Immunohistochemical detection of metallothionein

Gabriella Emri[1*], Eszter Emri[1], Livia Beke[2], Gábor Boros[1], Csaba Hegedűs[1], Eszter Janka[1], Emese Gellén[1], Gábor Méhes[2] and Éva Remenyik[1]

[1] Department of Dermatology, Faculty of Medicine, University of Debrecen, Nagyerdei krt. 98, H-4032 Debrecen, Hungary − European Union; E-Mails: emeszti@gmail.com; borosgabor27@gmail.com; hegeduscsaba88@gmail.com; janka.eszter.a@gmail.com; emesegellen@med.unideb.hu; remenyik@med.unideb.hu

[2] Institute of Pathology, Faculty of Medicine, University of Debrecen, Nagyerdei krt. 98, H-4032 Debrecen, Hungary − European Union; E-Mails: beke.livia@gmail.com; mehes.gabor@med.unideb.hu;

* Author to whom correspondence should be addressed; E-Mail: gemri@med.unideb.hu;
Tel.: +36-52-255-602; Fax: +36-52-255-736.

All human tissues consist of a wide variety of cells that have an impact on each other. Human diseases that are caused by genetic alterations and/or environmental factors can be characterized by morphological and functional changes in tissues. Both aspects are important to better understand the pathomechanism of a disease and to find new therapeutic targets. Immunohistochemistry identifies the expression, intracellular localization as well as tissue distribution of various proteins, while morphology of tissue can also be assessed precisely. It is used for routine diagnostics as well as for research. However, antigen specific assay standardization and the use of appropriate positive and negative tissue controls are very important to interpret the findings in a way that ensures the biological relevance. Abnormal metallothionein function and expression have been implicated in various human diseases, including cancer. Immunohistochemistry provides an excellent opportunity to gain an insight into the role of MT in the pathogenesis of diseases.

Keywords: metallothionein antibodies; metallothionein tissue expression; immunohistochemistry

1. Introduction

Zinc (Zn (II)) is an essential microelement, it has critical role in normal health and development [1]. Binding of Zn (II) to zinc coordination motifs in proteins stabilizes the structure or influences the function. The prevalence of genes encoding zinc proteins is estimated to be over 3% of the 32,000 identified genes. Over 300 Zn (II)-dependent enzymes have been defined and characterized [2]. It has been shown that Zn (II) can regulate the DNA-binding activity of zinc finger transcription factors [3]. The Zn (II)-metallothionein (MT)/thionein pair, which is an important component of cellular Zn (II) homeostasis, is critical to sequester or release Zn(II) depending on the local redox state, thereby influencing the function of numerous enzymes and transcription factors that control cell proliferation, apoptosis and signalling pathways [4,5]. Abnormal MT function and expression have been implicated in various human diseases, including cancer [6]. There are at least 10 isoforms of MT in human body, which are expressed in a tissue specific pattern and may play distinct roles in the various cell types. MT-I and MT-II isoforms are present in all cells throughout the body, MT-III was first isolated as a growth inhibiting factor (GIF) from brain neurons, MT-IV is located in stratified epithelium [6]. Transcription of MT-I and MT-II can be induced by inflammatory cytokines (IL-6, TNF-α, interferons), lipopolysaccharids, glucocorticoids, free radicals, antioxidants or

heavy metals. MT is a cytosolic protein in resting cells, but it can be translocated transiently to the cell nucleus during cell proliferation and differentiation [7].

Immunohistochemistry (IHC) identifies the expression, intracellular localization as well as tissue distribution of various proteins, while morphology of tissue can also be assessed precisely [8]. IHC detection of MT in tissue samples is a very important option to study its role in the pathogenesis of diseases. Special IHC methods such as multiple immunolabeling using serial sections of tissue blocks or double staining technique provide an opportunity to study correlations between MT expression and important cell and tissue functions [9]. Tissue microarray allows simultaneous examination of large number of tissues on a single microscope slide; therefore it is very suitable to evaluate the diagnostic, prognostic or predictive role of the MT expression [9].

2.Immunohistochemical detection of metallothionein

The IHC technique is a combination of immunologic and chemical reactions visualized with a photonic microscope [8]. It can be divided in pre-analytical, analytical and post-analytical phases. It starts with tissue fixation, embedding, and tissue sectioning, followed by deparaffinization, antigen retrieval, blocking of non-specific activities, incubation with the primary antibody, and labeling of the antigen-antibody reaction, and ends with slide counterstaining, coverslipping and evaluation (Figure 1.). Most commonly, formalin-fixation and paraffin-embedding (FFPE) is used for its ability to preserve tissue indefinitely for morphologic examination [8,10]. Heat-induced epitope retrieval was proved to restore the immunoreactivity of tissues fixed in formalin [8]
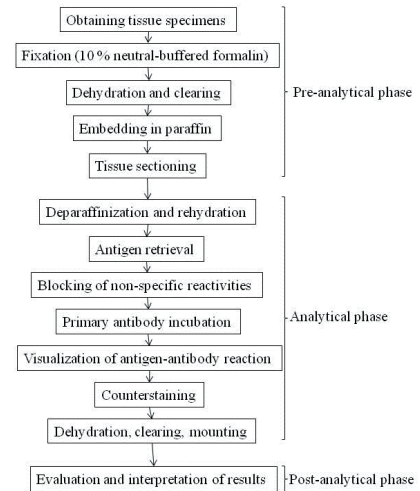
Figure 1: Outline of standard immunohistochemical protocol [8,11,12].

2.1 Pre-analytical phase of immunohistochemistry

The aim of fixation is to maintain morphological features and to preserve tissue suitable for a range of staining and IHC [11]. Adequate tissue fixation is very important to stabilize proteins and prevent tissue decay. Tissue fixation may be accomplished by physical (freeze) and/or chemical (coagulative and cross-linking) methods. For IHC purposes, the fixative of choice is a 10% neutral-buffered formalin solution. The specimen should be placed in fixative immediately after it is removed from the patient. The volume of formalin should be 10 to 20 times the volume of specimen. Delayed, too short or too long fixation can lead to false IHC results [8]. Formaldehyde penetrates the tissue very quickly, but fixes it very slowly; 16-24 h fixation time is required for a tissue specimen of 1-4 mm in diameter. Large specimens should be cut into slices 4 to 5 mm thick for further fixation [11].

Tissue processing refers to a series of steps that include removal of the extractable water and lipids from the tissue, usually in an automated manner, then infiltration and embedding in a support matrix (paraffin) so that the tissue can be stabilized and cut easily [11]. The specimens are cut on a rotary microtome into sections 3 to 5 μm thick. Sections that will be used for IHC

should be mounted on glass slides specially coated or charged to ensure better adherence [11]. Long-term storage of tissue sections is not recommended, because photo-oxidation of sections results in loss of antigenicity [8]. Detection of certain antigens has the limitation of requiring fresh tissue. In this case the specimen is obtained fresh and kept moist until it is oriented and embedded in optimal cutting temperature (OCT) compound, then the specimen is snap frozen [11]. The tissue is then sectioned at 6 µm. Occasionally, special tissue processing (e.g., decalcification, demelanization) is required prior to the immunostaining, but epitopes can be destroyed by an aggressive procedure [8]. The standard of protocol for tissue fixation and processing must be developed for each antigens and tissue type. It is worth to highlight that pre-analytical factors influence the success of a subsequent nucleic acid analysis as well [10].

2.2 Analytical phase of immunohistochemistry

Tissue sections mounted on coated or charged glass slides are deparaffinized and rehydrated before antigen retrieval (AR). Incomplete deparaffinization causes suboptimal or incomplete staining because of incomplete tissue penetration by the antibody [12]. Heat- and/or protease-induced AR is a procedure which reverses the structural changes induced by the fixation and tissue processing [8]. AR techniques are critical for antigen unmasking, optimal results require control of the pH and temperature of retrieval solutions and controlled enzymatic digestion [12]. The mechanism of heat to restore the immunoreactivity of formalin-fixed tissues is not completely clear, the dissociation of irrelevant proteins from target peptides, hydrolysis of methylene cross-links, restoring the native electrostatic charges, mobilization of trace paraffin etc. can be involved [8]. Enzymatic treatment is optimal only for few antigens and can alter tissue morphology or destroy epitopes [8].

Antigen epitopes consist of 5-6 amino acid residues, and are classified as linear or conformational [8]. Antigens may consist of multiple identical or distinct epitopes. Antigen-antibody binding involves hydrophobic, van der Waals,

and electrostatic interactions. The best diluents buffer for primary antibody is 0.05 to 0.1 M Tris buffer (pH 6.0) [8]. Background reactivity due to ionic interactions can be reduced by increasing the NaCl concentration in the buffer, but it can reduce the antigen-antibody binding [8]. Detergents (e.g., Tween 20) are also used to facilitate antigen-antibody binding by solubilizing membrane proteins; they are usually incorporated into dilution/rinse buffers [8]. Antibody concentrates and pre-diluted preparations must be optimized for usage at the correct dilution [12]. The affinity of an antibody for an antigen affects the sensitivity and specificity of an immunological reaction [8]. The overall binding intensity between antibodies and a multivalent antigen is described by avidity. Polyclonal antibodies usually have higher avidity, but lower specificity compared with monoclonal antibodies [8]. The high specificity, however, does not eliminate the possibility of cross-reactivity with other antigens, because the target epitopes can be part of multiple proteins and peptides.

The most frequent reason for the failure of IHC is the poor quality of the primary antibody used. However, nonspecific background staining, less than optimal specific staining, or no staining require a careful evaluation of all components involved in each step of the IHC technique [13].

2.2.1 Detection system

Primary (direct detection), secondary or tertiary (indirect detection) antibodies labelled with enzymes, metals or fluorescent compounds are used to visualize the antigen-antibody reaction [8,12]. The detection system must be compatible with the species tested. The most common label is the horseradish peroxidase, which produces a coloured precipitate at the site of the antigen-antibody reaction in the presence of its specific substrate and chromogens. Endogenous peroxidase activity has to be blocked before incubation with primary antibody. Chromogen entrapment, precipitation and contaminants may lead to false-positive interpretation of an IHC test [12]. Three-step techniques are more sensitive, e.g., the labelled streptavidin-biotin (LSAB) method, which is widely used. In this case, a biotinylated secondary antibody binds

to the primary antibody attached to the tissue antigen, and the biotinylated complex is detected by streptavidin that has been conjugated to the enzyme. Nevertheless, the endogenous biotin-associated background staining has led to increasing use of labelled polymer-based detection systems that are characterized by greater sensitivity, specificity, and suitable for manual and automated IHC platforms. Simultaneous or sequential multiple labelling using various detection systems can be used to localize different antigens in the same sections. Quantum dot labelling is a promising new tool for multispectral analysis [8].

Frozen sections can be more appropriate to examine the expression of certain proteins. In this case, immunofluorescence is used for detection of antigen-antibody reaction. Primary (or secondary) antibodies are linked to a fluorescent label such as fluorescein isothiocyanate to allow visualization using a fluorescence microscope [12]. Compared to frozen sections, paraffin-embedded tissues offer the advantage of better preservation of cellular details and permanency of the reaction [11].

2.3 Post-analytical phase of immunohistochemistry

Post-analytical phase of IHC includes assay standardization/validation, control performance, and interpretation of IHC results [8,14]. IHC assay standardization is important to obtain consistent and reproducible results within each laboratory and comparable results among laboratories [8]. Standardization is the process of optimizing the test method (reagents and protocols). Guidelines for antigen-, tissue- and species-specific standardization of IHC examinations should be based on currently available published evidence and modern understanding of quality assurance principles as applied to IHC in general [15]. Furthermore, diagnostic IHC laboratories must meet the ISO 15189 standards or standards of operation as defined by the Clinical Laboratory Improvement Amendments, respectively [16]. In non-accredited laboratories, IHC tests should be validated by documentation of internal and external quality assessments [17]. Use of quality controls

is required for technical calibration and analytical validation [14]. Positive tissue control is defined as tissue that is known to contain the antigen of interest. Positive and negative tissue controls must be fixed and stained in the same way as the tested specimen [8]. Positive tissue control in the tested specimen is designated as internal positive tissue control, e.g., MT immunoreactivity in basal layer of normal epidermis (Figure 2.). Negative reagent controls are used to confirm the specificity of the test and to assess the degree of nonspecific background staining present by omitting the primary antibody [14]. Validation detects any cross-reactivity of the selected antibodies with unrelated antigens, and cross-reactivity among different tissues and among different species, examines the variables that affect the IHC reaction, such as fixation time and storage of unstained tissue sections, and may include comparison of results among different laboratories using similar techniques [8]. Whenever possible, validation compares the sensitivity of IHC detection to the gold standard method of detection for the Ag in question.

According to the Clinical and Laboratory Standards Institute suggestions, the IHC report should include the cellular location and tissue distribution of the tested antigen, semi-quantitative evaluation of the immunoreactivity, along with an interpretation of the test results [8,13-15]. The report should contain demographic information, the tissue that was tested, disease characteristics, and the antibody used. IHC scoring schemes are based on a subjective assessment of the labelling intensity and percent positive cells by a pathologist. Recently, it has been demonstrated that software algorithms are able to properly indicate the disease-relevant regions in digitized tissue images and quantify the area and optical density of positive staining [18,19]. Advantages of an automated digital IHC image analysis are that it is unbiased, precise in ranges of staining that appear weak to the eye, and produces continuous data [18].

2.4 Antibodies for metallothionein immunodetection

In mammals, four tandemly clustered MT genes are known [20]. In humans, MT-I has

undergone duplication events that have resulted in 13 duplicate isoforms, five of which have been predicted to be no active forms [20]. All genes encode for conserved peptide chains that retain 20 invariant metal-binding cysteines. A total of 9 and 11 cysteines are required to form protein domains that bind three and four divalent metal ions [21]. MT-I and MT-II are 61-residue proteins, MT-III holds an extra residue in the β-domain and a six-residue long insertion in the α-domain, whereas MT-IV shares an additional residue in the β-domain [20]. In humans, the transcription of MT-I and MT-II isoforms is induced by various stimuli [21]. The expression of MT-II, MT-IE and MT-IX can be detected in most types of tissue, whereas the expression of other MT-I isoforms seems to be restricted to some tissues [20]. MT-III is constitutive tissue--specific isoform, it is expressed in human brain and in some other tissues [20]. MT-IV mRNA expression has been reported in stratified squamous epithelium in mouse, no data exist on the expression of MT-IV in humans [20].

In order to investigate protein expression, the most widely used primary antibody to detect MT-I/II proteins in FFPE tissues of a number of different species is a monoclonal mouse antibody (Clone E9, Dako) reacting with a single and highly conserved epitope formed by the last 5-7 residues of the N-terminus of the β-domain of MT-I and MT-II [22-25]. The immunogen was horse self-polymerized MT-I and MT-II. In previous studies a specific polyclonal antibody that was generated against rat liver MT in rabbits was used efficiently to detect MT expression also in humans [7,26,27]. Furthermore, due to the difference in the amino acid sequence, specific monoclonal and polyclonal antibodies against MT-III could be generated and used successfully in IHC tests [22,28-30]. Further progress in the development of primary antibodies against MT isoforms would be important for future investigations. Nevertheless, IHC studies to date have greatly contributed to the current knowledge on the functions of MT in the human body.

2.5 Tissue specific expression of metallothionein proteins

MT-I and MT-II can be expressed in all cells throughout the body, however, IHC examinations were able to reveal that not all cells express MT to the same extent in healthy tissues [27]. In addition, several factors, e.g., proliferation status, age, can influence the expression of MT. For example, in normal human skin, strong MT-I/II immunostaining can be detected in basal keratinocytes of epidermis and hair follicle outer root sheath, hair matrix cells and the secretory coil, but not in the exocrine portion of eccrine glands [31]. In hyperplastic epidermis the increased MT expression represents an increase in the germinative pool size [32]. Accordingly, the protein levels of MT-I and MT-II have been shown to decrease significantly with increasing age simultaneously with the decrease of keratinocyte proliferation [33]. Nartey et al. investigated the distribution of MT during human development [34]. They found that MT levels are higher in the fetal liver than in the adult liver; in addition, MT is localized in the nucleus and the cytoplasm of human fetal and neonatal hepatocytes, whereas MT is mainly expressed in the cytoplasm of adult liver cells. In the fetal and neonatal human kidney, MT is localized primarily in the nucleus and the cytoplasm of the proximal tubular epithelial cells, whereas in the adult kidney, intraluminal MT localization was also observed [34].

MT-III expression has been investigated by IHC in rats [28]. Some astrocytes in the deep layers of cortex, ependymal cells, some glomerular and tubular cells in the kidney, some glandular epithelial cells in the dorsolateral lobe of prostate, some Sertoli cells and Leydig cells in the testis, and taste bud cells in the tongue showed MT-III immunostaining [28].

2.5.1 Metallothionein expression in response to exogenous agents

It is thought that MTs exert cytoprotective effects against heavy metal toxicity and oxidative stress [27]. IHC is suitable method to investigate the expression of MT in various tissues in response to an exogenous agent. An example is the elevated nuclear and cytoplasmic

MT expression of periportal hepatocytes, proximal tubular epithelial cells, intestinal columnar epithelial cells and Paneth cells in rats during dietary copper or zinc overloads, and upon exposure to cadmium [25,35,36]. Furthermore, the hepatic MT levels were increased in both the nucleus and cytoplasm of hepatocytes in mice upon thioacetamide exposure [37], and in rats upon exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin [38]. Increased MT-I and MT-II expression in the renal proximal tubular cells of rats upon administration of doxorubicin might indicate a protective mechanism against the pro-apoptotic effect of this cytostatic agent [39]. The MT levels have been found to be elevated in the epidermis after acute ultraviolet radiation exposure [40].

2.5.2 Metallothionein expression in inflammatory conditions

MT knockout mice appear relatively healthy, but they show an impaired response to stress [5]. MT expression is induced by a variety of pro- and anti-inflammatory mediators including glucocorticoids, reactive oxygen species, antioxidants, endotoxin, acute phase cytokines, furthermore, MT have a wide range of functions in cellular homeostasis and immunity [41]. Altered expression of MT has been shown in many inflammatory conditions. For example, an IHC study on renal biopsies from patients with lupus nephritis showed the depletion of MT-I and MT-II protein expression in the proximal tubular epithelial cells compared with healthy kidney specimens [42]. Moreover, a tubular MT score below the median value of the cohort predicted a poor renal outcome. Tissue infiltrating MT-positive cells could be shown in colonic biopsies from patients with inflammatory bowel disease and acute infectious colitis [43]. In another study, the mucosal MT concentration in colonic tissue samples from patients with inflammatory bowel disease has been found to be lower compared with normal mucosa tissue samples suggesting an antioxidant imbalance in the intestinal mucosa of these patients [44,45]. In a dextran sulfate sodium-induced intestinal inflammation mouse model MT-positive cells were detected in the lamina propria and sub-

mucosal layer and were mainly co-localized in macrophages [46]. Nevertheless, the increase of colonic myeloperoxidase activity levels and pro-inflammatory cytokine production induced by dextran sulfate sodium was significantly higher in the MT-I/II knockout animals compared with the wild-type mice suggesting that MT plays a protective role against colitis [46].

MT expression was analyzed in the central nervous system of mice with experimental autoimmune encephalomyelitis [47]. MT-I/II seemed to be produced mainly by reactive astrocytes and activated macrophages. It was assumed that the elevation of MT expression level in tissue may act on the inflammatory microenvironment suppressing pro-inflammatory cytokine production of macrophages, decreasing apoptotic cell death in neurons and oligodendrocytes, and enhancing tissue repair [47].

2.5.3 Metallothionein expression in cancer

MT expression in tissues has been studied most intensively in human cancers [48]. The results suggest that change in MT levels might play a role in the conversion of a potentially malignant lesion to a malignant carcinoma. For example, significantly higher MT-I/II and MT-III expression was noted in actinic keratosis and cutaneous squamous cell cancer, as compared with normal skin epidermis, whereas very low levels of MT-III expression were found in basal cell cancer [22,29]. A significantly higher MT-I/II expression was also observed in oral squamous cell carcinoma tissues comparing with normal and oral leukoplakia epithelial tissues [49]. High MT expression was detected in pancreas adenocarcinoma tissues compared with pancreatic serous cystadenoma or healthy pancreatic tissue samples [50]. Analysis of MT-I/II expression in prostate epithelial cells showed higher MT expression in tissues derived from benign prostatic hyperplasia than in those derived from prostate cancer [51]. Furthermore, IHC analysis of specimens from normal colorectal mucosa, adenomas, carcinomas and lymph node metastases revealed the down-regulation of MT-I/II expression in association with colorectal cancer progression [52]. Janssen et al. confirmed that the MT concentrations

of colorectal adenomas, carcinomas and liver metastases are lower than that of corresponding normal mucosa, however, they have found that a relatively high MT content might be associated with the aggressiveness of colorectal cancers [53]. Meta-analysis of IHC studies led to the conclusion that significantly increased MT-I/II expression can be detected in head and neck, and ovarian cancers, respectively, compared with healthy tissues, but significantly decreased MT expression can be observed in liver tumours compared with normal liver tissue [54]. It seems that the expression of MT is not universal to all human tumours, but may depend on the differentiation status and proliferative index of tumours, along with other tissue factors and gene mutations [48]. The various tumours may also differ in the intracellular localization of MT. For example, we observed nuclear MT-I/II staining in benign melanocytic nevi and intensive cytoplasmic and nuclear staining in malignant melanomas (Figure 2.) [9]. Cytoplasmic and nuclear MT-I/II expression was significantly higher in endometrial cancer cells compared with cells in benign hyperplasia of endometrium, however, the nuclear MT expression correlated better with histologic grade [55].

Changes in MT expression (up- or down-regulation) may be associated with a more aggressive phenotype and therapeutic resistance, ultimately resulting in a worse prognosis [6,21]. Weinlich et al. have found that high MT levels in tumour cells are associated with reduced survival in patients with malignant melanoma [56]. We confirmed that high expression of MT-I/II in melanoma cells is significantly more frequent in primary cutaneous malignant melanoma with haematogenous metastases [9]. In bladder cancer patients a high MT expression in tumour tissues was linked to shorter tumour-specific survival and increased recurrence rates [57]. IHC analysis of specimens from renal cell carcinoma revealed that MT immunostaining is associated with significantly worse prognosis [58]. The absence of MT expression in ovarian cancer samples correlated with improved progression-free survival in patients treated with adjuvant platinum-based chemotherapy [59]. Nevertheless, the role of MT in metastasis

formation remains to be confirmed, and experimental evidence for its oncogenic role is still lacking.
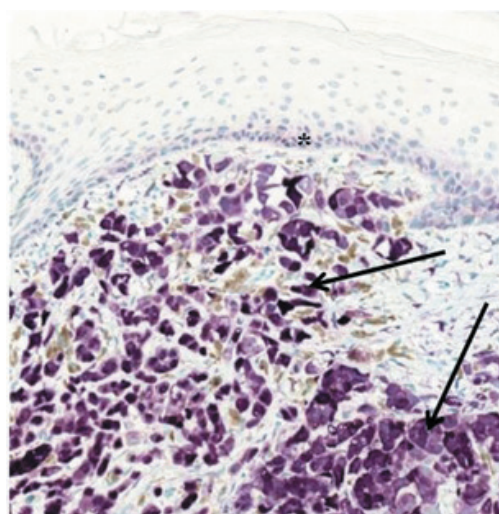
Figure 2: Expression of MT-I/II in primary cutaneous malignant melanoma cells (arrows). The keratinocytes (asterisks) of epidermal basal layer served as internal positive tissue controls. The IHC detection was based on an immunoperoxidase reaction using VIP chromogenic substrate. The slides were counterstained with methyl-green. Original magnification is x20.

3. Conclusions

Zn (II) is essential in fine-tuned orchestration of basic cell functions such as proliferation, gene expression and stress response. Besides metal detoxification, MT can release/bind Zn (II) with high affinity and regulates the availability of Zn (II) in various cell compartments thereby influencing the function of many transcription factors and enzymes [5]. It might explain why we can detect changes in the expression of MT in cancer, and calls for further investigations in this field. Moreover, the availability of zinc from MT is controlled by the local redox status [4]. Alterations in reactive oxygen species homeostasis are thought to be involved in the pathogenesis of various diseases, including cancers [6]. IHC is suitable to demonstrate MT expression in its morphological and functional

context; therefore, it is an important element of the investigations. Assay standardization and the use of appropriate positive and negative tissue controls are very important to interpret the findings correctly.

Acknowledgments

Conflicts of Interest

The authors declare no conflict of interest.

The authors declare they have no potential conflicts of interests concerning drugs, products, services or another research outputs in this study. The Editorial Board declares that the manuscript met the ICMJE „uniform reguirements" for biomedical papers.

References

1. Schwartz, J.R.; Marsh, R.G.; Draelos, Z.D. Zinc and skin health: Overview of physiology and pharmacology. Dermatologic surgery : official publication for American Society for Dermatologic Surgery [et al.] 2005, 31, 837-847; discussion 847.
2. Berg, J.M.; Shi, Y. The galvanization of biology: A growing appreciation for the roles of zinc. Science 1996, 271, 1081-1085.
3. Roesijadi, G.; Bogumil, R.; Vasak, M.; Kagi, J.H. Modulation of DNA binding of a tramtrack zinc finger peptide by the metallothionein-thionein conjugate pair. The Journal of biological chemistry 1998, 273, 17425-17432.
4. Maret, W. The function of zinc metallothionein: A link between cellular zinc and redox state. The Journal of nutrition 2000, 130, 1455S-1458S.
5. Davis, S.R.; Cousins, R.J. Metallothionein expression in animals: A physiological perspective on function. The Journal of nutrition 2000, 130, 1085-1088.
6. Thirumoorthy, N.; Shyam Sunder, A.; Manisenthil Kumar, K.; Senthil Kumar, M.; Ganesh, G.; Chatterjee, M. A review of metallothionein isoforms and their role in pathophysiology. World journal of surgical oncology 2011, 9, 54.
7. Cherian, M.G. The significance of the nuclear and cytoplasmic localization of metallothionein in human liver and tumor cells. Environmental health perspectives 1994, 102 Suppl 3, 131-135.
8. Ramos-Vara, J.A.; Miller, M.A. When tissue antigens and antibodies get along: Revisiting the technical aspects of immunohistochemistry--the red, brown, and blue technique. Veterinary pathology 2014, 51, 42-87.
9. Emri, E.; Egervari, K.; Varvolgyi, T.; Rozsa, D.; Miko, E.; Dezso, B.; Veres, I.; Mehes, G.; Emri, G.; Remenyik, E. Correlation among metallothionein expression, intratumoural macrophage infiltration and the risk of metastasis in human cutaneous malignant melanoma. Journal of the European Academy of Dermatology and Venereology : JEADV 2013, 27, e320-327.
10. Bass, B.P.; Engel, K.B.; Greytak, S.R.; Moore, H.M. A review of preanalytical factors affecting molecular, protein, and morphological analysis of formalin-fixed, paraffin-embedded (ffpe) tissue: How well do you know your ffpe specimen? Archives of pathology & laboratory medicine 2014, 138, 1520-1530.
11. Elenitsas, R.; Nousari, C.H.; Seykora, J.T. Laboratory methods. In Lever's histopathology of the skin, 9th ed.; Elder, D.E.; Elenitsas, R.; Johnson, J., B. L.; Murphy, G.F., Eds. Lippincott Williams & Wilkins: Philadelphia, USA, 2005; pp 59-67.
12. Ramdial, P.K.; Bastian, B.C.; Goodlad, J.; McGrath, J.A.; Lazar, A. Specialized techniques in dermatopathology. In Mckee's pathology of the skin, 4th ed.; Calonje, E.; Brenn, T.; Lazar, A.; McKee, P.H., Eds. Elsevier Saunders: 2012; Vol. 1, pp 32-37.
13. Ward, J.M.; Rehg, J.E. Rodent immunohistochemistry: Pitfalls and troubleshooting. Veterinary pathology 2014, 51, 88-101.
14. Ramos-Vara, J.A.; Kiupel, M.; Baszler, T.; Bliven, L.; Brodersen, B.; Chelack, B.; Czub, S.; Del Piero, F.; Dial, S.; Ehrhart, E.J., et al. Suggested guidelines for immunohistochemical techniques in veterinary diagnostic laboratories. Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc 2008, 20, 393-413.
15. Taylor, C.R. New revised clinical and laboratory standards institute guidelines for immunohistochemistry and immunocytochemistry. Applied immunohistochemistry & molecular morphology : AIMM / official publication of the Society for Applied Immunohistochemistry 2011, 19, 289-290.
16. Elliott, K.; McQuaid, S.; Salto-Tellez, M.; Maxwell, P. Immunohistochemistry should undergo robust validation equivalent to that of molecular diagnostics. Journal of clinical pathology 2015.
17. Nielsen, S. External quality assessment for immunohistochemistry: Experiences from nordiqc. Biotechnic & histochemistry : official publication of the Biological Stain Commission 2015, 90, 331-340.
18. Rizzardi, A.E.; Johnson, A.T.; Vogel, R.I.; Pambuccian, S.E.; Henriksen, J.; Skubitz, A.P.; Metzger, G.J.; Schmechel, S.C. Quantitative comparison of immunohistochemical staining measured by digital image analysis versus pathologist visual scoring. Diagnostic pathology 2012, 7, 42.
19. Varghese, F.; Bukhari, A.B.; Malhotra, R.; De, A. Ihc profiler: An open source plugin for the quantitative evaluation and automated scoring

of immunohistochemistry images of human tissue samples. PloS one 2014, 9, e96801.

20. Moleirinho, A.; Carneiro, J.; Matthiesen, R.; Silva, R.M.; Amorim, A.; Azevedo, L. Gains, losses and changes of function after gene duplication: Study of the metallothionein family. PloS one 2011, 6, e18487.

21. Pedersen, M.O.; Larsen, A.; Stoltenberg, M.; Penkowa, M. The role of metallothionein in oncogenesis and cancer prognosis. Progress in histochemistry and cytochemistry 2009, 44, 29-64.

22. Zamirska, A.; Matusiak, L.; Dziegiel, P.; Szybejko-Machaj, G.; Szepietowski, J.C. Expression of metallothioneins in cutaneous squamous cell carcinoma and actinic keratosis. Pathology oncology research : POR 2012, 18, 849-855.

23. Hifumi, T.; Miyoshi, N.; Kawaguchi, H.; Nomura, K.; Yasuda, N. Immunohistochemical detection of proteins associated with multidrug resistance to anti-cancer drugs in canine and feline primary pulmonary carcinoma. The Journal of veterinary medical science / the Japanese Society of Veterinary Science 2010, 72, 665-668.

24. Dincer, Z.; Jasani, B.; Haywood, S.; Mullins, J.E.; Fuentealba, I.C. Metallothionein expression in canine and feline mammary and melanotic tumours. Journal of comparative pathology 2001, 125, 130-136.

25. Mullins, J.E.; Fuentealba, I.C. Immunohistochemical detection of metallothionein in liver, duodenum and kidney after dietary copper-overload in rats. Histology and histopathology 1998, 13, 627-633.

26. Nartey, N.; Cherian, M.G.; Banerjee, D. Immunohistochemical localization of metallothionein in human thyroid tumors. The American journal of pathology 1987, 129, 177-182.

27. Thirumoorthy, N.; Manisenthil Kumar, K.T.; Shyam Sundar, A.; Panayappan, L.; Chatterjee, M. Metallothionein: An overview. World journal of gastroenterology : WJG 2007, 13, 993-996.

28. Hozumi, I.; Suzuki, J.S.; Kanazawa, H.; Hara, A.; Saio, M.; Inuzuka, T.; Miyairi, S.; Naganuma, A.; Tohyama, C. Metallothionein-3 is expressed in the brain and various peripheral organs of the rat. Neuroscience letters 2008, 438, 54-58.

29. Pula, B.; Tazbierski, T.; Zamirska, A.; Werynska, B.; Bieniek, A.; Szepietowski, J.; Rys, J.; Dziegiel, P.; Podhorska-Okolow, M. Metallothionein 3 expression in normal skin and malignant skin lesions. Pathology oncology research : POR 2015, 21, 187-193.

30. Sens, M.A.; Somji, S.; Lamm, D.L.; Garrett, S.H.; Slovinsky, F.; Todd, J.H.; Sens, D.A. Metallothionein isoform 3 as a potential biomarker for human bladder cancer. Environmental health perspectives 2000, 108, 413-418.

31. van den Oord, J.J.; De Ley, M. Distribution of metallothionein in normal and pathological human skin. Archives of dermatological research 1994, 286, 62-68.

32. Karasawa, M.; Nishimura, N.; Nishimura, H.; Tohyama, C.; Hashiba, H.; Kuroki, T. Localization of metallothionein in hair follicles of normal skin and the basal cell layer of hyperplastic epidermis: Possible association with cell proliferation. The Journal of investigative dermatology 1991, 97,

97-100.

33. Ma, C.; Li, L.F.; Chen, X. Expression of metallothionein-i and ii in skin ageing and its association with skin proliferation. The British journal of dermatology 2011, 164, 479-482.

34. Nartey, N.O.; Banerjee, D.; Cherian, M.G. Immunohistochemical localization of metallothionein in cell nucleus and cytoplasm of fetal human liver and kidney and its changes during development. Pathology 1987, 19, 233-238.

35. Santon, A.; Giannetto, S.; Sturniolo, G.C.; Medici, V.; D'Inca, R.; Irato, P.; Albergoni, V. Interactions between zn and cu in lec rats, an animal model of wilson's disease. Histochemistry and cell biology 2002, 117, 275-281.

36. Tanimoto, A.; Hamada, T.; Higashi, K.; Sasaguri, Y. Distribution of cadmium and metallothionein in cdcl2-exposed rat kidney: Relationship with apoptosis and regeneration. Pathology international 1999, 49, 125-132.

37. Oliver, J.R.; Jiang, S.; Cherian, M.G. Augmented hepatic injury followed by impaired regeneration in metallothionein-i/ii knockout mice after treatment with thioacetamide. Toxicology and applied pharmacology 2006, 210, 190-199.

38. Nishimura, N.; Miyabara, Y.; Suzuki, J.S.; Sato, M.; Aoki, Y.; Satoh, M.; Yonemoto, J.; Tohyama, C. Induction of metallothionein in the livers of female sprague-dawley rats treated with 2,3,7,8-tetrachlorodibenzo-p-dioxin. Life sciences 2001, 69, 1291-1303.

39. Chmielewska, M.; Symonowicz, K.; Pula, B.; Owczarek, T.; Podhorska-Okolow, M.; Ugorski, M.; Dziegiel, P. Expression of metallothioneins i and ii in kidney of doxorubicin-treated rats. Experimental and toxicologic pathology : official journal of the Gesellschaft fur Toxikologische Pathologie 2015, 67, 297-303.

40. Ablett, E.; Whiteman, D.C.; Boyle, G.M.; Green, A.C.; Parsons, P.G. Induction of metallothionein in human skin by routine exposure to sunlight: Evidence for a systemic response and enhanced induction at certain body sites. The Journal of investigative dermatology 2003, 120, 318-324.

41. Lynes, M.A.; Hidalgo, J.; Manso, Y.; Devisscher, L.; Laukens, D.; Lawrence, D.A. Metallothionein and stress combine to affect multiple organ systems. Cell stress & chaperones 2014, 19, 605-611.

42. Faurschou, M.; Penkowa, M.; Andersen, C.B.; Starklint, H.; Jacobsen, S. The renal metallothionein expression profile is altered in human lupus nephritis. Arthritis research & therapy 2008, 10, R76.

43. Devisscher, L.; Hindryckx, P.; Lynes, M.A.; Waeytens, A.; Cuvelier, C.; De Vos, F.; Vanhove, C.; Vos, M.D.; Laukens, D. Role of metallothioneins as danger signals in the pathogenesis of colitis. The Journal of pathology 2014, 233, 89-100.

44. Kruidenier, L.; Kuiper, I.; Van Duijn, W.; Mieremet-Ooms, M.A.; van Hogezand, R.A.; Lamers, C.B.; Verspaget, H.W. Imbalanced secondary mucosal antioxidant response in inflammatory bowel disease. The Journal of pathology 2003, 201, 17-27.

45. Ioachim, E.; Michael, M.; Katsanos, C.; Demou, A.; Tsianos, E.V. The immunohistochemical expression of metallothionein in inflammatory

bowel disease. Correlation with hla-dr antigen expression, lymphocyte subpopulations and proliferation-associated indices. Histology and histopathology 2003, 18, 75-82.

46. Tsuji, T.; Naito, Y.; Takagi, T.; Kugai, M.; Yoriki, H.; Horie, R.; Fukui, A.; Mizushima, K.; Hirai, Y.; Katada, K., et al. Role of metallothionein in murine experimental colitis. International journal of molecular medicine 2013, 31, 1037-1046.

47. Espejo, C.; Carrasco, J.; Hidalgo, J.; Penkowa, M.; Garcia, A.; Saez-Torres, I.; Martinez-Caceres, E.M. Differential expression of metallothioneins in the cns of mice with experimental autoimmune encephalomyelitis. Neuroscience 2001, 105, 1055-1065.

48. Cherian, M.G.; Jayasurya, A.; Bay, B.H. Metallothioneins in human tumors and potential roles in carcinogenesis. Mutation research 2003, 533, 201-209.

49. Pontes, H.A.; de Aquino Xavier, F.C.; da Silva, T.S.; Fonseca, F.P.; Paiva, H.B.; Pontes, F.S.; dos Santos Pinto, D., Jr. Metallothionein and p-akt proteins in oral dysplasia and in oral squamous cell carcinoma: An immunohistochemical study. Journal of oral pathology & medicine : official publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology 2009, 38, 644-650.

50. Sliwinska-Mosson, M.; Milnerowicz, H.; Rabczynski, J.; Milnerowicz, S. Immunohistochemical localization of metallothionein and p53 protein in pancreatic serous cystadenomas. Archivum immunologiae et therapiae experimentalis 2009, 57, 295-301.

51. Lee, J.D.; Wu, S.M.; Lu, L.Y.; Yang, Y.T.; Jeng, S.Y. Cadmium concentration and metallothionein expression in prostate cancer and benign prostatic hyperplasia of humans. Journal of the Formosan Medical Association = Taiwan yi zhi 2009, 108, 554-559.

52. Arriaga, J.M.; Levy, E.M.; Bravo, A.I.; Bayo, S.M.; Amat, M.; Aris, M.; Hannois, A.; Bruno, L.; Roberti, M.P.; Loria, F.S., et al. Metallothionein expression in colorectal cancer: Relevance of different isoforms for tumor progression and patient survival. Human pathology 2012, 43, 197-208.

53. Janssen, A.M.; van Duijn, W.; Oostendorp-Van De Ruit, M.M.; Kruidenier, L.; Bosman, C.B.; Griffioen, G.; Lamers, C.B.; van Krieken, J.H.; van De Velde, C.J.; Verspaget, H.W. Metallothionein in human gastrointestinal cancer. The Journal of pathology 2000, 192, 293-300.

54. Gumulec, J.; Raudenska, M.; Adam, V.; Kizek, R.; Masarik, M. Metallothionein - immunohistochemical cancer biomarker: A meta-analysis. PloS one 2014, 9, e85346.

55. Jagielski, L.; Jelen, M.; Kobierzycki, C.; Jagielska, G.; Blok, R. Increase of nuclear expression of metallothionein i/ii in neoplastic transformation of the endomnetrium. Ginekologia polska 2015, 86, 182-187.

56. Weinlich, G.; Eisendle, K.; Hassler, E.; Baltaci, M.; Fritsch, P.O.; Zelger, B. Metallothionein - overexpression as a highly significant prognostic factor in melanoma: A prospective study on 1270 patients. British journal of cancer 2006, 94, 835-841.

57. Hinkel, A.; Schmidtchen, S.; Palisaar, R.J.; Noldus, J.; Pannek, J. Identification of bladder cancer patients at risk for recurrence or progression: An immunohistochemical study based on the expression of metallothionein. Journal of toxicology and environmental health. Part A 2008, 71, 954-959.

58. Tuzel, E.; Kirkali, Z.; Yorukoglu, K.; Mungan, M.U.; Sade, M. Metallothionein expression in renal cell carcinoma: Subcellular localization and prognostic significance. The Journal of urology 2001, 165, 1710-1713.

59. Woolston, C.M.; Deen, S.; Al-Attar, A.; Shehata, M.; Chan, S.Y.; Martin, S.G. Redox protein expression predicts progression-free and overall survival in ovarian cancer patients treated with platinum-based chemotherapy. Free radical biology & medicine 2010, 49, 1263-1272.