



## Review Article

# Basic principles of immunohistochemistry and epithelial immunohistochemical markers

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## ABSTRACT

Dr. Albert Coons et al in 1941 first initiated immunohistochemistry to identify cellular or tissue constituents by means of antigen and antibody interactions. The site of antibody binding can be identified either by direct labeling of antibody or by use of secondary labeling method.

Immunohistochemistry (IHC) involves the use of labeled antibodies to localize the antigens in the tissues through antigen-antibody interactions and are visualized either by fluorescent dye, enzyme, radioactive element or colloidal gold. IHC usage has an obvious advantage over the traditional one, which uses special enzyme staining technique that identify only limited number of proteins, enzymes and certain tissue structure. IHC can be commonly employed to distinguish the tissue of epithelial and mesenchymal differentiation, cellular changes which are normally not visible in regular staining techniques, and earliest changes in transformed tissues. IHC is relatively rapid and simple method to better determine the specific neoplastic tissue origin, the behavior or progression of neoplasm. Hence IHC have gained importance in the medical research and has been considered as a crucial & widely used technique in research laboratories as well as in clinical diagnostics.

In present review IHC markers for epithelial origin that can be utilized to diagnose the tumor or to determine the prognosis of the tumor..

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## 1. Introduction

Immunohistochemistry (IHC) is a process of localizing cellular or tissue antigens by exploiting the principle of antibody specifically binding to antigen within the biological tissues. IHC has derived its name from the roots “immuno,” used in reference to antibodies, and “histo” meaning tissue.<sup>1,2</sup> Antibody binding site can either be done by direct labeling of the antibody or by use of secondary labeling method.<sup>1</sup> Antigen-antibody interactive reactions can be visualized by a marker such as fluorescent dye, enzyme, radioactive element or colloidal gold.<sup>3</sup>

Because of its specific antigen-antibody reactions, immunohistochemistry has apparent advantage over

traditionally used staining techniques that identify only a limited number of proteins, enzymes and tissue structures. Therefore, immunohistochemistry has become a crucial technique and is widely used in many medical research laboratories as well as clinical diagnostics.<sup>3,4</sup> Localization of the antigens can be performed by variety of methods based on the parameters like specimen type and degree of sensitivity required.<sup>3</sup>

IHC aids in identification of abnormal tumor cells. Specific molecular markers, mark the various events like cellular proliferation or the cell death (apoptosis) along with the usage in basic research to understand the distribution and localization of biomarkers.<sup>3</sup>

Antibodies used for IHC purpose are either monoclonal antibodies or polyclonal antibodies. Monoclonal antibodies are the product of an individual clone of plasma cells.

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Antibodies from a given clone are immunochemically identical and react with a specific epitope on the antigen against which they are raised. Monoclonal antibodies are very useful tools for diagnostic and research techniques.<sup>5,6</sup> Polyclonal antibodies are produced by different cells and in consequence, are immunochemically dissimilar. They react with various epitopes on the antigen against which they are raised.<sup>7</sup> [Figure 1]

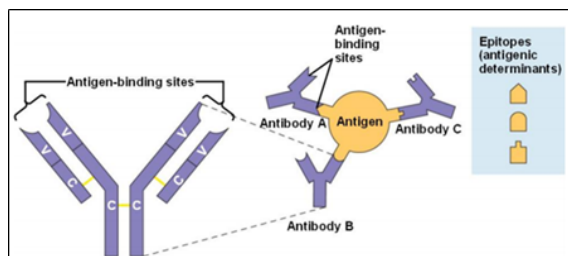


Fig. 1: Antigen– Antibody reaction

Labels play an important role in immunohistochemistry techniques. These substances are attached to the primary, secondary or tertiary antibodies of a detection system to allow visualization of the immune reaction. A variety of labels have been used, including fluorescent compounds, enzymes, and metals.<sup>1</sup> 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.

### 1.1. Merits of IHC

1. It works in routine conditions
2. It is compatible with standard fixation and embedding procedure
3. It can be performed retrospectively in archival material
4. It is sensitive and specific and is applicable to almost any immunogenic molecule and it is interpreted in morphologic context

### 1.2. Immuno-histochemical Techniques

**Direct traditional method:** is the oldest technique where an enzyme-labeled primary antibody reacts with the antigen in the tissue and subsequent use of substrate-chromogen concludes the reaction sequence. Because this method utilizes only one antibody, it could be completed quickly, and nonspecific reactions are limited<sup>1</sup> and the method is no longer considered as sensitive.<sup>7</sup> The primary antibody is conjugated directly to the label. Fluorochrome, horse-radish peroxidase and alkaline phosphatase are the popular direct conjugates.<sup>1</sup>

New enhanced polymer one step method in which a large number of primary antibody molecules and peroxidase enzymes are attached to a dextran polymer “back bone” with enhanced sensitivity achieved by avidin-

biotin technology but with fewer steps. Currently the directly labeled reagents are supplied optimally diluted for incubation up to 1 hour.<sup>1</sup>

**Indirect technique:** in which the unconjugated primary antibody is applied, followed by labeled antibody directed against the first antibody. Horse radish peroxidase labeling is most commonly used and with the chromogen substrate, is a more sensitive technique than the equivalent traditional direct method. It is also rapid and relatively inexpensive.<sup>1</sup>

**Unlabeled Antibody Enzyme-Complex Technique (PAP AND APAAP):** The staining sequence of this technique consists of the use of an unconjugated primary antibody, a secondary antibody, the soluble enzyme-anti-enzyme complex and substrate solution. The primary antibody and the antibody of the enzyme immune complex must be made in the same species. The secondary antibody must be directed against immunoglobulins of the species producing both the primary antibody and the enzyme immune complex. The secondary antibody is added in excess so that one of its two Fab sites binds to the primary antibody leaving the other site available for binding the antibody of the enzyme immune complex. [Figure 2]

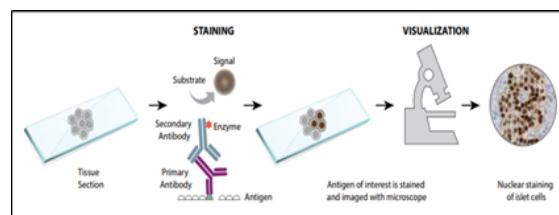


Fig. 2: BasicImmuno-Histochemical technique (Indirect method)

**Soluble enzyme-anti-enzyme immune complex techniques** were named after the particular enzyme immune complex they used. For example, the PAP method utilized a peroxidase-antiperoxidase complex APAAP used an alkaline phosphatase-antialkaline phosphatase complex. The PAP complex consists of three molecules of peroxidase and two antibodies and the APAAP complex has two molecules of alkaline phosphatase and one antibody.<sup>7</sup>

**Immunogold technique** Colloidal gold is a very electron-dense particle which can be seen both with the light and electron microscope. With light microscopy, it appears as a pink or red color depending on the density and size of particles.<sup>8</sup>

**Avidin Biotin technique** Avidin, with a molecular weight of 68000 has four binding sites for biotin and biotin has only one binding site for avidin. Several biotins can be bound to an immunoglobulin molecule without seriously hampering its binding affinity for antigens. Biotin-avidin conjugates have been used as tools in a number of systems since few decades.<sup>8</sup>

**Hapten labeling technique** Bridging techniques using haptens such as dinitrophenol and arsanilic acid have been

advocated. In this technique, the hapten is linked to the primary antibody and a complex is built up using an anti hapten antibody or hapten labeled PAP complex and this technique has similar sensitivity to avidin biotin system.<sup>1</sup>

**Mirror image complementary antibody labeling technique (Mica)** Sequential use of mutually attractive antibodies, which is achieved by raising antibodies against each antibody species, which enables each antibody to be both antigen and antibody with respect to each other. The first antibody is directed against the tissue or cell bound antigen which can be of mouse anti human, the second antibody will be sheep anti mouse followed by peroxidase conjugated donkey anti-sheep followed by sheep anti-donkey and then peroxidase conjugated donkey anti-sheep antibodies.<sup>1</sup> These steps attach a large number of peroxidase enzymes to the antibody-antigen and are 60 times more sensitive than conventional avidin biotin systems.[Figure 3]

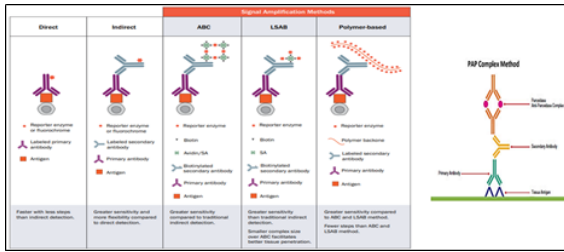


Fig. 3: Different IHC techniques

1.3. Epithelial immunohistochemical markers

1.3.1. Cytokeratin's

Cytokeratin's (Keratins) are the main structural proteins in epithelial cells belonging to the complex members of the intermediate filament protein family are found within the cytoplasm of all epithelial cells. Collection of more than 20 proteins are described till date. Based on the molecular weight cytokeratin's are grouped into acidic (type I or A) and basic (type II or B) subfamilies, or by their usual pattern of expression in simple or complex epithelium.<sup>9</sup> Normally, each acidic cytokeratin is co-expressed with a specific basic one, as a "keratin pair". Cytokeratin's are the cytoplasmic expression of a defined set of keratin polypeptide in different epithelial cell types at different stages of differentiation.<sup>10</sup>

1.3.2. Cytokeratin expression in normal oral mucosa

Simple epithelial keratins primarily express CK 8 and 18 are not normally found in stratified squamous epithelium, but rather in single cells, such as glandular tissue (e.g., salivary glands). Occasionally CK8 positive cells in Merkel cells (of neuroectodermal origin) rather than epithelial cells.

In stratified squamous epithelium, basal cells express CK 5 and 14 and non-keratinized epithelium expresses CK19. Suprabasal cell layers of non-keratinized epithelium

(buccal mucosa, ventral tongue) express CK 4 and 13, suprabasal cell layers of keratinized (hard palate, dorsal tongue) epithelium express CK 1 and 10. In regions of increased proliferation CK6 and CK16 are found.

Odontogenic epithelium express CK 8 and 19, basal cells of salivary gland epithelium express CK 5, 14 and 17 and luminal cells express CK 7, 8, 18 and 19.

Keratins as tumor markers have two main applications, in distinguishing epithelial from non- epithelial tumors and in distinguishing the type of epithelial tumor. [Figure 4]

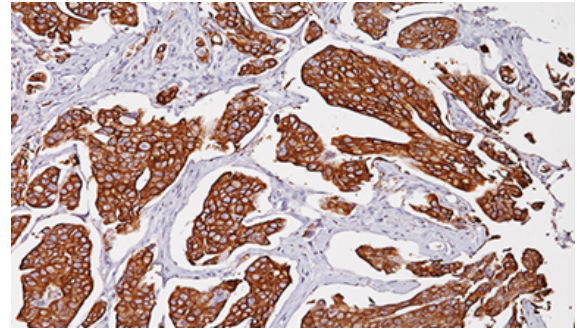


Fig. 4: Cytokeratin expression

1.4. Epithelial membrane antigen

Epithelial membrane antigen (EMA) is an incompletely characterized antigen that is present in a group of carbohydrate-rich, protein-poor, high-molecular-weight molecules present on the surface of many normal types of epithelium such as those in the pancreas, stomach, intestine, salivary gland, respiratory tract, urinary tract, and breast.<sup>9</sup>

Both the epithelial and the spindle cell components of biphasic synovial sarcoma show pancytokeratin and EMA positivity. In monophasic tumors, scattered spindle cells are positive for both markers. EMA may be a more sensitive marker than keratins for monophasic and poorly differentiated synovial sarcomas, and most cases show patchy or streaky reactivity.<sup>11</sup> [Figure 5]

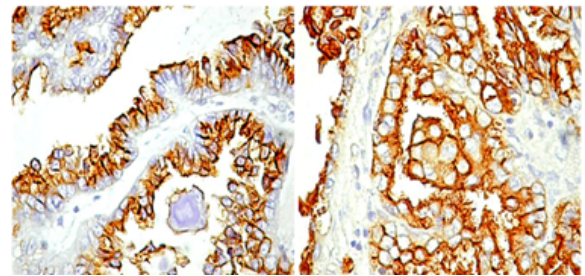
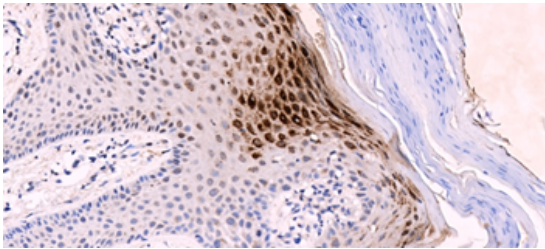


Fig. 5: EMAexpression

### 1.5. Filaggrin

A basic, intracellular marker histidine-rich, keratin filament-aggregating protein synthesized by cells of keratinizing epithelia that sub-serves major physiological functions in maturing epidermis.<sup>12</sup>

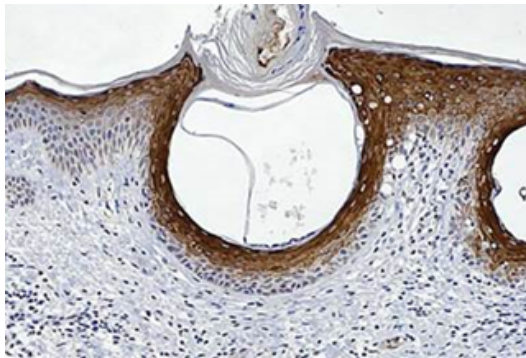
Normal squamous cervical epithelium exhibits a positive homogeneous immunoperoxidase stain in the upper parabasal, intermediate and superficial cell layers.<sup>13</sup> Localization of filaggrin in lower cornified cells correlates precisely with the formation of aggregated keratin filaments, and the disappearance of filaggrin in upper cornified cells correlates precisely with the loosening of keratin filaments.<sup>14</sup> [Figure 6]



**Fig. 6:** Filaggrin expression

### 1.6. Involucrin

An intracellular keratinocyte protein that first appears in the cell cytosol, but ultimately becomes cross-linked to membrane proteins by transglutaminase.<sup>15</sup> It is a sensitive and specific marker for squamous and urothelial differentiation.<sup>16</sup> Most of the involucrin-positive cells are located at the central parts of tumor in the cell clusters of squamous cell carcinomas.<sup>17</sup> Immunostaining for involucrin is common in squamous carcinomas but is also found in adenocarcinomas, adenosquamous carcinomas, large cell carcinomas and carcinosarcomas. Small cell carcinomas, carcinoid tumors and mesotheliomas are negative.<sup>18</sup> [Figure 7]



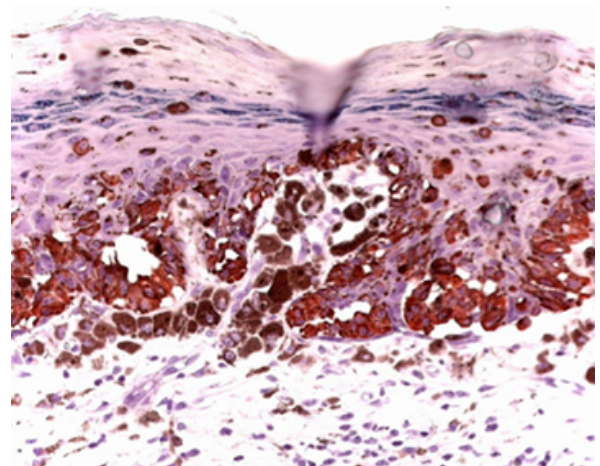
**Fig. 7:** Involucrin expression

### 1.7. Markers for melanocyte differentiation

#### 1.7.1. HMB 45

HMB 45 was first described by Gown et al in 1986. HMB 45 (Homatropine methyl bromide) is a monoclonal antibody originally obtained from an extract of malignant melanoma which identifies an oncofetal glycoconjugate associated with immature melanosomes and probably related to the tyrosinase enzymatic system.<sup>19</sup>

HMB-45 is generally negative in nevi and resting melanocytes but is expressed in approximately 85% of melanomas. Fewer than 10% of desmoplastic melanomas are HMB-45 positive.<sup>9</sup> Anti-HMB-45 is regarded more specific but less sensitive than S-100 protein. It is very helpful in distinguishing the differentiated melanomas from other non-melanocytic malignancies. Expression of HMB-45 has been detected in primary and metastatic melanomas by several investigators. However, HMB-45 is also expressed in a few tumors other than melanomas, such as breast carcinomas, plasmacytomas, angiomylipomas, and pigmented nerve sheath tumors.<sup>20</sup> [Figure 8]



**Fig. 8:** HMB 45 expression

#### 1.7.2. MELAN - A

The anti-Melan-A mouse monoclonal antibody A103 was generated from immunizing mice with recombinant Melan-A protein by Jungbluth et al. in 1998. Melan - A is a 20-22-kDa component of the premelanosomal membrane. Its function is unknown. Like HMB-45, Melan-A is a marker of melanosomes, not melanomas, it is also present in perivascular epithelioid cell tumors (PEComas). Melan-A, is the product of the MART-1 gene (melanoma antigen recognized by T cells).<sup>19</sup>

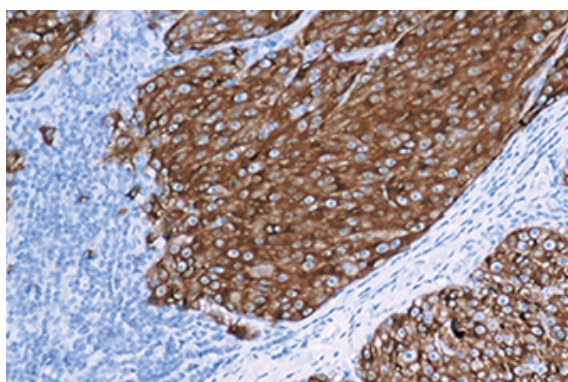
Unlike HMB-45, Melan-A is positive in resting melanocytes and nevi. Melan-A is expressed by approximately 85% of epithelioid melanomas and has been reported to be present in upward of 50% of desmoplastic melanomas, although the true rate is almost certainly far

**Table 1:** Cytokeratin expression in various epithelium

Cytokeratin Expression In Various Epithelium	
Type of epithelium	Cytokeratins expressed
<b>Simple epithelium</b>	Primarily expresses cytokeratins 8 and 18 and often cytokeratins 7, 19 and 20.
<b>Stratified epithelium</b>	
Basal layer	Cytokeratins 5 and 14.
Suprabasal cell layers of non keratinized epithelium	Cytokeratins 4 and 13
Suprabasal cell layers of keratinized epithelium	Cytokeratins 1 and 10
<b>Transitional epithelium</b> of the urinary bladder	Cytokeratins 4 and 13 together with cytokeratins 7, 8, 18, 19 and 20.
<b>Odontogenic epithelium</b>	Cytokeratins 8 and 19
<b>Salivary gland epithelium</b>	Cytokeratins 5, 14 and 17
Basal cells	
Luminal cells	Cytokeratins 7, 8, 18 and 19

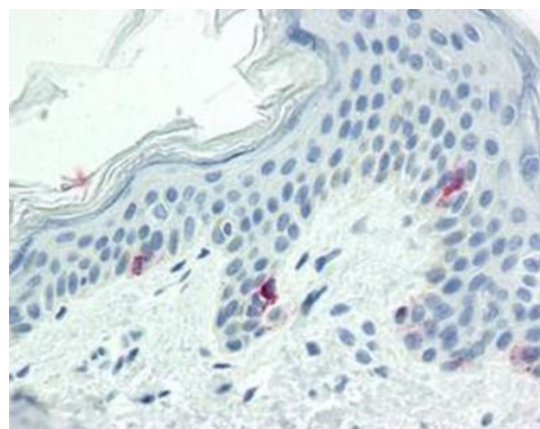
lower and is probably the same as for HMB-45.

Anti-Melan-A antibody has been shown to stain both benign and malignant melanocytic lesions in a very similar pattern to that of anti-S-100 with the exception of desmoplastic, spindle cell, and metastatic melanomas. This antibody is also useful for the distinction of primary epitheloid melanocytic tumors from histologic mimics, such as histiocytomas.<sup>20</sup>[Figure 9]

**Fig. 9:** Melan A expression

### 1.7.3. Tyrosinase

Tyrosinase is an enzyme involved in the synthesis of melanin. Antibodies to tyrosinase have recently been shown to have a sensitivity and specificity that is roughly equivalent to that of HMB-45 and Melan-A. In general, we reserve the use of tyrosinase for cases strongly suspected of representing melanoma, which are negative with HMB-45 or A103.<sup>9</sup>[Figure 10]

**Fig. 10:** Tyrosinase expression

## 2. Conclusion

Immunohistochemistry is the indispensable tool for pathologists and are adjuvant to the regular histopathological techniques used for added precision and sensitivity in the diagnosis of the diseases, which were earlier difficult to diagnose. Immunohistochemical stains are basically focused on markers of specific cell and/or tumour type as it aids in the diagnosis of specific tumors. With the advent of IHC, it has become easy to understand the different nature of the tissues including their origin, behavior, monitoring the course of the disease and their response to treatment.

## 3. Conflicts of Interest

All contributing authors declare no conflicts of interest.

## 4. Source of Funding

None.

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