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REVIEW ARTICLE

Usefulness of immunohistochemical indicators for diagnosis and prognosis of poorly differentiated tumours

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Abstract

Immunohistochemical studies have become an indispensable element of establishing the correct histopathological diagnosis of poorly differentiated lesions, proving particularly suitable, and occasionally indispensable, for diagnosis of poorly differentiated neoplastic tumours. Knowledge of the mechanism of action and normal reaction of individual proteins is required in selection of the antibody pattern for a given tissue and in evaluation of the obtained results. This paper aims to promote the application of immunohistochemical techniques in routine diagnosis, especially in cases of poorly differentiated or undifferentiated tumours.

Keywords: domestic animals, neoplasms, immunohistochemistry, poorly differentiated tumours.

Introduction

The incidence of neoplastic diseases in domestic animals continues to increase. In parallel, enormous progress in knowledge of the possible diagnostic techniques for these diseases has also been made. In order to diagnose tumours, the material can be sampled in the form of a thin- or large-needle bioptate or a tissue section. Histopathological examination represents one of the most reliable techniques allowing for correct diagnosis. However, particularly in cases of poorly differentiated lesions, its result increasingly often proves to be insufficient for conclusive diagnosis, and additional histochemical or immunohistochemical (IHC) tests become necessary. IHC tests allow the definition of the histogenesis of a neoplastic lesion and its proliferative and metastatic potential. Moreover, they permit a more precise prognosis and appraisal of the efficacy of planned treatment.

IHC reactions aim to demonstrate the location of antigen–antibody binding. For visualisation of the location, immunoenzymatic techniques are most frequently used, employing chemically labelled or enzyme-labelled antibodies. An antibody labelled in such a manner yields a colour reaction, which can be examined using a light microscope.

Immunohistochemical techniques permit the detection of various proteins, located either in healthy cells or in neoplastically transformed cells. Choosing an appropriate antibody, we can define the origin of tumour cells and their proliferative and metastatic potentials as well as the intensity of the angiogenetic process in a tumour. This paper presents data on immunohistochemical techniques and clarifies the potential provided by respective studies in veterinary medicine. Moreover, the authors demonstrate the suitability of IHC for diagnosis of undifferentiated tumours, for presenting the mechanism and types of immunohistochemical reactions, and for assisting in selection of a panel of antibodies appropriate for diagnosis of tumours frequently manifested in dogs. Additionally the paper describes the most frequent problems encountered by pathomorphologists in their immunohistochemical studies, and presents ways to prevent them.

Table 1. Expression of cell markers in undifferentiated tumours of different origin. Results do not invalidate the potential for exceptions (*i.e.* sarcocarcinoma, in which co-expression of vimentin and cytokeratin can be detected)

	Epithelial tumour	Mesenchymal tumour	Round cell tumour		
			lymphoma	mast cell tumour	histiocytoma
cytokeratin	+	-	-	-	-
vimentin	-	+	+	+	-
CD3	-	-	+/-	-	-
CD79α	-	-	+/-	-	-

Origin of tumour cells. The first step for establishing correct diagnosis involves determination of the tissue type from which the tumour cells originate. This seems particularly important in metastatic lesions and poorly differentiated neoplasms, in which cells are morphologically similar and, therefore, one cell type is difficult to discern from another (Table 1).

In both normal and pathologically altered cells of epithelial origin, expression of two epitopes present in most epithelial cytokeratins (CK) can be demonstrated (43). Cytokeratins belong to intermediate filaments constituting one of the most important elements of the cytoskeleton in human and animal epithelial cells (10). Previous studies used an AE1/AE3 kit associating two monoclonal antibodies, and epitope expression was demonstrated in most cytokeratins (29, 43). The clinical value of cytokeratin expression also relies on the early detection of recurrence and the fast assessment of treatment efficacy (3).

Vimentin is a protein that also belongs to the group of intermediate filaments, but it is typical for cells of mesenchymal origin. Expression of respective epitopes is detected in sarcoma, melanoma, or lymphoma cells. Unfortunately, vimentin is not an ideal marker for differentiation of tumours of epithelial and non-epithelial origin. Another cellular marker – calretinin – has recently been used for this purpose. It should be mentioned that co-expression of vimentin and cytokeratin was demonstrated in normal cells and in neoplastically altered cells, particularly in cancer cells. Such a situation is observable in carcinoma of the kidneys, adenocarcinoma of endometrium, in certain sarcomas, and in sarcocarcinoma. Currently, both antibodies should be incorporated in studies on metastases and in differential diagnosis of tumours (2).

Desmin is another important intermediate filament. It is a muscle-specific protein, which is expressed in skeletal and smooth muscles or within neoplasms with muscle differentiation (30). Desmin may be used in the various antibody panels determining tumour origin in spindle-shaped cellular lesions.

Calretinin is an intracellular protein which is capable of binding calcium, and is present in several types of normal and pathologically altered cells, including nerve cells, renal tubule cells, endocrine glands, and mesothelial cells. The function of calretinin has not yet been elucidated in detail, but it probably plays an important role in securing cell survival during disturbances in calcium homeostasis (19). In humans it is used in medicine primarily for distinguishing pulmonary adenocarcinoma from malignant mesothelioma, and in veterinary medicine it is employed in diagnosis of tumours in the testes, nervous tissue, and mammary gland (36).

Expression of S-100 protein, belonging to the family of low-molecular-weight proteins in vertebrate cells, can be detected in approximately 90%–100% of melanocytic lesions. The protein is present in both normal melanocytes and melanomas. It is important to realise that in up to 4% of malignant melanomas, S100 immunolabelling can be negative (1). Its expression can also be present in various tumours: smooth muscle neoplasms, carcinomas (but not in squamous cell carcinoma), myoepithelial tumours, and others.

Lymphomas are one of the most common tumours of the lymphoid system. Upon implementation of treatment it is significant to distinguish lymphomas originating from B cells from those originating from T cells. Proteins CD3 and CD79α are most frequently used for this purpose. Expression of CD3 is already shown by T lymphocytes at the stage of early thymocytes and this represents one of the first signs of cell affiliation to the cell line. Therefore, reactions for the protein are detected in most tumours originating from T cells, although negative reactions can be encountered in tumours of high malignancy. CD79α is used to detect B lymphocytes. Its expression persists throughout the entire period of lymphocyte differentiation, but its expression is markedly reduced upon activation of already mature B lymphocytes. It should be noted that a small proportion of plasma cells manifest a positive reaction in staining. The two markers are frequently used in parallel and their co-expression is used in diagnosis of lymphoblastic leukaemias and lymphomas consisting of T lymphocytes (33).

Proliferative potential and apoptosis. Evaluation of a tumour's proliferative potential represents a very significant element in the definition of its malignancy and, at a further stage, in specifying the prognosis for the patient. A high proliferative potential is linked to a higher rate of tumour growth, and frequently to its

ability to develop local recrudescence and distant metastases.

The most frequently employed marker of cell proliferation is the Ki-67 antigen. Its presence can already be detected in the G_1 phase of the cell cycle; it increases in phases S and G_2, reaching peak expression in the M phase and abruptly decreasing thereafter. In the G_0 phase, in which cells remain inactive, it is undetectable. It represents a valuable and frequently used cell proliferation index (31).

Another marker linked to the proliferative potential of a tumour is proliferative cell nuclear antigen (PCNA). The protein is important for maintenance of cell cycle continuity; its activity can be detected from the G_1 phase up to the M phase with peak activity noted in the S phase of the cell cycle. Expression of PCNA is associated also with DNA repair processes, it manifests a prolonged half-life, and its activity can also be detected after completion of mitosis. Therefore, the protein is gradually ceasing to be used as a quantitative marker of cell proliferative activity (31).

Useful markers in immunohistochemical examination of tumours also include proteins p53 and p21; expression of the latter can be stimulated by p53 or it can appear in tissue in a p53-independent manner. One of the phosphoproteins present in several normal cells is p53 protein. In neoplastic processes mutations frequently develop in the gene coding for the protein, making it more stable, and therefore causing it to begin to accumulate in tissues. Physiologically, the protein is responsible for, *i.a.*, control of the cell cycle and apoptosis; it functions as a suppressor of tumour development. Its mutated form behaves as a dominant oncogene, taking part in cell neoplastic transformation (41). The other protein, p21, belongs to the Cip/Kip family of cyclin-dependent kinases (CDK). Complexes of cyclin/CDK facilitate passage of cells from the G_1 phase to the S phase of the cell cycle. This protein fulfils two functions: it may act both as an inhibitor of cyclin/CDK complex activity and as a stimulator of the complex formation. A significant reduction or a complete absence of p21 was demonstrated in neoplastic cells, but an augmented expression of the protein does not necessarily lead to cessation of tumour growth. Despite earlier assumptions and expectations, no relationship has been demonstrated till now between expressions of p53 and p21 (14, 16).

In recent years, studies have often been performed which attempt to clarify the control mechanisms of DNA synthesis. Proteins controlling the processes are useful indices of tumour growth. The indices include proteins of the minichromosome maintenance (MCM) group, such as MCM 2–9 proteins (27). The proteins initiate DNA duplication and, by doing so, they mark the cells with the ability to replicate. In the course of the G_1 phase of the cell cycle, an inactive MCM protein binds to the pre-replication complex at sites of replication initiation. Under the effect of DBF4-dependent kinase (DDK) and CDK, the protein becomes active. The activity can be detected throughout the duration of the entire cell cycle, in phases G_1, S, G_2, and M. MCM becomes inactive again when the cell reaches resting condition, differentiates, or becomes senescent (26).

Caspases are a family of genes important for maintaining homeostasis through regulating cell death. They are endoproteases that hydrolyse peptide bonds. Caspase-mediated processing can result in substrate inactivation; it may also generate active signalling molecules that participate in ordered processes such as apoptosis and inflammation (28). It is highly expressed in the lungs, spleen, heart, liver, and kidneys.

Cytokine receptor. CD117 or c-kit is a tyrosine-protein kinase that acts as cell-surface receptor for the cytokine KITLG/SCF. It plays a key role in the regulation of cell proliferation and survival, as well as haematopoiesis, stem cell maintenance, gametogenesis, mast cell development, migration and function, and in melanogenesis (42). The importance of c-kit expression in many tumours, *i.e.* mast cell tumour, gastrointestinal stromal tumours, or seminomas has previously been described. It could be used as a predictor of carcinogenesis or a helpful marker to establish appropriate tumour therapy.

Metastatic potential. Cellular ability to get released from the parent tumour is associated, among other factors, with the strength of tumour cell binding to the sublayer and to other cells, the attachment secured to a large extent by adhesion proteins, including catenins and cadherins.

β-catenin is a cytoplasmic protein which plays a crucial role in cadherin-mediated anchorage of cells, cytoskeleton organisation, and control of gene expression. It also provides a key link in the Wingless/Wnt/β-catenin signalling cascade, important in embryogenesis and neoplastic transformation (9). β-catenin interacts with the promoter site of genes linked to activation of cell proliferation and inhibition of the apoptotic phenomenon, which may lead to neoplastic transformation. The neoplastic process is accompanied by degradation of β-catenin, resulting in its accumulation in cytoplasm and in the cell nucleus, linked to intensified tumour progression, development of metastases to lymph nodes, and to a less favourable prognosis (9, 37).

E-cadherin is a trans-membrane adhesion protein, dependent on calcium ions and, together with β-cadherin, responsible for cellular adhesion (32). Genetic disturbances result in lost or lowered expression of the protein, which is linked to increased invasiveness of tumour cells, increased malignancy, and increased frequency of tumour metastases (9).

Intensification of angiogenetic process. Neoangiogenesis involves formation of new blood vessels from the vascular endothelium of pre-existing blood vessels. The process of vascular development plays a key role in the neoplastic process, and is a pre-

requisite for tumour growth and for development of metastases (6, 34).

Vascular endothelial growth factor (VEGF) is regarded as the most powerful and most specific growth factor for endothelial cells. It plays a very important role in the process of neoangiogenesis: it is the principal controller of angiogenesis and vasculogenesis, stimulates endothelial proliferation, promotes cell migration, inhibits apoptosis, and affects permeability of blood vessels. The factor binds to high-affinity receptors belonging to the tyrosine kinase family. Its increased expression has been confirmed in several neoplastic tumours in animals as well as in cell cultures (5, 6).

Another marker used to evaluate the neoangiogenesis level in tumours is von Willebrand factor VIII. It is a glycoprotein; its synthesis takes place in the endothelium and in blood platelets. It participates in blood clotting, in which it is regarded as serving as a carrier and stabiliser of FVIII; it is indispensable for platelet adhesion to collagen in sites of vascular injury. The factor undergoes expression first of all in blood vessel endothelial cells (in healthy or neoplastically transformed tissue) and in lymphatic vessels. Moreover, presence of the factor is detected in megakaryocytes and blood platelets (34).

CD31 or platelet/endothelial cell adhesion molecule 1 (PECAM-1) may serve as another example of a good marker allowing detection of endothelial cells in animals. It is a trans-membrane protein of the immunoglobulin family. CD31 is present at the surface of blood platelets, monocytes, macrophages, and neutrophils. Moreover, it plays an important role in the course of inflammatory processes, specifically during adhesion between endothelial and inflammatory cells. It should be noted that it undergoes expression in the vascular endothelium, including arteries, arterioles, venules, veins, and capillary blood vessels (24, 34).

Inflammatory mediators. From recent studies on animal models, strong evidence points to prostaglandins playing a key role in tumour promotion. An excessive expression of cyclooxygenase (Cox) and an increase in its enzymatic product, prostaglandin E2 (PGE2), seem to be involved in the development of various tumours. Deregulation of this enzymatic pathway appears to be an important part of the neoplasm progression, affecting cell proliferation, apoptosis, immune surveillance, and angiogenesis (7). Recent data suggest that inflammation may be one of the most important processes during malignancy (18). Overexpression of inflammatory cell markers might play an important role in tumour growth, neoangiogenesis, and the tissue repair process. It might help to reveal high-risk patients and improve accuracy of predictions and implementation of treatment.

Cox or prostaglandin-endoperoxide synthase (PTGS) is an enzyme which is responsible for formation of prostanoids including thromboxane and prostaglandins such as prostacyclin. Both cyclooxygenases are constitutively expressed by most cells, but Cox-2, in contrast to Cox-1, is the mitogen-inducible isoform. Cox-2 may play a crucial role in promoting neoangiogenesis and increases tumour cell motility and invasiveness (21).

Prostaglandin E2 (PGE2) is one of the primary cyclooxygenase products of arachidonic acid and one of the most widely investigated prostaglandins. It is produced by a variety of cells and tissues and has a broad range of biological activities. Three prostaglandin E synthase (PGES) enzymes have been identified: microsomal PGES-1 (mPGES-1), PGES-2 (mPGES-2), and cytosolic PGES (cPGES) (11).

Tumour necrosis factor alpha (TNF-α) is mainly secreted by macrophages and can induce the death of certain tumour cells. Under certain conditions, it can also increase cell proliferation and induce cell differentiation (22). It is a potent pyrogen, causing fever by direct action or by stimulation of interleukin-1 secretion and is implicated in the induction of cachexia. TNF-α overexpression is linked with both disease severity and mortality.

Multidrug resistance marker. One of the most important factors implicated in treatment efficacy is tumour cell multidrug resistance (MDR). Exploring the mechanisms of this phenomenon and methods of effective treatment are two of the trends of current cancer research.

P-glycoprotein (P-gp) is synthesised by multidrug-resistance genes. It is an ATP-dependent drug efflux pump, reducing intracellular drug concentrations and thereby producing resistance to a large number of structurally unrelated cytotoxic agents (12). Overexpression of P-gp results in decreased accumulation of a drug within the tumour cells. Multidrug-resistance-associated protein's (MRP) chemical structure is similar to P-gp (15). Increased expression of MRP may result in accumulation of a drug in neoplastic cells and consequently cause drug resistance (23).

Heat shock proteins. Heat shock proteins (HSP) are important components of signalling pathways that regulate growth and development. They are connected with disease aggressiveness and with resistance to anticancer therapies, and can be also used as targets for therapies.

HSP70 and HSP90 are molecular chaperones expressed constitutively to maintain protein homeostasis and are induced upon environmental stress. They promote the maturation, structural maintenance, and proper regulation of specific target proteins involved for instance in cell cycle control and signal transduction (25).

Hormone receptors. Neoplastic cells with these receptors depend on hormones to grow. They help to determine a patient's risk of relapse and whether the tumour can be treated with hormonal therapy.

In veterinary medicine, there is little knowledge of the role of various proteins in canine mammary tumours (CMT). Oestrogen and progesterone receptors may be good prognostic markers in CMT, but some studies do

not confirm this finding (20, 40). Human epidermal growth factor receptor 2 (HER2)'s relevance in the pathogenesis and prognosis of CMT is still controversial (4). In contrast, epidermal growth factor receptor (EGFR) may contribute to malignant epithelial transformation of neoplasms. Queiroga *et al.* (35) suggest that EGFR may represent a therapeutic target in cases of high histological aggressiveness and especially in cases of metastatic phenotype and poor prognosis. The role of androgen receptor (AR) is still unknown. Recent studies have demonstrated the presence of this receptor in normal and neoplastic canine mammary tissue. An overexpression of AR may be involved in the tumoural invasion process (13, 20).

Immunoscoring. Expression of cell markers with nuclear reaction should be assessed using a semi-quantitative scale, evaluating the percentage of positive cells: 0%–5%, no reaction (-); 6%–25%, weak reaction (+); 26%–50%, moderate reaction (++); >50%, intense reaction (+++) (Fig. 1).

The scale, which takes into account both the percentage of positive cells (A) and intensity of reaction colour (B), is useful in detection of proteins with cytoplasmic or membranous reaction. The final result represents a product of the parameters and ranges from 0 to 12 pts: 0 pts, no reaction (-); 1–2 pts, weak reaction (+); 3–4 pts, moderate reaction (++); 6–12 pts, intense reaction (+++) (Fig. 1). The assessment is difficult and subjective. The examination should be performed in repeated iterations by a few pathologists and the results averaged.

Lately it has been suggested that automated computer-based evaluation can provide a consistent and objective evaluation of immunohistochemical slides. The method processes microscopy images from tissue slides with a multistage algorithm. Evaluation includes steps of colour pixel classification, nuclei segmentation, and cell membrane modelling, and extracts quantitative, continuous measures of cell membrane staining intensity and completeness.

Expression of markers in the vascular endothelium may be evaluated on the basis of mean vessel number per visual field (hot spots) using magnification of 200× or 400× (Fig. 1).

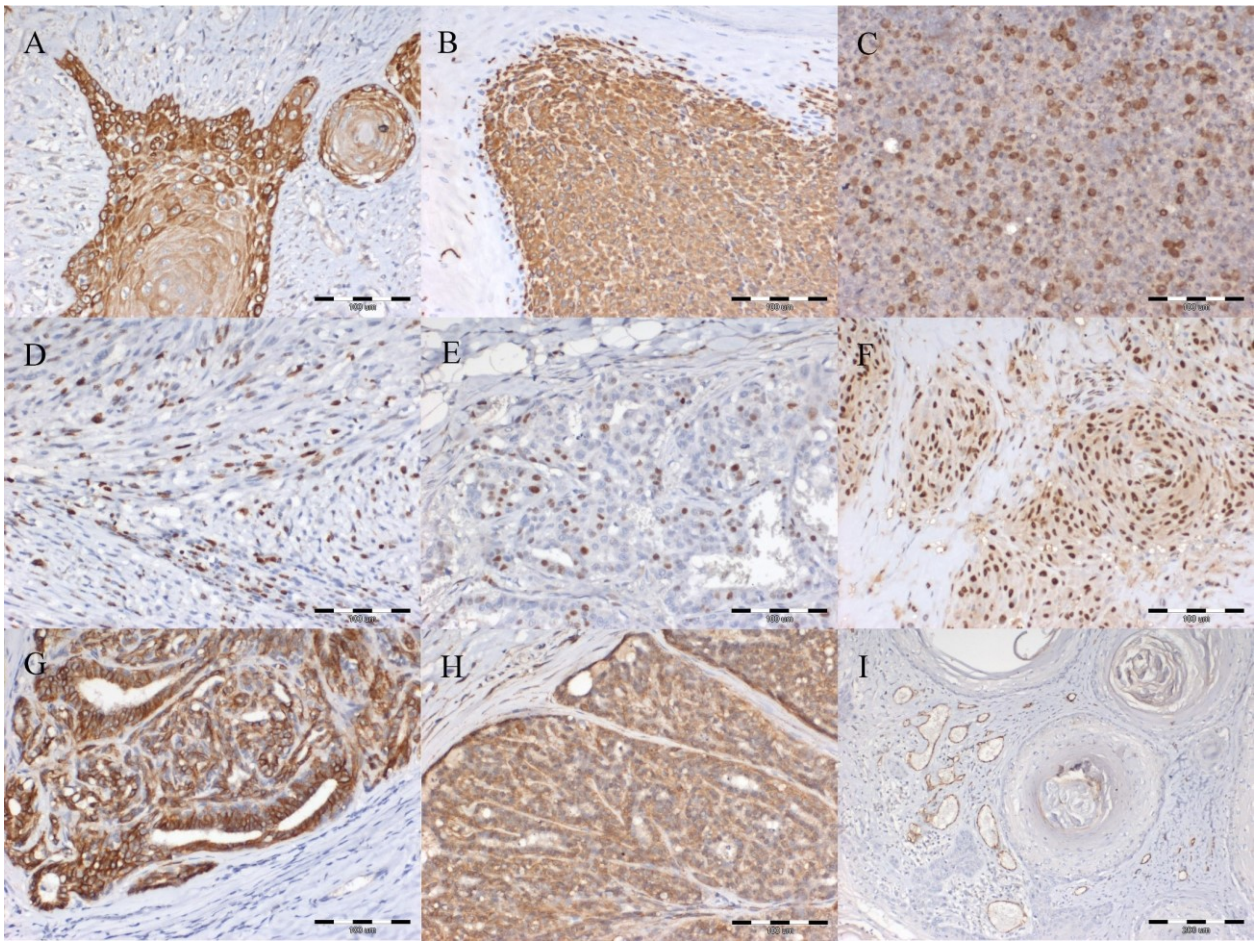

Fig. 1. Examples of immunohistochemical reactions. A–C – cells labelled by the antibody display staining confined to the cytoplasm. A – expression of cytokeratin., bar = 100 µm. B – expression of vimentin, bar = 100 µm. C – expression of CD3, bar = 100 µm. D–E – nuclear reaction. D – expression of Ki-67, bar = 100 µm. E – expression of MCM-3, bar = 100 µm. F – nuclear and cytoplasmic staining. Expression of PCNA, bar = 100 µm. G–H –cytoplasmic and membrane reaction. G – expression of E-cadherin, bar = 100 µm. H – expression of β-catenin, bar = 100 µm. I – immunolabelled blood vessels. Expression of CD31, bar = 200 µm

Common problems. Fixation is the first variable which undoubtedly enormously affects the result of studies. Until now, no single universal fixative has been formulated which could be used for every tissue or antigen type. Most frequently formalin is used, which allows for relatively high immunoreactivity of antigens. Occasionally, however, formalin induces alterations within the studied epitope which abolish antibody binding and therefore lead to a false negative result. Sometimes it reflects an excessively prolonged fixation (44).

Another cause of abnormal IHC reaction may involve inappropriate tissue dehydration. The problem may be bypassed by always using fresh alcohol solutions.

Inappropriately selected microscope glasses may manifest poor adhesiveness while imprecise sectioning may result in bending of sections and formation of grooves, which may cause non-uniform staining of the preparation (Fig. 2A).

Abnormal results are also obtained using outdated reagents and antibodies. False positive reactions are observed upon nonspecific adsorption of antibodies or markers by the tested tissue, which may be prevented, among other ways, by using purified reagents, diluted antibodies, or changing buffer solutions (17). In veterinary medicine, the use of antibodies designed to be used for human tissues brings the potential for non-specific reactions linked to certain differences in proteins forming cellular structures.

There are two methods of antigen retrieval – enzymatic and heat-mediated. During each method a sufficient volume of water or buffer must be used to cover the slides. Enzyme activity can be reduced by cold slides (they will lower the temperature of the solution). Enzymatic retrieval can sometimes damage the morphology of the tissues, so the concentration and treatment time need to be tested.

Performing negative and positive controls must not be neglected. The negative control involves application of the same technique, with identical reagents, but on healthy tissue, possibly of the same origin. In this way the possibility of non-specific reaction between studied antibodies and the employed tissue is eliminated. The positive control, in which the antibody is omitted in the procedure, allows the exclusion of abnormal reactions between the used reagents and tumour tissue (17).

Methodical errors can be suspected when one of the following nonspecific staining patterns is encountered: the absence of staining of studied tissue and of positive control, the absence of staining of studied tissue and normal staining in positive control, weak staining of studied tissue and in positive control, staining of background in the studied tissue and/or in the positive control, or the presence of artefacts in the studied tissue and/or the control (Fig. 2B). Moreover, the studied material should manifest the staining pattern previously defined in specification of individual antibodies (8).

When the absence of staining is noted in the studied tissue and positive control, the appropriacy of all reagents and the sequence of their use in all stages of staining should be checked. Attention should be devoted also to correct titration and dilution of antibodies. Double absence of staining tends to be accompanied by an improper pH of buffers or incorrectly prepared chromogen solution (39).

Absence of staining of the studied tissue and correct staining in the positive control indicates that the experimental technique and employed reagents were faultless. The problem may pertain to the tissue itself, which might be improperly prepared (44).

If studies demonstrate weak staining of the studied tissue and normal staining of the positive control abnormal tissue fixation or improper performance of procedures may be suspected. Apart from the causes mentioned in the case of the complete absence of the reaction, an improper concentration of the antibodies may also be put under consideration (39).

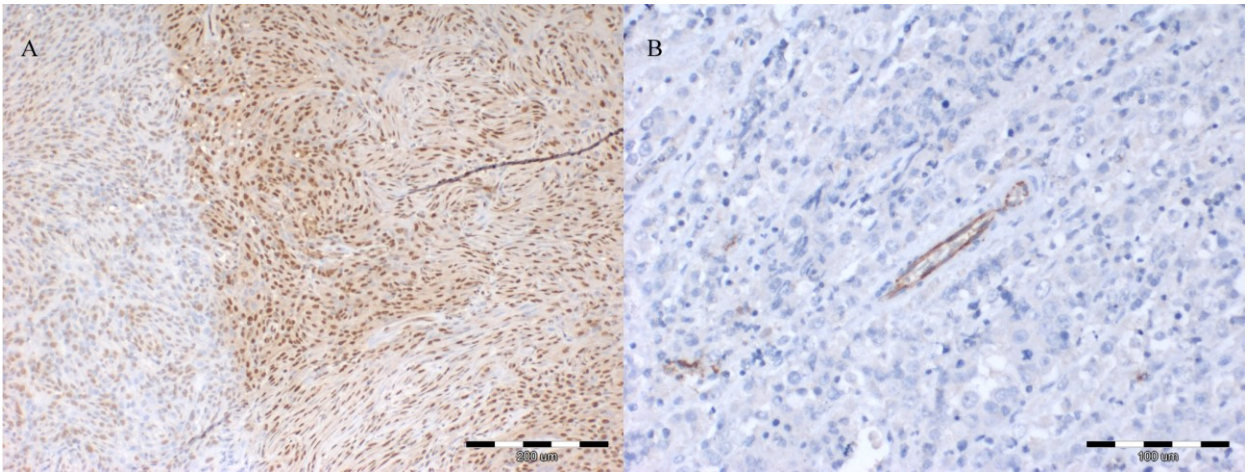

Fig. 2. Typical errors during immunohistochemical staining. A – grooves as a result of imprecise cutting, which causes non-uniform staining, bar = 200 µm. B – presence of artefacts, bar = 100 µm

If the background of preparations is stained it is indispensable to perform the negative control. Frequently, erroneous staining of this type results from non-specific ionic binding between antibodies and elements of the studied tissue, *e.g.* collagen fibres. In such cases, the error can be eliminated using another antibody from a serum of a non-immunological character originating from the same animal species. It is also useful to use salts in a buffer solution. Background staining may also result from abnormally fixed tissue or from the presence of necrosis foci in the preparation (38).

Chromogen artefacts or those originating from tissue contrasting stains may be eliminated by filtration. Occasionally, the presence of precipitates reflects the presence of endogenous stains, such as haemosiderin or melanin and their manifestation will be detected in the negative control (38).

Immunohistochemical studies are routinely used in medicine and they are applied in veterinary medicine with increasing frequency. There is broad knowledge of the mechanisms of action manifested by appropriate antibodies and of their potential use in various types of tumours as well on possible cross-reactions with the animal tissue, and with this knowledge antibodies can supplement diagnosis and facilitate the work of clinicians and veterinary pathologists. The use of precisely selected panels of antibodies provides the key for a correct diagnosis and for more accurate determination of the prognosis and therapeutic potential. Moreover, studies employing immunohistochemical techniques and detection of new markers promote more accurate and more rapid diagnosis of neoplastic lesions and deeper recognition of carcinogenesis phenomenon.

As does any multi-stage test, immuno-histochemical studies require appropriate preparation and equipment. Their result to a significant extent depends on appropriate fixation of tissues, appropriate performance at individual stages of examination, and an appropriately selected staining technique.

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