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MALIGNANT MARKERS OF MELANIC TUMORS IN ANIMALS

Summary of PHD Thesis

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INTRODUCTION

Melanoma is a devastating disease frequently encountered within both veterinary and human medicine. Molecular changes linked with neoplastic transformation of melanocytes include mutations in genes that encode proteins intrinsic to the regulatory pathways of two tumor suppressor proteins (retinoblastoma protein and p53), proto-oncogene mutation to oncogenes, altered expression of epithelial cadherin and CD44 adhesion molecules, and upregulation of angiogenic factors and other growth factors. Histologic evaluation of the primary mass is the most common means of diagnosis, with cytology used more frequently to document metastasis. Melanoma’s highly variable histologic and cytologic patterns can make diagnosis by either method problematic. Adherent epithelioid morphology, including signet ring forms, and nonadherent round and spindle forms are recognized, with pigmentation an inconsistent finding. The site of the tumor, the thickness of the primary tumor or depth of invasion, and the number of mitotic figures per high-power field or per millimeter are used histologically to predict biologic behavior, whereas site and degree of pleomorphism are typically used for cytologic preparations. Diagnosis of amelanotic melanoma can be aided by ancillary diagnostic techniques. Immunohistochemical and Immunofluorescence methods were more important for identify and prognosis of melanic tumors particularly amelanotic tumors by using two groups of markers 1. Identify markers 2- malignant markers.

Aim of thesis
In this thesis, I have been studied the evaluation of melanic tumors in dog and horse by using Epidemic, Macroscopic, Histologic, Cytologic, Immunohistochemical and Immunofluorescence methods in order to Identify and distinguish between these tumors and other tumors and for distinguish between malignant and benign melanic tumors.

EPIDEMIOLOGICAL STUDY OF MELANOCYTIC NEOPLASMS IN DOGS AND HORSES

Introduction
Melanocytic neoplasms have a high incidence in canines, horses and they originate in melanocytes (Hsu et al., 1981). Epidemiological studies of melanocytic neoplasms concentrate on the USA on the decade of the 1990s. According to data obtained in different US States, dog was most often affected, mainly in the oral cavity (Bergman et al., 2007-Oyamada et al., 2007), and gray horse mainly in: the inferior side of the tail, and the perineum (Baba et al., 1981- Durand et al., 1927).

Aim of study
This study aimed to complement epidemiological information regarding melanocytic neoplasms in dogs and horses. Whereas the main epidemiological data extracted from registers regarded the affected population (age, gender and breed) and tumor characteristics (anatomic localization and macroscopical aspects).
Material and Methods
The database of the faculty Veterinary Medicine of Cluj-Napoca, discipline of pathologic anatomy, diagnostic Laboratory was searched for cases of canine melanic tumors, whereas 2857 canine cases were diagnosed from April 2001, until October, 2010, 630 cases (510 malign, 120 benign) were diagnosed as tumoural cases 46 (43 malign, 3 benign) of them were canine melanic tumors, and 2 horse melanic tumors were diagnosed in the period between 1992 to 1998. Also other database of 9 cases was taken of horses affected on melanoma from Alexandria slaughtered horses in the period between 5-7 may 2010.

Results
The percentage of melanic tumors of all tumors types was 7.30% but the percentage of melanic tumors of all skin tumors was 45.54%. The most frequently tumors of all tumors were mammary tumors 27%, followed in decreasing order by the skin tumors without melanic tumors 8.7%, melanoma 7.3%, hemangioma tumors 6.34%, osteosarcoma 2.7%, lymphosarcoma 2.5% and other tumors 43.96%. The melanoma was 6.66% of all tumors and 8.23% of all malignant tumors. The melanocytoma was 0.63% of all tumors and 3.33% of all benign tumors. In malignant tumors in both percentage tumors of all tumors and type of tumors, the most frequently tumors were mammary tumors followed in decreasing order by melanoma, hemangiosarcoma, the skin tumors without melanoma, osteosarcoma, and lymphosarcoma. In benign tumors in both percentage tumors of all tumors and type of tumors, the most frequently tumors were, the skin tumors without melanoma, melanoma, and lymphoma. The main skin tumors were: melanoma, squamous cell carcinomas, papilloma, basal cell carcinoma. The most frequently affected sites are the skin (80.5%), oral cavity (6.5%), digit (6.5%), lip (4.4%), and eye (2.1%). The more frequently on the head (15 cases) the next on the ventral abdomen (8 cases) then the scrotum (3 cases). 11 horse melanic tumors cases were localized as 4 cases were in the tail, 5 cases were around anus region and genital region, 1 case was in the tail and genital region and one metastatic melanoma. The breed of dogs was Metis (5 cases), German shorthaired pointer (2 cases), Cocker spaniel (3 cases), vizsla Hungarian (1 case), Boxer (4 cases), Airedale terrier (2 cases), Tekel (3 cases), Giant Schnauzer (2 cases), Schnauzer (4 cases), Tosa ken (3 cases), Doberman (2 cases), Dog de Bordeaux (1 case), English Bulldog (1 case), Irish setter (1 case), Rottweiler (2 cases), and German shepherd (2 cases). In this study, melanoma in German shepherd and Schnauzer were at increased risk of developing melanoma of the lip. Melanoma was in cocker spaniel, schnauzer and Airedale terrier that were at increased risk of developing subungual melanoma. Melanoma was in Irish setter and Doberman that were at increased risk of developing oral melanoma. All horse melanic tumors were in gray horse. Dog melanocytoma was between 6-9 years. Dog melanoma was between 2-13 years, whereas 28 cases were over 7 years and 11 cases were under 7 years. The range of age of melanoma cases was 8 to 15 years, and the range of age of melanoma cases was 8 to 13 years and melanocytoma cases were 10 to 15 years. The genders of dog affected with melanic tumors were 19 female and 25 masculine and 3 cases unknown their gender.
The genders of horse affected with melanic tumors were 6 female and 5 male. All melanocytoma horse cases were in male gender.

**Conclusions:**

1. The percentage of melanic tumors of all tumors was 7.30%, but the percentage of melanic tumors of all skin tumors was 45.54%.
2. The melanic dog tumors were found most frequently on the skin, and much less in other body regions.
3. All mucocutaneous junctions regions include (lip, eyelid, nose, subungual) were malignant dog melanic tumors.
4. The most frequently dog melanic tumors were in the breeds: boxer, Schnauzer, Tosa ken Teckel, Metis and Cocker spaniel.
5. All melanic tumors diagnosed in horses originate from gray individuals.

**MACROSCOPICAL AND HISTOLOGICAL STUDY IN CANINE MELANIC TUMORS**

**Introduction**

In the dog, it is a common diagnosis, accounting for 7% of all malignant tumors (Cotchin et al., 1955). It is the most common malignant neoplasm of the oral cavity and the second most frequent subungual neoplasm (Marino et al., 1995). As in domestic animals, it is generally very advanced when detected and has only a 5% 5-year survival rate (Rogers et al., 1997).

**Aim of study**

The aim of this study was: the extended histological and macroscopical study of canine melanic tumors through study of tumoural localization, tumoural aspect, cell type, mitosis..etc, and use them for comparative between benign and malign tumors.

**Materials and methods**

The database of the faculty Veterinary Medicine of Cluj-Napoca, discipline of pathologic anatomy, diagnostic Laboratory was searched for cases of canine melanic tumors, whereas 33 canine melanic tumors and 1 metastatic melanoma (melanoma with unknown primary site located in lymph nodes or internal organs) were diagnosed from July, 1998 to October, 2010. Macroscopic examine: study of these cases was epidemiologically about (age, sex, breed) and macroscopically about diameter of tumor, tumor surface color, section color, period of growth this tumor and tumor palpation (thick, thin, smooth, coarse) using for this study register book. Microscopic examine: Melanoma biopsies were treated by formalin fixed (formalin 10% tamponed, PH7) during of 24 hours, and then paraffin embedded by standard procedures. These paraffinized biopsies cut to sections 4-5 microns by microtum Leica RM2125 RT, after that these section stained with Hematoxylin & Eosin Stain protocol, then these biopsies examined by microscope Olympus BX 51. Imagines were taken by a digital Olympus SP 350 and analyzed with a program adecvat-Olympus DP soft.

**Results**

The color of melanic tumors was white, pink, ashen-black, blackish-red to black. The diameter was between 0.5 to 6 cm, but the diameter in majority cases was between 0.5 to 4 cm. The tumoural aspect was dens in majority cases and friable in some cases
with pedicel in one case, and the majority cases were solitary without one case had three nodules. One metastatic melanoma with a multiple dimensions (1-13) Cm (Fig 1.A). 30 dog melanomas were formed of epithelioid type cells in 11 cases, spindle type cells in 7 cases, mixed epithelioid and spindle type cells in 8 cases, dendritic cells in 2 cases, mixed dendritic and spindle type cells in 1 case and balloon cell type in one case. Melanocytoma cases were formed of 4 cases, 3 cases were spindle type cells and one case was dendritic type cell (Fig 1.C). In 30 melanoma cases, 5 cases had high grade of mitotic index figures, 4 cases had over medium grade of mitotic index figures, 14 cases had medium grade of mitotic index figures and 7 low grade of mitotic index figures, while 4 melanocytoma cases were with low grade of mitotic index figures. Epithelioid type cells had numbers of following mitotic index figures (3, 11, 14, 17, 40). Spindle type cells had a number of following mitotic index figures (4, 5, 8, 34). Mixed spindle and epithelioid type cells had a number of following mitotic index figures (1, 6, 18, 41, 53). Dendritic type cells had a number of following mitotic index figures (4, 18). Mixed dendritic and spindle type cells had a number of following mitotic index figure (15). Balloon type cell had a number of following mitotic index figures (3). The necrotic zones in melanoma cases were extended in 9 cases, moderate in 2 cases, reduced in 12 cases and absent in 7 cases. The infiltrated lymphocytes in melanoma cases were intense in 9 case, moderate in 14 cases and absent in 7 case. The necrotic zones increase with increase grade of mitotic index figures whereas the aggressively of tumors increase with extend necrotic zones and inverse with small and absent necrosis tumors. 28 melanoma cases had 4 grade of Clark's level, 2 melanoma cases had 5 grade of Clark's level. 3 melanocytoma cases had 4 grade of Clark's level, one melanocytoma case with 1 grade. The junctional activity was in 24 dog melanoma cases and was absent in 6 dog melanoma cases, also junctional activity was in 3 dog melanocytoma cases, and absent in 1 dog melanocytoma case.

Conclusions:
1. The shape of malignant cells in dog melanoma were epithelioid, fusocellular, balloon, mixed of epithelioid and dendritic, mixed of epithelioid and fusiform and dendritic cells, but the shape of melanocytoma cells were spindle and dendritic cells.
2. The aggressive rate increase with reduced infiltrative lymphocytes and extend necrotic zones in dog melanic tumors.
3. The mitotic index is much higher in the case of dog melanomas than in melanocytomas.

HISTOLOGICAL AND MACROSCOPICAL STUDY OF HORSE MELANOMA

Introduction
In the horses, the tumors represent 6-15% of skin tumors. A relation between the incidence of these tumors and the gray color is found, although horses of other colors are occasionally affected. There is an increased tendency to the development of melanic tumors in gray horses with age approximately 80% of gray horses over 15
years old develop skin melanic growths that are clinically detectable (Simth et al., 2002).

**Aim of study**
The aim of this study was: the extended histological and macroscopical study of equine melanic tumors through study of tumoural localization, tumoural aspect, cell type, mitosis., etc, and use them for comparative between benign and malign tumors.

**Material and Methods**
11 Tissue samples were collected from Alexandria slaughtered horses in the period between 5 -7 may 2010, and from the discipline of morphopathology and necropsy, faculty of veterinary medicine USAMV Cluj-Napoca, in the period between 1992 to 1998. Macroscopic examine: study of these cases was epidemiologically about (age, sex, breed) and macroscopically about diameter of tumor, tumor surface color, section color, period of growth this tumor and tumor palpation (thick, thin, smooth, coarse) using for this study register book. Microscopic examine: Melanoma biopsies were treated by formalin fixed (formalin 10% tamponed, PH7) during of 24 hours, and then paraffin embedded by standard procedures. These paraffined biopsies cut to sections 4-5 microns by microtom Leica RM2125 RT, after that these section stained with Hematoxylin & Eosin Stain protocol, then these biopsies examined by microscope Olympus BX 51. Imagines were taken by a digital Olympus SP 350 and analyzed with a program adecvat-Olympus DP soft.

**Results**
In this study the melanic tumors were nodules in all cases, 6 cases with small nodules (1-2 Cm) and 5 cases with big nodules (2-15 cm) composed from 1 to 2 nodules. 2 cases were ulcerated in the covered skin of tumor in the tail (Fig 1.B). One metastatic melanoma was multiple nodular formations in variable dimensions, from a wheat bean to a head of man, had a black color with thick-elastic structure, localized in liver mass, kidney, ovary, lung and spleen. 8 dog melanomas were formed of spindle type cells in 5 cases and dendritic cells in 3 cases. Horse melanocytoma cases were formed of 3 cases, one case was spindle type cells and two cases were dendritic type cells (Fig 1.D). The melanoma was medium grade of mitotic index figures in all cases, while melanocytoma was low grade of mitotic index figures. The melanoma cases were extended of necrotic zones in 1 case, moderate in 2 cases, reduced in 4 cases and absent in 1 case, while melanocytoma cases were reduced necrotic zones in all cases. Dendritic type cells had a number of following mitotic index figures (11, 1, 3, 7, 2). Spindle type cells had a number of following mitotic index figures (1, 10, 7, 4, 3, 5). Melanoma cases were intense infiltrated lymphocytes in 1 case, moderate in 3 cases and absent in 4 case while melanocytoma cases were absent infiltrated lymphocytes in all cases. The moderate 3-14 mitotic index figures was in (1 extend, 2 moderate, 4 reduced and 1 absent) necrosis (1 intense, 3 moderate, 0 reduced and 4 absent) infiltrate lymphocytes. The junctional activity was in all horse melanoma cases and all horse melanocytoma cases. 5 melanoma cases had 4 grade of Clark's level, and 3 melanoma cases had 5 grade of Clark's level.
Conclusions:
1. The primary and metastatic melanoma was multiple nodular formations in variable dimensions.
2. In horses the majority of melanomas showed spindle type cells, and the majority of the melanocytomas had dendritic type cells.
3. The mitotic index is much higher in the case of horse melanomas than in melanocytomas.
4. The relationship between infiltrated lymphocytes and necrotic zones was inverse legation with mitotic index in horse melanic tumors.

CYTOLOGICAL STUDY OF HORSE MELANIC TUMORS

Introduction
The cytopathology is used more frequently than biopsies for monitoring of metastasis in both human patients and animals (Basler GC et al., 1997-Wilcock BP et al., 1986). Melanomas are highly variable in their cytological appearance and may resemble round cell, epithelial, or spindle cell neoplasms (Fournel-Fleury et al., 1994), whereas in horses are cells have a melanic content, they are either fusiform or dendritic about 30 microns in diameter (Levene et al., 1971).

Aim of study
The aim of this work was cytologic study of the melanic cells (cytoplasm, nuclei, nucleoli, chromatin and melanic pigments), and benefit of these aspects to distinguishing benign from malignant tumor.

Material and methods
9 tissue samples were collected from Alexandria slaughtered horses in the period between 5 -7 may 2010. The samples were taken from the melanic tumors in skin of the ventral tail and perineum. The methods were: 1-Remove melanin by melanin bleaching solution 2-Stain cells by Diff Quick stain. The samples examined by microscope Olympus BX 51. Imagines were taken by a digital Olympus SP 350 and analyzed with a program adecvat-Olympus DP soft.

Results
The nuclei in all cases were round to oval shape with polymorphic in shape and size in malignant melanoma and approximately round to oval uniform in melanocytoma (Fig 1.E), but the chromatin was dispersed and fine in other cases and nucleoli were a little evidence with 1 to 3 nucleoli in nucleus, eccentric and big in some cases, the nuclear to cytoplasmic ratios were moderately high to very high in some cases, but in some other cases were moderate. Two cases had diffusion neutrophils and ulceration in tumoural surface and a few macrophages in some other cases. There were four spindle type cells, five round type cells to polygonal in one case, only one spindle cell melanocytoma and two round cell melanocytomas. These cells had a strong melanin in cytoplasm that removed by appropriate bleaching solution. The round cells were
round shape with a little irregular round in contour while the spindle cell were spindle shape with slightly prolonged. All cases had strong melanin with punctuate, to spherical Pigment granules and very fine with slightly elongated in some cases The cytologic aspect of malignant melanoma was appeared in all cases as anisocytosis and anisokaryosis with a little giant nuclei, but in melanocytoma cases the cytologic aspect in some cases was a little differentiation in size and shape of nuclei and cytoplasm, but in others the nuclei and cytoplasm were uniform. All cases were containing big melanic pigments.

Conclusions:

1. The shape of cells was spindle and round type in malignant melanoma and melanocytoma with a strong melanin in cytoplasm.
2. The cytologic aspect of malignant melanoma was anisocytosis and anisokaryosis with some giant nuclei, while the Cytologic aspect melanocytoma was approximately uniform aspect of nuclei and cytoplasm.

IMMUNOHISTOCHEMICAL EXPRESSION OF MELAN-A IN CANINE MELANIC TUMORS

Introduction
MART-1 / Melan-A is a protein antigen found on melanocytes. Antibodies against the antigen are used in the medical specialty of anatomic pathology in order to recognize cells of melanocytic differentiation, useful for the diagnosis of a melanoma (Kawakami, et al., 1994). In a study examining immunohistochemical staining of canine melanic tumors, Melan A was considered a specific and sensitive marker for canine melanomas (Ramos-Vara, et al., 2000). It is a protein of unknown function that is expressed mainly by melanocytes (Chen, et al., 1996).

Aim of study
The aim of this study was to use computerized image analysis to measure Melan-A antibody in series of canine melanocytic tumors to assess density of marked cells by this antibody, and to correlate percentages of marked cells with macroscopic and microscopic aspect, in order to an importance this marker for identification melanic tumors.

Materials and Methods
In the period 2001–2010 were diagnosed in Pathology Department USAMV Cluj-Napoca, 12 dog cutaneous melanomas, one dog cutaneous melanocytoma and one metastatic melanoma in intestine, in order to Melan-A expression study. Macroscopic study included: breed, age, sex, localization of tumor and size of tumor. Histological aspect was by formalin-fixed, paraffin-embedded tissue sections were used. Four-micrometer sections on slides and stained by Hematoxylin and eosin stain. Immunohistochemical method: staining the tissue sections by primary antibody
Melan-A, and develop process with DAB Chromogen and alkaline phosphatase Chromogen. Images were captured by using a microscope (Olympus BX51).

**Results**

**Immunoreactivity for Melan-A in melanomas.** The percentages of marker stain were between (40.43% - 97.89%). Melan-A reactivity was demonstrated in 10/12 cases (83.3%) of canine melanomas, in additional to metastatic melanoma negative primary sites was positive (1/1 cases), also 1/1 canine melanocytoma had been positive staining for Melan-A (Fig 2.B). The percentage of Melan-A reactivity was in all cases (85.71%). Intense classification of Melan-A in these cases: 2 cases (14.28%) were negative, zero (0%) had 5–10% positive cells, 3 (21.42 %) had 11–50% positive cells, 3 cases (21.42 %) had 51–80% positive cells, and 6 cases (42.85 %) had more than 80% positive cells. In canine cutaneous malignant melanoma, the intensity of staining was high in 6 cases (50 %), over moderate in 1 case (8.3%), moderate in 3 cases (25%) weak in 0 case (0%), and absent in 2 cases (16.6%), but the staining was over moderate in the metastatic melanoma (59.27%) and in melanocytoma (67.49%). Nuclear staining was visible in 0 canine melanoma case, and was staining visible in some cells and invisible in others in 6 melanoma cases, one metastatic melanoma and one melanocytoma case but was no visible in 4 melanoma cases. All tumor cell types demonstrated reactivity for Melan A. The staining in majority cases was heterogeneous with areas weren’t stained in 5 melanoma cases and 1 melanocytoma case, while 5 melanoma cases were homogenous stain in the tissue section, but metastatic melanoma was heterogeneous with this stain. There was no obvious relationship between breed, sex, age, localization of tumors and diameter of tumors reactivity for Melan A. Epithelioid cells were a tendency for high staining in 3 melanoma cases while 2 melanoma cases were moderate stain. Mixed epithelioid and spindle cells were a tendency for high staining in 2 melanoma cases while one melanoma case was moderate stain. Spindle cells were in general over moderate stain in (3/3) one of them was melanocytoma. One balloon melanoma type cells was a high stain with Melan-A. In all tumoural cases cells were reacted diffusely in cytoplasm with Melan-A and there aren’t any cells with polar or punctuate staining in cytoplasm. There was no obvious relationship between tumoural type (malign & benign) and Melan-A. The melanotic melanic tumors were intensity immunostaining in 3 cases, over moderate in 1 case, moderate in 1 case and absent in 1 case, while the amelanotic melanic tumors were intensity immunostaining in 3 cases, over moderate in 2 cases, moderate in 1 case and absent in 1 case.

**IMMUNOHISTOCHEMICAL EXPRESSION OF S100 MARKER IN THE CANINE MELANIC TUMORS**

**Introduction**

S100a marker is an isoform of a protein restricted to neuroectodermal cells. S100 is normally present in cells derived from the neural crest (Schwann cells, melanocytes, and glial cells…..etc), (Donato et al, 2003). S100 protein family are useful as markers for certain tumors and epidermal differentiation. It can be found in melanomas
S100 protein is demonstrated in more than 50% of melanomas. 

Aim of study
The aim of this study was to use computerized image analysis to measure S100 antibody in series of canine melanocytic tumors to assess density of marked cells by this antibody, and to correlate percentages of marked cells with macroscopic and microscopic aspect, in order to an importance this marker for identification of melanic tumors.

Materials and methods
In the period 2001 – 2010 were diagnosed in pathology department USAMV Cluj-Napoca, 11 dog cutaneous melanomas, one dog cutaneous melanocytoma and one metastatic melanoma in intestine, in order to S100 expression study. Macroscopic study included: breed, age, sex, localization of tumor and size of tumor. Histological aspect was by formalin-fixed, paraffin-embedded tissue sections were used. Four-micrometer sections on slides and stained by Hematoxylin and eosin stain. Immunohistochemical method: staining the tissue sections by primary antibody S100 and develop process with DAB Chromogen and alkaline phosphatase Chromogen. Images were captured by using a microscope (Olympus BX51).

Results
S100 reactivity was demonstrated in 12/12 cases (100%) canine melanomas (Fig 2.A), in additional to metastatic melanoma negative primary sites was positive with S100 (1/1 cases), also 1/1 canine melanocytoma had been positive staining for S100. Classification of intense of S100 in these cases: zero (0%) had 5–10% positive cells, zero (0 %) had 11–50% positive cells, 10 cases (76.92%) had 51–80% positive cells and 3 cases (23.07 %) had more than 80% positive cells. In canine cutaneous malignant melanoma the intensity of staining was high in 3 cases (25 %), over moderate in 9 cases (75%), moderate in zero (0 %), weak in zero (0%) cases, and absent in 0 (0%) cases, but, the staining was over moderate in the metastasis (79.84%) and in melanocytoma (54.94%). Nuclear staining was visible in 0 canine melanoma, and was staining visible in some cells and invisible in others in 10 melanoma cases and wasn't visible in 2 melanoma cases, while one melanocytoma case and one metastatic melanoma were staining visible in some cells and invisible in others. All tumor cell types demonstrated reactivity for S100. The staining in majority cases was heterogeneous with areas weren't stained in 9 melanoma cases, and 1 melanocytoma case while 3 melanoma cases were homogenous stain in the tissue section. There was no obvious relationship between breed, sex, age, localization of tumors and diameter of tumors reactivity for S100. Epithelioid cells were a tendency for over moderate staining in 3 melanoma cases while 2 melanoma cases were intensity stain. Mixed epithelioid and spindle cells were an over moderate staining in 4 melanoma cases. One balloon melanoma type cells was a over moderate stain, and spindle cells were stained of over moderate staining in (2/2) one of them was melanocytoma, and other melanocytoma was intensity staining (91.31%). The melanotic melanic tumors were
over moderate in 6 cases, while the amelanotic melanic tumors were intensity immunostaining in 3 cases, and over moderate in 4 cases. In all cases tumoural cells were reacted diffusely in the cytoplasm with S100 and there weren’t any cells had polar or punctuate staining in cytoplasm. High percentages of marker stain accompany with medium of number of mitosis, that was observed in (2) cases. There was no obvious relationship between melanoma and melanocytoma with S100 marker.

The aim of this study was to use computerized image analysis to measure S100 antibody in series of canine melanocytic tumors to assess density of marked cells by this antibody, and to correlate percentages of marked cells with macroscopic and microscopic aspect, in order to an importance this marker for identification of melanic tumors.

**IMMUNOHISTOCHEMICAL EXPRESSION OF THE VIMENTIN MARKER IN DOG MELANIC TUMORS**

**Introduction**

Vimentin is the most ubiquituous intermediate filament protein and the first to be expressed during cell differentiation. Vimentin is expressed in a wide variety of mesenchymal cell types fibroblasts, endothelial cells etc (Katsumoto et al., 1990). Vimentin is present in many different neoplasms but is particulary expressed in those originated from mesenchymal cells Sarcomas e.g., fibrosarcoma, malignt fibrous histiocytoma, angiosarcoma, and leio- and rhabdomyosarcoma, as well as lymphomas, malignant melanoma and schwannoma (Lang et al., 2002- Niveditha et al.,2003-Ramos-Vara et al., 2000). This type of intermediate filaments can distinguish melanoma from undifferentiated carcinoma, but not from lymphoma or sarcoma (Koenig et al., 2001).

**Aim of study**

The aim of this study was to use computerized image analysis to measure vimentin antibody in series of canine melanocytic tumors to assess density of marked cells by Vimentin, and to correlate percentages of marked cells by Vimentin with macroscopic and microscopic aspect. In order to an importance this marker for identification of melanic tumors.

**Materials and methods**

In the period 2001 – 2010 were diagnosed in pathology department USAMV Cluj-Napoca, 12 dog cutaneous melanomas, two dog cutaneous melanocytomas and one metastatic melanoma in intestine, in order to Vimentin expression study. Macroscopic study included: breed, age, sex, localization of tumor and size of tumor. Histological aspect was by formalin-fixed, paraffin-embedded tissue sections were used. Four-micrometer sections on slides and stained by Hematoxylin and eosin stain. Immunohistochemical method: staining the tissue sections by primary antibody Melan-A and develop process with DAB Chromogen and alkaline phosphatase Chromogen. Images were captured by using a microscope (Olympus BX51).

**Results**
Immunoreactivity for Vimentin in melanic tumors. Vimentin reactivity was demonstrated in 11/12 cases (93.33%) of canine melanomas (Fig 2.C), in additional to metastatic melanoma negative primary sites was positive for Vimentin (1/1 cases), also 2/2 canine melanocytoma had been positive staining for Vimentin. The percentages of marked cells by Vimentin were between (58.95% – 97.40%). Classification of Vimentin intense in these cases were: 1 case (6.66%) negative, zero case (0%) had 5–10% positive cells, 0 case (0%) had 11–50% positive cells, 6 cases (40%) had 51–80% positive cells, and 8 cases (53.33%) had more than 80% positive cells. In canine cutaneous malignant melanoma the intensity of staining was high in 7 cases (58.33%), over moderate in 4 cases (33.33%), moderate in 0 case (0%), weak in 0 case (0%), and absent in one case (8.3%), while the staining was over moderate in the metastatic melanoma (58.95%) and in one melanocytoma (65.18%), but other melanocytoma (93.87%). Nuclear staining was visible in 0 canine melanoma case, and was staining visible in some cells and invisible in others in 5 melanoma cases, and one melanocytoma case but wasn't visible in 6 melanoma cases, one metastatic melanoma and one melanocytoma. All tumor cell types demonstrated reactivity for Vimentin. The staining in majority cases was homogeneous in 8 melanoma cases, and 1 melanocytoma case while 3 melanoma cases were heterogeneous stain in the tissue section, also metastatic melanoma and one melanocytoma were heterogeneous with this stain. There wasn't obvious relationship between breed, sex, age, localization of tumors and diameter of tumors reactivity for Vimentin. Epithelioid cells were tendency for staining of high stain in 3 melanoma cases while 1 melanoma case and 1 melanocytoma were over moderate stain. Mixed epithelioid and spindle cells were tendency for staining of over moderate in 2 melanoma cases while one melanoma case was high stain. Spindle cells were a tendency for staining high stain in 3 melanomas, 1 melanocytoma and over moderate in one melanocytoma. One balloon melanoma cell type was high stain with Vimentin. All tumoural cells were reacted diffusely in cytoplasm with Vimentin and there weren’t any cells stained polar or punctuate in cytoplasm. The high percentages of marked cells were in amelanotic tumors, 6 cases were intensity stain while 2 cases were over moderate stain, but the moderate percentages were in melanotic tumors 4 cases were over moderate stain while 2 cases were intensity stain. I didn't find any legation between percentage of Vimentin and tumor types (benign or malignant).

COMPARATIVE STUDY OF IDENTIFY MARKERS MELAN-A, S100 AND VIMENTIN IN DOG MELANIC TUMORS

Introduction
Melan-A, S100 and Vimentin have a significant diagnosis in pathology laboratory in identify on melanic tumors, whereas Vimentin is expressed in a wide variety of mesenchymal cell types fibroblasts, endothelial cells etc. (Katsumoto et al., 1990), S100a marker is an isoform of a protein restricted to neuroectodermal cells. S100 is normally present in cells derived from the neural crest (Schwann cells, melanocytes, and glial cells…..etc), (Donato et al, 2003), and MART-1 / Melan-A is a protein antigen found on melanocytes. Antibodies against the antigen are used in the medical
specialty of anatomic pathology in order to recognize cells of melanocytic differentiation, useful for the diagnosis of a melanoma (Kawakami, et al., 1994).

**Aim of study**
The purposes of this study were extended comparative study of melanoma identical markers Melan-A, Vimentin and S100 through of sensitivity of staining, localization of stain in the cells and the tissue sections and comparative them with some sign histological aspects as shape of cells and type of tumors.

**Materials and Methods**
In the period 2001 – 2010 were diagnosed in pathology department USAMV Cluj-Napoca, 10 dog cutaneous melanomas, one dog cutaneous melanocytoma and one metastatic melanoma in intestine, in order to Melan-A, Vimentin, S100 expression study. Macroscopic study included: breed, age, sex, localization of tumor and size of tumor. Histological aspect was by formalin-fixed, paraffin-embedded tissue sections were used. Four-micrometer sections on slides and stained by Hematoxylin and eosin stain. Immunohistochemical method: staining the tissue sections by primary antibody Melan-A, S100 and Vimentin and develop process with DAB Chromogen and alkaline phosphatase Chromogen. Images were captured by using a microscope (Olympus BX51).

**Results**
Immunostaining of melanic tumors with Vimentin, S100 protein, and Melan-A. All melanic tumors (100%) were positive for S100 protein (12/12 cases), (91.6%) were positive for Vimentin (11/12 cases), and (83.3%) were positive for Melan A (10/12 cases). One Vimentin case (8.4%) and two Melan-A cases (16.7%) were negative staining. One of 12 cases (8.4%) melanic tumors were negative for both Melan A and vimentin. One of 12 cases (8.4%) melanic tumors were positive for Vimentin and negative for Melan-A, (0 %) melanic tumors were negative for both Melan A and S100. One of 12 cases (16.7 %) melanic tumors were positive for Melan-A stain were (40.43 to 97.89%), the percentages of S100 stain were (54.06% to 98.1%), and the percentages of S100 stain were (58.95% to 96.04%). The Melan-A stain was absent in 2 cases, moderate in 2 cases, over moderate in 3 cases and intense in 5 cases, the S100 stain was over moderate in 9 cases and intense in 3 cases and the Vimentin stain was absent in one case, over moderate in 5 cases and intense in 6 cases. The epithelioid type cells were intensity stain in one case, over moderate in 3 cases and negative in one case with Vimentin stain, they were intensity stain in two cases, over moderate in one case and negative in two cases with Melan-A stain and they were intensity stain in two cases and over moderate in 3 case with S100 stain. The mixed epithelioid and spindle type cells were intensity stain in one case and over moderate in 2 cases with Vimentin stain, they were intensity stain in two cases and moderate in one case with Melan-A stain and they were over moderate in 3 case with S100 stain. The spindle type cells were intensity stain in 3 cases with Vimentin stain, they were moderate stain in one cases and over moderate in two cases with Melan-A stain and they were intensity stain in one case and over moderate in 2 cases with S100 stain.
One balloon cell case was intensity in Melan-A and Vimentin but over moderate in S100 stain. The melanic tumors were intensity stain in 3 cases, over moderate in 2 cases, moderate in one case and absent in one case for Melan-A stain, they were intensity stain in 2 cases and over moderate in 5 cases for S100 stain and they were intensity stain in 2 cases, over moderate in 4 cases and absent in one case for Vimentin stain. The amelanotic tumors were intensity stain in 2 cases, over moderate in 1 case, moderate in one case and absent in one case for Melan-A stain, they were intensity stain in 1 case and over moderate in 4 cases for S100 stain and they were intensity stain in 4 cases and over moderate in 1 case for Vimentin stain. The distribution of marker in tissue sections were homogeneous in 5 cases and heterogeneous in 5 cases in Melan-A marker, while the distribution of Vimentin was homogeneous in 7 cases and heterogeneous in 4 cases and the distribution of S100 was homogeneous in 3 cases and heterogeneous in 9 cases. The staining was cytoplasmic and nuclear in some cases for S100, Melan-A and Vimentin protein whereas the nuclei were positive in areas and negative in others in Melan-A (7/10), S100 (6/11) and Vimentin (6/12). There weren't any relationship between these markers and tumoural types (malign & benign).

Conclusions of Comparative markers
1. The staining of Melan-A and S100 was heterogeneous and inversely with Vimentin in majority cases.
2. The staining of nuclei was more frequently in Melan-A then S100 and then Vimentin.
3. The intensity of immunostaining of melanic tumors was tended in Melan-A then S100 and then Vimentin but in amelanotic tumors the intensity was tended in Vimentin then Melan-A and then S100, while S100 was more sensitivity in melanotic tumors then amelanotic tumors inversely in Vimentin and Melan-A was approximately equal in melanotic tumors and amelanotic tumors.

IMMUNOHISTOCHEMICAL EXPRESSION OF THE VASCULAR MARKER CD31 IN DOG CUTANEOUS MELANOMA

Introduction
Angiogenesis is a complex multistep process characterized by the formation of new capillaries from the preexisting vascular network. In neoplasms, it is essential for tumor growth and metastasis (Blood et al., 1990). Endothelial cells proliferate 30–40-fold faster in tumor blood vessels than in the vasculature of normal tissues (Hobson et al., 1984). Furthermore, several studies have demonstrated a significant correlation between marked angiogenesis, evidenced by a high microvessel density (number of microvessels per square millimeter), metastasis, and poor prognosis in several human tumor types, including breast (Fox et al., 1993). CD31 is a transmembrane protein of 135 kDa molecular mass that belong to immunoglobulin family, and has an important
role in adhesion between endothelial cells or between endothelial cells and leucocytes (Graham et al., 1999).

**Aim of study**

The aim of this study was to use computerized image analysis to measure angiogenesis in series of canine melanocytic tumors to assess the microvessel density and, in addition, the area and perimeter of each vessel, and to correlate these parameters with histological signs and malignant degree of melanic tumors.

**Materials and methods**

In the period 2001 – 2010 were diagnosed in pathology department USAMV Cluj-Napoca, 10 dog cutaneous melanomas, 4 dog cutaneous melanocytomas and one metastatic melanoma in intestine, in order to CD31 expression study. Macroscopic study included: breed, age, sex, localization of tumor and size of tumor. Histological aspect was by formalin-fixed, paraffin-embedded tissue sections were used. Four-micrometer sections on slides and stained by Hematoxylin and eosin stain. Immunohistochemical method: staining the tissue sections by primary antibody CD31 and develop process with DAB Chromogen and alkaline phosphatase Chromogen. Images were captured by using a microscope (Olympus BX51).

**Results**

The tumoural microvessel density was between (3.33 to 11.6) vessels/high power field (Fig 2.D). The tumoural microvessel density was in 11 melanoma cases between (3.33 to 7.2) vessels/high power field, one of them was metastatic melanoma with 5.6 vessels/field, while in melanocytoma cases was one negative and 3 positive cases (5-5.66-11.6) vessels/high power field. The percentages of vessel area/total field area in malignant melanoma were between (1.37%-8.7%) and in metastatic melanoma was (9.61%), while in melanocytoma were between (0 to 4.98%). All malignant melanoma cases were between (4-5) clack's level and were positive with CD31. All ulcerated tumors were positive with CD31. In malignant melanoma the low number of mitotic index figures was in cases had between (3.66 to 5.6) vessels/high power field and between (2.18% to 9.61%) Percentage of vessel area/total field area, but the moderate to high number of mitotic index figures was in cases had between (3.33 to 7.2) vessels/high power field and between (1.37% to 8.7%) Percentage of vessel area/total field area. In melanocytoma, the percentage of vessel area/total field area was between (0 to 4.98%). The area average in malignant melanoma was between (94.52 µm² to 769.56 µm²), and (608 µm²) in metastatic melanoma, but the area average in melanocytoma was between (0 to 335.04 µm²). The perimeter average in malignant melanoma was between (56.7 µm to 118.08 µm), and 117.82 µm in metastatic melanoma, but the area average in melanocytoma was between (0 to 90.16 µm²).
Introduction

PCNA is synthesized in early G1 and S-phases of cell cycle (Takahashi et al., 1993). Tight linkage to cell proliferation has led to the investigation of its role in the evaluation of tumors for prognosis. Takahashi noted a progressive increase in percent of PCNA positive cells in melanomas with increasing tumor thickness, but didn't correlate PCNA staining with clinical outcome (Takahashi et al., 1991).

Aim of study

The aim of this study is to use computerized image analysis to measure PCNA and CD31 antibodies in a series of canine melanocytic tumors to assess density of marked cells by these antibodies, and to correlate density of marked cells with malignant degree of these tumors through comparative study between CD31, PCNA and microscopic aspect.

Materials and Methods

In the period 2001 – 2010 were diagnosed in pathology department USAMV Cluj-Napoca, 9 dog cutaneous melanomas, two dog cutaneous melanocytomas and one metastatic melanoma in intestine, in order to PCNA expression study, and 10 samples of them were treated with CD31 marker by immunohistochemical for comparative study. Histological aspect was by formalin-fixed, paraffin-embedded tissue sections were used. Four-micrometer sections on slides and stained by Hematoxylin and eosin stain. Immunohistochemical method: staining the tissue sections by primary antibody CD31 and develop process with DAB Chromogen and alkaline phosphatase Chromogen. Immunofluorescence method: staining the tissue sections by primary antibody PCNA and develop process with fluorochrome-conjugated secondary antibody-Rhodamine. Images were captured by using a microscope (Olympus BX51).

Results

*PCNA expression study:* all dog melanic tumors were positive with PCNA Marker (Fig 2.E). All cases were in low grade of PCNA according on John's classification. (7 of 12 cases) 58% were positive according of Proniewska's classification. The malignant melanoma cases had a range between (4.6% to 21.88%), while the melanocytoma cases had a range between (9.8% to 10.5%). The high values of mitosis in these cases had big percentages of PCNA than other cases (14, 53, 14) mitotic index figures had respectively (21.88%, 13, 60%, 17.93%). The spindle type cells had (4.6%, 9.8%, 10.5%, 16.31%) it means between (4.6% to 16.31%), whereas in Proniewska's classification, the percentage of positive markers was 50%. The epithelioid type cells had (8.5%, 12.05%, 17.93%, 21.88%) it means between (8.5% to 21.88%), in Proniewska's classification percentage of positive marker was 75%. The mixed epithelioid and spindle type cells had (5.05%, 6.7%, 13.6%) it means between (5.05% to 13.6), in Proniewska's classification percentage of positive marker was 33.3%. All cases had 4 Clack's level. There weren't any relationship between necrotic zones & infiltrated lymphocytes and PCNA percentages of these cases.

Study of the relationship between PCNA and CD31: all cases were positive for PCNA marker while 9 of 11 cases were positive for CD31 marker. Percentages of
PCNA marker were between (4.6% to 21.88%), while numbers of vessels/field were between (2.8 to 7.2). Percentages of PCNA marker in malignant melanoma were between (4.6% to 21.88%), while numbers of vessels/field were between (2.8 to 7.2). One melanocytoma case had 9.8% PCNA percentage and 5 number of vessels field. One metastatic melanoma case had 5.6% PCNA percentage and 12.05 number of vessels/field. The high percentages of PCNA had in majority cases big numbers of microvessels/field (16.31%, 21.88%, 13.6%, 12.05%, 17.93%) percentages of PCNA had respectively (4.2, 7.2, 6, 5.6, 5.66 microvessels/field), whereas one of them had a high percentage of PCNA (21.88%) and big number of vessels (7.2). There weren't any relationship between grade of PCNA and percentage of vessel area/total area, Average of perimeter and average of vessel area.

Conclusions of malignant markers:
A. The percentage of vessel area / total field area, microvessels, average area and perimeter were an increase in malignant melanoma comparatively with melanocytoma.
B. The high values of mitosis and microvessels concurrent approximately with big values of PCNA percentages in majority cases.
C. The malignant melanoma had high PCNA percentages than melanocytoma.
D. The malignant melanoma had big number of vessels and high percentages of PCNA than melanocytoma.

General Conclusions
A. The aggressive rate increase with reduced infiltrative lymphocytes and extend necrotic zones in melanic tumors.
B. The mitotic index is higher in horse and dog melanomas than in melanocytomas.
C. The Vimentin marker more intensity than S100 and then Melan-A
D. The values of vessels labeled by CD31 and percentages of positive nuclei by PCNA were an increase in malignant melanoma comparatively with melanocytoma.
E. PCNA and CD31 markers has the significant in evaluation of aggressive of tumors.

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