused on markers of specific cell of origin and tumour type as aids in the diagnosis of specific tumours.

Key Words: oral cancer, IHC, immunohistochemistry markers, IHC procedures

INTRODUCTION:

Immunohistochemistry (IHC) is the method of using antibodies (Abs) to detect specific antigens (Ags) in tissues. Histopathologic evaluation of diseases has been altered and enhanced by the adjuvant use of IHC, and some sophisticated techniques have been replaced by IHC due to its easy and versatile immunohistochemical techniques. And of course, disorganized IHC application could be misleading the diagnosis. Immunohistochemistry is based on specifi c Ab-Ag interactions. Although histologic analysis of hematoxylin and eosin stained tissue sections remains at the core of the practice of head and neck surgical pathology, immunohistochemistry have additionally become a powerful tool to the pathologist[1].It affords a significant advantage in the diagnosis of difficult and confusing tumors. Immunohistochemistry has also provided knowledge into tumor histopathogenesis and has contributed to more accurate determination of patient disease prognosis. Therefore, Immunohistochemistry is important in diagnosis, investigation, and determining the behavior and pathogenesis of oral tumors in addition to conventional histopathological techniques.

IMMUNOHISTOCHEMISTRY PRINICPLE

It is a technique for identifying cellular or tissue constituents (antigens) by the help of antigen-antibody interactions. In identifying the antibody binding site, two different methods are under practice, either by direct labelling of the antibody or by use of a secondary labelling method[2]. The Antibodies which are used to detect Antigen(s) are called primary Antibodies. Primary Abs are linked to enzymes which is the main part of chromogenic system through an another Antibody called link Antibody. This linkage to enzymes is mediated by polymers or some molecules such as streptavidin-biotin complexes. In immunohistochemistry,Peroxidase is the enzyme mostly used aparat from peroxidase, Alkaline phosphatase is also used but less frequently.

The choice of antibodie selection for the immunohistochemical testing is made on the basis of their tumor specificity and the likelihood that they will react with the tumor under evaluation using the specific antibody. Once after the tissue sections are incubated with the prospective antibodies, positive tumor antigen- antibody binding reaction are identified via the application of one among the several detection systems. Those that have the highest sensitivity use a secondary antibody, reactive against the primary antibody, which is linked to an enzyme marker. This system tends to be very sensitive because it allows for the attachment of a relatively large number of enzyme molecules, such as peroxidise, at the antigen site. The color of the reaction is determined by the selection of a precipitating chromogen, usually diaminobenzidine (brown) or aminoethylcarbazole (red), with which the enzyme reacts[3].

APPLICATIONS OF IMMUNOHISTOCHEMISTRY

- Prognostic markers in cancer
- In finding Tumors of uncertain histogenesis
- Prediction of response to therapy or treatment prognosis
- In case of infections – to confirm infectious agent in tissues
- In genetics - to determine the function of specific gene products
- Identifying the presence of chromosomal translocations.
- Neurodegenerative Disorders
- Brain Trauma
- IHC in muscle diseases
- Research application

BASICS OF AN IHC EXPERIMENT

In this multi-step application, multiple variables may determine the staining and requires the careful optimization of new assays for each target antigen. For example, when investigating a high abundance protein in formaldehyde-fixed tissue, heat induced antigen retrieval step may be used in the IHC protocol and use a directly labeled primary antibody. For another example, the optimal protocol for staining a low abundance protein in a methanol fixed, frozen liver section may require blocking of endogenous biotin and a signal amplification technique[4]. Since a number of variables can cause artifacts or interfere with a successful outcome of the procedure, appropriate controls are necessary for accurate interpretation of IHC result outcomes. Therefore a brief guide is intended to serve as a reference for researchers to understand, perform, and various trouble in IHC protocols encountered during the development and optimization of new IHC assays.

SAMPLE PREPARATION AND FIXATION:

IHC can be broadly classified into two forms based on the type of tissue processing involved: IHC- formalin-fixed, IHC-paraffin-embedded (FFPE) and IHC-frozen (Fr). Often the preservation method is closely associated with the type of fixation. Formalin-fixed tissues are commonly paraffin-embedded following fixation, while frozen tissue sections can be fixed with formaldehyde or alcohol prior to or following cryosectioning[5].

	IHC- Formalin fixed, paraffin-embedded	**IHC- Frozen**
Fixation	Performed before embedding into paraffin wax.	Can be performed before or after cryo-sectioning.
Fixative	Formaldehyde	Formaldehyde or Alcohols
Sectioning	Microtome, 4-10 μm sections	Cryostat/Cryotome, 5-20 μm sections

Storage	Ideally fresh sections should be cut after 4 weeks due to loss of antigenic epitopes. Fresh cut sections should not be used after 1 month. For long term storage (several years), coating of slide in paraffin is recommended.	Short-term1 year at -80 o C.
Advantage	Ease of handling and preserves structural morphology. Blocks can be stored long term.	Preserves enzyme & antigen function. Useful for study of post-translationally modified protein, DNA, or RNA.

IHC STAINING PROCEDURE

Development of the new modern techniques, have replaced the traditional old ones with added advantages of sensitivity, specificity, low cost efficiency and reliability[6].

The techniques employed are

1. Traditional direct technique
2. New direct
3. Indirect technique
4. New indirect - Unlabeled antibody enzyme-complex techniques (PAP and APAAP)

   -Immunogold silver staining technique (IGSS).

   -Avidin-biotin techniques.
5. Hapten labeling technique

STAINING PROTOCOL

A sensitive IHC test is one that detects a small amount of antigen. It depends on the following important steps: -

Blocking endogenous enzyme activity

This procedure is to block the nonspecific interactions, in order to obtain sensitive result outcome. The block is an inhibitor that prevents an enzyme that originates within a cell or tissue from causing a reaction with substrate. Specifically, this is done for the visualization of the end product by converting colorless chromogens into colored end products. Antibody-based applications is dependent on the specific binding of an antibody to the target epitope for generating accurate expression data. The same forces that govern specific interactions can also contribute to non-specific binding including hydrophobic interactions, ionic interactions, and interaction between the hydrogen bonding. Common buffers that are used to block non-specific interactions are serum, BSA, casein, or commercial buffers[7].

Antigen retrieval :

For antigen to be retrieved from the tissue a high-temperature heating method needs to be used to recover the antigenicity of tissue sections that had been masked by formalin fixation. While the majority of antigens from formalin fixed tissue require an antigen retrieval step, some targets are negatively impacted by it. But for example in some cases tissue section those are polyclonal primary antibody ,IHC-Frozen sectioned tissue, this procedure can be skipped.

• The process of antigen retrieval on frozen tissue may be too harsh and can damage the tissue.

• A polyclonal antibody may enhance antigen detection compared to a monoclonal due to its ability to bind multiple epitopes.

• A change in pH or cationic concentration of an antibody diluent; or a simple change in the incubation conditions of the primary antibody can also improve antibody affinity for an antigen[8].

Blocking non-specific antibody binding:

Blocking other reactive epitopes and quenching endogenous enzymatic reactions in tissue samples prior to the primary antibody incubation step also prevents non-specific binding and false positive staining. Autofluorescence can impact imaging for immunofluorescence (IF), especially for tissue samples with elevated levels of flavins or porphyrins[7,8]. Non-specific staining observed when using common detection reagents and tips on how to minimize high background signal. The choice of blocking buffer is also contingent on the method of detection used. For example, if using an alkaline-phosphatase (AP) conjugated secondary antibody, the blocking serum should be diluted in tris-buffered saline (TBS). whereas PBS will interfere with the alkaline phosphatase reaction.

Optimal primary antibody dilution and incubation time

Diluents for antibody solution – For the purpose of preparing a working antibody reagent these are inert fluids or reagents used in immunohistochemistry to dilute a particular antibody stock solution. Since a number of unknown factors influencing the overall stability of diluted antibodies there is less chance for a general and safe recommendation for how long a diluted antibody will be stable. Some of the available diluents are TrisHCl, Bovine serum albumin ,and normal saline[9]. Incubation Time and Temperature- There is an inverse relationship between incubation time and antibody titer: The higher the antibody titer, the shorter the incubation time required for optimal results. Commonly used incubation time is 30 minutes at room temperature using humid chamber. In case a very dilute antibody used to save reagent, then a overnight incubation may be used [10].

Secondary antibody /Polymer incubation

WASH BUFFERS

In order to remove the excess or unwanted reagents or complexes which are formed during each step of the IHC procedure the wash buffer is useful. Commonly used wash buffers that are commercially available include Tris Buffered Saline (TBS) and Phosphate Buffered Saline (PBS)[8,9] .

SECONDARY ANTIBODY

Selection of the best secondary antibody can improve immunostaining of the tissue and reduce false positive or false negative staining in immunohistochemistry procedure[9,10] .

STAINING PROTOCOL

The standard protocol is intended for use as a guideline in developing antibody-specific procedures. Different antibodies and tissues may require changes to this procedure.

Controls validate immunohistochemical results.

(i) Positive controls:-

It is defined as tissue that is known to contain the antigen of interest detected by identical IHC methods to those used in diagnostic cases. Positive tissue controls must be fixed and stained in the same way as the diagnostic case tissue had been fixed and stained for every antibody and procedure used. Positive elements within test sections,for example normal reactive lymphocytes when staining with an antibody to the leukocyte common antigen to identify a suspected lymphoma, are the best form of positive control[10].

(ii) Negative controls:-

Negative tissue control is defined as tissue which does not contain the antigen of interest. At least one ancillary test (e.g. PCR, virus isolation) performed on the tissues or the organ systems of the same animal should be used to find the presence of the antigen of interest. This also involves the omission of the primary antibody from the staining schedule of the replacement of the specific primary antibody by an immunoglobulin which is directed against an unrelated antigen[10]. Commonly, the primary antibody is replaced by antibody diluent, same species non-immune immunoglobulin of the same dilution and immunoglobulin concentration, an irrelevant antibody or buffer. These methods will decide the degree of cross-reactivity of the primary antibody, and the degree of non-specific binding by the labeling the secondary antibody and detection system[9].

(iii) Internal positive tissue controls:-

Internal positive tissue controls are present in diagnostic case tissues. In this type of control there is no fixation variable when comparing between the control and the diagnostic case tissue.An example is the detection of smooth muscle markers or vimentin in normal blood vessels. The presence of positive staining in these areas expresses the specific immunoreactivity[10].

(iv) Tissue Artifact Control :-

In particular tissue the background staining may be more. Because high background can mask positive signal from low abundance antigens or result in artifacts being mistaken for specific staining, IHC data can be misinterpreted. Before commencing staining, tissues should be examined under a bright-field for chromogenic labels or fluorescence microscope for flurorescent lable therefore to ensure that the signal is not due to the inherent properties of the tissue.

INTERPRETATION OF RESULTS

Diagnostic IHC tests can be divided in two main types; qualitative and semi-quantitative. The majority of IHC tests are qualitative, where the resulting stain is interpreted only as positive or negative. These types of stains can to some extent involve quantitation in the form of a cut-off point or threshold for positivity. For example greater than 10% stained cells is indicative of a positive result. Interpretation of the qualitative IHC tests is focused on the correct cellular localization of the staining reaction and staining of the correct tissue structures. Appropriate positive and negative controls are highly importatnt for optimization of these tests[11,12].

MOLECULAR MARKERS:

Epithelial marker	keratins
General mesenchymal markers	vimentin
Muscle markers	desmin, actins, myoglobins, myogenin
Neural markers	S100, GFAP, neurofilaments, CD57
Endothelial markers	CD31, CD34, factor VIII related antigen
Lymphoid markers	κ and λ, CD3, CD15, CD20, CD30,CD45, CD68, CD79a,ALK-1 and TdT
Neuroendocrine markers	synatophysin, chromogranin
Metastatic tumor markers	CK7, CK20 and villin

Minor salivary gland tumor markers	S-100 protein, actins1

CONCLUTION:

Several recent developments emphasize the increasingly important role IHC will play in the coming years. These include genomic IHC for diagnosis, search for proteins for targeted therapy, methods to develop better monoclonal antibodies with recombinant technology, "technician free" automation of the IHC procedures, and "pathologist free" microscopic image analysis technology for interpretation of high throughput results. IHC is a well-established ancillary technique to facilitate the diagnosis of infectious and neoplastic processes in animals[13]. Immunohistochemical studies have traditionally focused on markers of specific cell and/or tumour type as aids in the diagnosis of specific tumours. However, as our knowledge of the molecular basis of tumours has increased, IHC is being used with increasing frequency to identify underlying molecular changes or the presence of specific molecular markers in tumours, both as an aid to diagnosis and as a guide to appropriate therapy.

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