Immunohistochemistry in Bone and Soft Tissue Tumours

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8.1 Introduction

Immunohistochemistry (IHC) is an indispensable tool in the diagnosis of sarcomas. Sarcomas are a vast, diverse and complex group of neoplasms arising from mesenchymal origin. They present a diagnostic challenge as they are rare and have many overlapping appearances under the microscope. Accurate diagnosis of sarcomas is necessary for deciding management and prognostication of the disease. Historically, the categorisation of sarcomas has been based on their presumed line of differentiation [1, 2]. In some cases, the histomorphology is straightforward and distinct; however, in other cases, the histogenesis can be ambiguous, and further investigation is required to assist with the diagnosis [3]. To compound this, benign mesenchymal lesions and non-sarcomatous tumours are frequently encountered as differential diagnoses to sarcomas and thus present an additional diagnostic challenge [4]. The use of ancillary pathological techniques, therefore, is essential in the evaluation of mesenchymal tumour samples. IHC is a microscopy-based method that utilises immunological and biochemical principles to detect protein expression in tumour cells. Since its discovery in 1942, IHC has progressively improved through development of new antibodies. Nowadays, it has established itself as a valuable adjunctive step in the pathological diagnosis of surgical specimens of bone and soft tissue tumours.

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8.2 History of IHC: "Putting Tail Lights on Antibodies"

While IHC was discovered by Dr. Albert Coons in 1942, the story of its development is vast and involves the contribution of many scientists. Its inception can be traced back to the late 1800s, when Dr. Emil von Behring first described antibodies in the context of passive immunisation against diphtheria and tetanus and successfully treated his first patient [5]. It was through this work that earned him the first Nobel Prize in physiology and medicine in 1901 [6]. Following this, many other scientists contributed to research into antigen-antibody interactions, including Professor Paul Ehrlich, who further characterised the antigen-antibody interaction [7]; Dr. Kraus, who developed the precipitin test, a technique for detecting antigen-antibody complexes in solution [8]; and Dr. John Marrack, who attached dye to antibodies in order to visualise these complexes [9].

One important catalyst in the development of IHC occurred in the early 1920s, when Michael Heidelberger and Oswald Avery produced coloured antigen-antibody complexes to demonstrate that antigens were polysaccharides [10]. Then, by attaching a purple azo dye to antigens, Heidelberger and another associate, Forrest Kendall, were able to produce coloured antigen-antibody complex precipitates [11].

In 1942, Dr. Albert Coons developed fluorescein-labelled antibodies that could be detected by light microscopy and, thus, discovered the technique of IHC [12]. Immunofluorescence, however, was unable to be detected by electron microscopy, so subsequent developments aimed to address this limitation. In 1959, Dr. S. J. Singer detected antigens by electron microscopy by using ferritin-antibody [13]. Later, Graham and Karnovsky pioneered a new immunoenzyme labelling method by tagging antibodies with enzymes [14]. Through this work, Elizabeth Leduc, Stratis Avrameas and, separately, Paul Nakane, developed new immunoperoxidase techniques, which allowed detection of antigens and both light and electron microscopy levels [15, 16]. This technique was further modified by Ludwig Sternberger with the peroxidase-antiperoxidase (PAP) technique, thus improving sensitivity and efficacy [17]. In 1971, W. Page Faulk and G. Malcolm Taylor used colloidal gold as a label to detect antigens by electron microscopy [18]. Jurgen Roth, Moise Bendayan and Lelio Orci contributed further improvements to this protocol over the next decade through introduction of protein A-coated colloidal gold and thus developed the technique that is widely used today [19].

8.3 Procedure, Technical Considerations and Possible Limitations

The general concept of IHC involves using antibodies attached with a chromogenic enzyme to highlight and visualise a specific antigen of interest. The process of IHC is heavily protocolised to ensure strict standardisation and reproducibility of results [20]. The process of IHC is lengthy and complex, with each step contributing to its overall accuracy. The quality assurance of IHC is comprised on many critical components, including tissue handling, fixation, processing, sectioning, testing and interpretation of results [21].

The IHC protocol can be broadly simplified to three main players: (1) the tissue sample in question, (2) the antibodies to be used for testing and (3) the method of detection and analysis. The careful consideration of each of these main components determines the reliability of the IHC test. Each factor, their technical considerations and possible limitations will be discussed in further detail below.

8.3.1 Preparing the Tissue Sample

The first critical task is ensuring that enough viable tumour tissue is obtained in order to perform all necessary histology and ancillary tests. It is essential that the surgeon captures sufficient cells representative of the tumour to make an accurate diagnosis. This can be challenging when performing a biopsy. It has been shown that open biopsy has the greatest diagnostic accuracy when compared with fine needle aspiration and core biopsy [22]. Open biopsy, however, is more invasive, has higher risk of contamination and carries higher costs, so core biopsy is most often preferred as the next most accurate technique [23].

The pre-analytical phase of IHC is critical for accurate analysis. This includes tissue handling, fixation, paraffinisation, sectioning, storage and antigen retrieval, and each of these steps influences the quality of the result later. Firstly, the time from resection to fixation of a tissue sample can affect the detection of proteins by IHC due to the length of ischaemic time that the tissue undergoes [24]. During this period of time, the tissue undergoes 'cold ischaemia', in which proteins, RNA and DNA are degraded as a result of anoxic damage [25]. Studies of various cancer types have shown differences in IHC results when there is a delay in fixation [26–28]. Fixation itself is another area of potential variability or error. Factors including the duration, formula of the formalin solution and the tissue to fixative ratio all influence the quality of IHC [24, 29]. The tissue, once fixed, then undergoes further processing that includes washing and removal of excess fixative, dehydration and clearing and paraffin impregnation. These paraffin blocks are sectioned into thin slices and stored. Thickness of the sections may also influence IHC results and increase intensity of immunostaining. Tears sustained during the cutting process may cause artefact or loss of protein staining [25]. Storage of these sections also has impact on IHC, with studies demonstrating a loss of p53 staining with prolonged storage of sections [30, 31].

8.3.2 Selecting Useful Antibodies

In order to perform IHC, pathologists firstly need to select a panel of suitable antibodies that will help guide the diagnosis. The selection, application and interpretation of useful antibodies are discussed in further detail in this chapter. There are also some antibodies that have been shown to be non-specific and, as a result, do not provide diagnostic relevance. Vimentin, for example, is a marker that is widely expressed in almost all tumour types, mesenchymal and non-mesenchymal and therefore not recommended for use [3, 32, 33]. Similarly, histiocytic markers

alpha-1-antitrypsin and alpha-1-antichymotrypsin demonstrate widespread expression and have been replaced by the more specific CD68 [4, 33]. Myoglobin was previously used for detection of rhabdomyosarcoma, however is only expressed in approximately 60% of cases [34, 35]. Nowadays, myogenin is used in its place as a marker in rhabdomyosarcoma.

At the manufacturer level, it is necessary to enforce strict protocols for quality control in order to ensure standardisation of all reagents. There are also various technical considerations regarding storage of antibody reagents. Improper storage, for example, can be responsible for greater than 50% of IHC failures [36].

8.3.3 Detecting and Analysing the Reaction

Once the antigen-antibody reaction has taken place, there needs to be an adequate system to visualise and analyse the IHC results in a reproducible and reliable fashion. This part of the process can be broadly broken up into two broad steps: (1) detection of immunoreactivity and (2) clinical interpretation of results.

Detection systems are necessary to visualise whether an antigen-antibody reaction has taken place, since antibodies alone cannot be seen under light or electron microscopy. In order to do this, labels are attached to the antibodies. Common detection systems include direct-conjugate-labelled antibody method, indirect procedure, avidin-biotin complex method, streptavidin-biotin systems, phosphatase anti-phosphatase label system, polymer-based detection and tyramine amplification system [37]. These are all various techniques used to attach a chromogenic label to the antibodies. Polymeric- and tyramine-based amplification methods are beneficial in that they greatly improve sensitivity; however, they are also associated with more complex protocols that result in worse standardisation and reproducibility [21]. In order to better highlight the immunoreaction, counter staining can also be performed to provide further contrast to the antibody labels. Haematoxylin is the most common counterstain used for IHC, although eosin, methylene blue, methylene green and toluidine blue can also be used [21, 37].

In order to accurately interpret the results, pathologists also need to understand the relative sensitivities and specificities of the reagents. It must be emphasised that, while IHC is a powerful diagnostic tool, there is no single antibody or antibody combination that is completely unique to tumour type [38]. The interpretation of these markers in the context of bone and soft tissue tumours will be discussed in detail in this chapter.

8.4 Immunohistochemical Markers

8.4.1 Broad-Spectrum Markers

In sarcoma diagnosis, a panel of antibodies is routinely used in the initial instance to analyse a tissue sample. These markers can either confirm a diagnosis or characterise cell phenotypes to further guide immunohistochemical or molecular

testing. Frequently used cell-typic markers include cytokeratins, epithelial membrane antigen (EMA), S100 protein, desmin, smooth muscle actin (SMA) and CD34. These antibodies are largely non-specific, which means that they are expressed in multiple sarcoma subtypes. The interpretation of these markers, therefore, is most useful in combination with the wider clinical, radiological and histopathological picture.

8.4.1.1 Cytokeratins

Keratins are a family of proteins expressed in normal epithelial tissue. As such, the detection of keratins, in particular low-molecular-weight keratins, may indicate epithelial differentiation of soft tissue tumours [33]. There are 20 described keratin protein types, of which 8 have basic or higher isoelectric points (Type I; KRT 1–8) and the remainder has acidic or lower isoelectric points (Type II; KRT 9–20) [39]. AE1/AE3 are broad-spectrum immunohistochemical antibodies that are most commonly used to detect keratins in surgical pathology. AE1 contains antibodies to Type I keratins (KRT 10, 14–16 and 19), while AE3 recognises Type II keratins (1–8) [40]. This antibody cocktail is used as a first-line immunohistochemical investigation for spindle cell, pleomorphic, round cell and epithelioid tumours. They are also extremely useful in the differentiation between sarcoma and its carcinoma mimics [41].

8.4.1.2 Epithelial Membrane Antigen

EMA is a transmembrane glycoprotein that is widely expressed in normal epithelial tissue and their neoplastic counterparts. In addition to carcinomas, some soft tissue tumours demonstrate recurrent EMA expression. These tumours typically include epithelioid sarcoma [42], synovial sarcoma [43–46] and myoepithelioma [47, 48] but are widely absent in many other soft tissue tumours [49].

8.4.1.3 S100 Protein

The S100 protein describes a multigene family of 21 proteins that demonstrate close structural similarity but widely varying function that includes participation in proliferation, migration, inflammation and differentiation [50–52]. It is commonly positive in a range of soft tissue tumours, including melanomas, benign peripheral nerve sheath tumours (PNST), clear cell sarcoma and myoepitheliomas.

8.4.1.4 Desmin

Desmin, a muscular marker, is an intermediate filament that is normally expressed in skeletal muscle and smooth muscle cells [53, 54]. In surgical pathology, its main utility resides in the identification of rhabdomyosarcomas and leiomyosarcomas, as well as their benign counterparts [53, 55]. Desmin is, however, positive in a number of other sarcomas, including desmoplastic small round tumours [56, 57], myofibroblastic tumours [53, 54, 58] and tenosynovial giant cell tumours [59]. Its interpretation is best made alongside other myogenic markers, including SMA and more specific markers myogenin and MyoD1, in the differentiation and diagnosis of muscular tumours.

8.4.1.5 Smooth Muscle Actin

SMA is another muscular marker that is expressed in normal smooth muscle cells. It is a useful marker in diagnosis of smooth muscle tumours and myofibroblastic tumours. It is also expressed in normal myofibroblasts, myoepithelial cells and smooth muscle-related pericytes and glomus cells and, therefore, positive in the tumours of the respective lineages [60].

8.4.1.6 CD34

CD34 is a transmembrane glycoprotein that is widely expressed in many soft tissue tumours, particularly spindle cell and epithelioid cell tumours [4]. Its expression is encountered consistently in malignant vascular tumours [61, 62], solitary fibrous tumour (SFT), dermatofibrosarcoma protuberans and spindle cell lipomas. Variable expression is seen in GISTs, epithelioid sarcoma and MPNST.

8.4.1.7 CD99

CD99, also known as MIC2, is a transmembrane glycoprotein normally expressed on the cell surface of T lymphocytes [63]. In surgical pathology, it is a non-specific marker that is useful in the classification of round cell tumours. It is particularly useful in the identification of Ewing's sarcoma/PNET, in which it demonstrates strong membranous staining [64–66].

8.4.2 Novel Markers

The advent of molecular techniques has allowed the genetic characterisation of soft tissue tumours and discovery of recurrent mutations including reciprocal translocations, amplifications and point mutations. An understanding of these genetic aberrations and their respective protein products have allowed pathologists to develop targeted immunohistochemical surrogates for the identification of genetic mutations in tumours samples that can aid in its diagnosis.

8.4.2.1 FLI-1

In translocations, these surrogate markers do not detect the fusion itself but rather identifies the resultant overexpression of specific proteins [67].

Ewing's sarcoma/primitive neuroectodermal tumours (PNET) are a class of small, blue, round cell tumours that share a similar histomorphology with tumours such as neuroblastoma, rhabdomyosarcoma and poorly differentiated synovial sarcomas [68]. A specific t(11;22) translocation is found in approximately 90% of all Ewing's sarcoma/PNET, resulting in an EWSR1-FLI-1 fusion product [69–71]. The sc-356 immunohistochemical stain is a polyclonal antibody to the carboxy-terminal of FLI that has demonstrated 71% sensitivity and 92% specificity for Ewing's sarcoma/PNET in previous studies [68, 72].

8.4.2.2 MDM2 and CDK4

Atypical lipomatous tumours/well-differentiated liposarcomas (ALT/WDLPS) and dedifferentiated liposarcomas (DDLPS) are two separate types of fatty tumours that are both characterised by complex genomes, resulting in supernumerary ring and giant marker chromosomes and the amplification of 12q13–15 gene locus [73, 74]. MDM2 and CDK4 are two genes within this locus, and their amplified protein products can be detected by IHC [75, 76].

8.5 Application and Interpretation of IHC

IHC has a well-established role in the diagnosis of bone and soft tissue tumours. The frequent overlapping histological features of sarcomas require the use of ancillary techniques, such as IHC, to help further distinguish tissue characteristics. As described in Fig. 8.1, IHC plays three key roles in the differential diagnosis of bone and soft tissue tumours: (a) in establishing any rare or atypical benign mesenchymal lesions that may resemble malignant tumours; (b) the identification of malignant lesions of non-mesenchymal origin; and (c) the characterisation of specific sarcoma subtypes, in particularly distinguishing one sarcoma type from histologic mimics. Ultimately, accurate diagnosis of mesenchymal neoplasms is essential in providing prognostic information for patients and guiding appropriate therapeutic care.

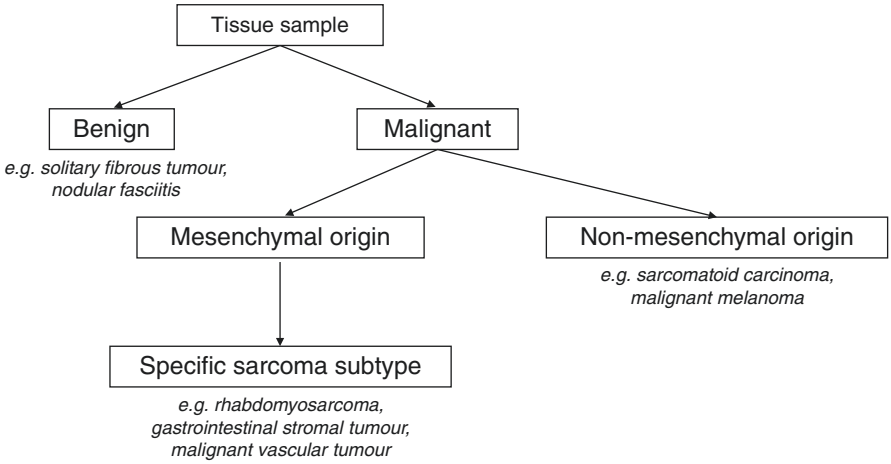

Fig. 8.1 A schematic overview of the application of immunohistochemistry (IHC) in the diagnostic process of sarcomas

8.5.1 Identification of Benign Tumours

While IHC alone cannot definitively differentiate all benign and malignant tumours [38], it can be extremely useful in identifying atypical benign lesions that share similar histological appearances with a malignancy. Clarification of whether a tumour is benign or malignant may influence the course of treatment, for example, the type of excision (marginal vs wide local excision) or the addition of neoadjuvant therapy. Tumours that may mimic malignancies include SFT and benign PNST.

8.5.1.1 Solitary Fibrous Tumour

SFT is a benign soft tissue tumour of spindle cell morphology. Its histologic features can be difficult to distinguish from various other soft tissue tumours, including mesothelioma or other spindle cell tumours [4, 77]. IHC reveals CD34 and Bcl-2 positivity in most cases, and these markers are therefore highly sensitive for SFT [78, 79]. Negative staining for both markers would strongly suggest an alternative diagnosis [80]. IHC for STAT6 can be used as a surrogate marker of the NAB2-STAT6 fusion product highly characteristic for SFT [81]. STAT6 is therefore a highly specific marker for SFT and is useful in distinguishing from histologic mimics [82, 83]. Other valuable markers in the diagnosis of SFT include CD99 and beta-catenin [78, 84].

8.5.1.2 Nodular Fasciitis

Nodular fasciitis (NF) typically presents as a rapidly growing, poorly circumscribed mass that reveals dense cellularity and high mitotic activity on pathological examination. It is not uncommon for NF to be misdiagnosed as sarcoma, such as dermatofibrosarcoma protuberans, low-grade myofibroblastic sarcoma or malignant peripheral nerve sheath tumour (MPNST), thus warranting IHC for definitive diagnosis [85–87]. IHC demonstrates positive staining for SMA in almost all cases with consistent negativity for desmin, h-caldesmon, S100 and beta-catenin [88, 89].

8.5.2 Exclusion of Non-mesenchymal/ Non-sarcomatous Tumours

Once a benign lesion has been ruled out and the tumour has been classified as malignant, the pathologist must exclude the diagnosis of a non-mesenchymal tumour. There are various non-sarcomatous lesions that may resemble sarcomas due to their overlapping histological features. Common examples include sarcomatoid carcinoma, melanoma, lymphoma and mesothelioma.

8.5.2.1 Sarcomatoid Carcinoma

Histologically, sarcomatoid carcinomas may be confused with undifferentiated spindle cell or pleomorphic sarcomas [60]. It is most often associated with primary breast carcinoma, renal cell carcinoma and mucosal or cutaneous squamous cell carcinoma, although it can present at any site [41]. In these cases, it is important to

use IHC to identify areas of epithelial differentiation, which will support a diagnosis of carcinoma [90]. Broad-spectrum keratins, such as AE1/AE3 and pan-cytokeratin and EMA, are expressed in almost all sarcomatoid carcinoma, allowing distinction from histologically similar sarcomas [91–94].

8.5.2.2 Malignant Melanoma

Malignant melanoma can prove a diagnostic challenge for pathologists as they often mimic sarcomas, even in their immunohistochemical profile. Primary malignant melanoma, for example, can appear histologically like MPNST and clear cell sarcomas. Both MPNST and malignant melanoma have been shown to express S100 [95–99], as has clear cell sarcoma [100–102]. In this situation, the pattern of staining carries significance in delineating these entities. S100 expression is more commonly diffuse in melanoma, compared with MPNST, in which it is usually focal or multifocal. In clear cell sarcoma, staining for HMB45 is generally more intense or diffuse than S100, which is not the case in melanoma [41].

8.5.3 For Diagnosis of Mesenchymal Tumours

In some sarcomas, IHC forms a crucial part of diagnosis, where the immunohistochemical profile of a tissue sample may be diagnostic or highly suggestive of a sarcoma subtype. Examples discussed further below are rhabdomyosarcoma, gastrointestinal stromal tumours (GIST) and malignant vascular tumours.

8.5.3.1 Rhabdomyosarcomas

Rhabdomyosarcomas encompass multiple subtypes, of which embryonal rhabdomyosarcoma (ERMS) and alveolar rhabdomyosarcoma (ARMS) are the most common. Desmin, alongside more lineage-specific markers MyoD1 and myogenin, is useful for diagnosis [103, 104]. These markers are positive in almost 100% of ERMS and ARMS and up to 90% of all rhabdomyosarcomas [53, 103, 105, 106]. The expression pattern of myogenin is also of significance in these tumours. For instance, myogenin staining is often stronger and more uniform in ARMS than ERMS [107]. A diffuse expression of myogenin has also been correlated with poor survival in paediatric patients with rhabdomyosarcoma [108].

8.5.3.2 Gastrointestinal Stromal Tumours

Identification and diagnosis of gastrointestinal stromal tumours (GIST) is crucial for patient outcomes as there is a highly effective treatment available [109]. KIT (CD117) is a receptor tyrosine kinase that is activated in 85–90% of GISTs through a gain-of-function mutation [110–113]. This results in the constitutive activation of KIT receptor tyrosine kinase in a ligand-independent manner [114]. Immunohistochemical detection of CD117, therefore, is highly supportive of a GIST diagnosis. This finding is particularly significant given the availability of targeted therapies such as imatinib, an inhibitor of KIT-tyrosine kinase [115, 116]. It is important to note, however, that CD117 can be positive in other tumours

including Ewing sarcoma [117–119] and angiosarcoma [120–122], so the immunohistochemical results must be interpreted in the context of clinical and radiological findings. The small subset of GISTs that are KIT-negative is often positive for DOG1, also called anoctamin-1 (Ano-1). DOG1/Ano-1 is considered the antibody of choice in addition to CD117 in the immunohistochemical testing for GISTs [123], with similar sensitivities and specificities between DOG1/Ano-1 and CD117 [124].

8.5.3.3 Malignant Vascular Tumours

Malignant vascular tumours encompass a broad class of tumours including angiosarcoma, epithelioid and spindle vascular tumours and Kaposi sarcoma. IHC plays a valuable role in diagnosing malignant vascular tumours because due to their wide spectrum of histopathological patterns that are not easily identifiable on histology alone. In assessing these tumours, CD31, CD34 and Fli-1 are useful immunohistochemical markers. CD31 is considered the gold-standard marker in the diagnosis of malignant vascular tumours, as it demonstrates positivity in angiosarcomas, Kaposi sarcomas and epithelioid haemangioepitheliomas [61, 125, 126]. ERG is also a highly sensitive vascular marker in the diagnosis of angiosarcoma [126]. CD34 is often positive in angiosarcoma and Kaposi sarcoma but variably expressed in epithelioid vascular tumours [61, 127]. A more recent marker, Fli-1, also demonstrates good sensitivity for spindle and epithelioid tumours [128]. Additionally, Kaposi sarcoma is an atypical vascular lesion that is uniquely defined by the presence of human herpes virus-8 (HHV-8), which can be detected by IHC [129, 130].

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