Bulletin UASVM, Veterinary Medicine 68(1)/2011
pISSN 1843-5270; eISSN 1843-5378

Immunohistochemical Expression of Melan-A in Canine Melanic Tumors

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Abstract. We evaluated the expression of Melan A/MART-1 (melanoma antigen recognized by T cells). Material and methods: 12 clinical specimens of dog melanic tumors were diagnosed with Melan-A by immunohistichemmical method. Results: Melan-A reactivity was demonstrated in (10-12, 83.3%) melanoma, one metastatic melanoma and one melanocytoma were positive. 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. 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. Conclusion: The percentages of marker stain were between (40.43%- 97.89%), Melan-A reactivity was staining in majority cases. The intensity of Melan-A stain was between moderate to intense in melanoma, while in melanocytoma and metastatic melanoma cases were over-moderate. The staining in majority cases was heterogeneous. All tumor cell types demonstrated reactivity for Melan A. In all tumoural cells of cases were reacted diffusely in their cytoplasm with Melan-A,

Keywords: Melanoma. Immunohistochemical. Melan-A.

INTRODUCTION

Dermal melanomas account for 9–20% of skin tumors in dogs and generally follow a benign course. Melanomas are the most common malignant tumor of the oral cavity and digits in dogs (Aronsohn, et al., 1998- Marino, et al., 1995), at least 90% of these tumors are malignant (Moulton,et al., 2000), and many have metastasized by the time of diagnosis. However, few oral melanomas follow a benign course, and some dermal melanomas are malignant, making definitive prognostication for these tumors difficult. Melanocytes arise from embryonic neuroectoderm and, as such, retain the ability to differentiate into spindled or epithelioid cells, making a diagnosis of canine melanoma challenging in poorly differentiated amelanotic tumors. In these cases, determining the presence of constituent proteins whose expression is restricted to melanin-producing cells or cells arising from neuroectodermal tissues can assist in the diagnosis. 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), 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. The same name is also used to refer to the gene which codes for the antigen. The names MART-1 and Melan-A were coined by two groups of researchers who independently sequenced the gene for this antigen in 1994, whereas both names are currently in common use (Kawakami, et al., 1994). At the National Cancer Institute coined the term MART-1, which stands for "Melanoma Antigen Recognized by T-cells (Coulie, et al., 1994), of Belgium called the gene Melan-A, presumably an abbreviation for "melanocyte antigen. The MART-1 / Melan-A antigen is specific for the melanocyte lineage, found in normal skin, the retina, and melanocytes, but not in other normal tissues. It is thus useful as a marker for melanocytic tumors (melanomas) with the caveat that it is normally found in benign nevi as well. MART-1/ Melan-A is a putative 18 kDa transmembrane protein consisting of 118 amino acids. It has a single transmembrane domain (Kawakami, et al., 1994).

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

The database of our investigation was constituted of cadavers from the discipline of morphopathology and necropsy in faculty of medicine veterinary Cluj-Napoca, and also as samples sent from the surgery clinic and private practitioners, for diagnostic purpose. From all cadavers and samples examined between 2001– 2010. were initially selected and reviewed to determine their suitability for the study. Cases with small samples or no tissue remaining were excluded. Those cases in which the morphologic diagnosis was not definitive were reviewed to establish. This review process resulted in selection of 14 cases were diagnosed with 12 dog cutaneous melanomas and one melanocytoma and one metastasis melanoma in intestine, for detailed study. The macroscopic study included: breed, age, sex, localization of tumor and size of tumor, and histological aspect was by formalin-fixed, paraffin-embedded tissue sections were used. Four-micrometer sections on slides and stained by Hematoxylin and eosin stain (Mayer’s Hematoxylin: Dako), in order to study aspect of cells, nuclei, nucleoli, tumoural type (benign, malignant), localization of the tumoural cells in tissue section, and others that were compared with Melan-A Marker by immunohistochemical method.

For immunohistochemical method: the paraffin-embedded tissue sections in positive charge slides were processed according this protocol, pretreatment with a steamer, heating the slides in antigen retrieval citrate buffer solution at pH 6.0, then the primary antibody Melan-A (monoclonal mouse anti human Clone A 103 Code M7196. Dako) was incubated for overnight at temperature 4 °C then for diaminobenzidine DAB method (brown color) secondary antibody, Streptavidin peroxidase, Substrate-chromogen solution, (LSAB+System-HRP Edition 06/07 Code K0679, Code K0690) Dako with the same incubation times (30 minutes) at room temperature (RT) everyone but with chromogen only between 20 sec to 1 min. For more details see index nr.4, but for alkaline phosphatase method (red color) the secondary antibody (15 min) Streptavidin peroxidase (15 min) Substrate-chromogen solution (alkaline phosphatase 20 min), (Dako REAL™ Detection System, Alkaline Phosphatase/RED, Rabbit/Mouse Code K5005). For more details see index nr.5. In some

cases which the amount of melanin obscured partial the immunologic reaction, tissues were counterstained with Azure B stain for 3 min *(Kamino, et al., 1991).*

Number of positive cells was assessed randomly by choosing immunolabeled cells on a 400x field (40x objective and 10x ocular) and using an automated image analysis system (Olympus cell B). Five fields per tumor were examined.

Images were captured by using a microscope (Olympus BX51) connected to a video camera (Olympus DP25), stored in the digital memory, and shown on the monitor.

Statistics: Independent group *t*–tests, x2 tests of independence or Fisher's exact test were used to compare two groups in regard to the categorical data, by using (Epi-Info software) and Microsoft Excel.

RESULTS AND DISCUSSIONS

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%) canine melanomas examined, in additional to metastases of Melan-a negative primary sites was positive (1/1 cases), while 1/1 canine melanocytoma had been positive staining for Melan-A. 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 cells of cases were reacted diffusely in their 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.

In the retrospective analysis of sporadic canine melanic tumors, Melan A/MART-1 identified 85.71 % of the pigmented tumors (fig 2.3, diagram 1). Melan A/MART-1 has been described as a sensitive and specific marker for cells of melanocytic origin (Busam.,et al., 1999). In one study, 89.1% of canine melanomas were positive for Melan A/MART-1 (Ramos-vara.,et al., 2000) and other study 90% of canine melanomas were positive for Melan

A/MART-A (Koenig.,et al., 2001), in humans, results of immunostaining for Melan A/MART-1 seem to vary widely depending on the type of lesion. (Bergman et al., 2000) (Hofbauer, et al.,1998). In multiple studies, normal melanocytes exhibited more homogeneous staining and a higher percentage of Melan A/MART-1-positive cells than the malignant melanocytes or cells in melanoma metastases (Curry, et al., 1999- de Vries et al., 1997- Kageshita, et al., 1997). The reasons for the lower percentage of Melan-A positive tumors in some cases are unclear but as for the cell lines, may be related to issues of pigment production, selection of variants with high proliferative potential and low immunogenicity (Koenig.,et al., 2001),the ABC detection method (which may be less sensitive than other methods), or other phenotypic features of these tumors that cannot be classified at this time. For example, the more detailed classification scheme for human melanocytic lesions may help explain some of the disparity in immunostaining between canine and human melanomas. Two melanoma cases were negative with Melan-A marker, one of them a weak melanotic melanoma, that may to evaluate causes by the author Koenig that said: Melan A/ MART-1 may be important for melanin synthesis in pathways that are distinct from tyrosinase; some pigmented tumors lack immunoreactivity against tyrosinase (de Vries, et al., 1997), or other melanoma-specific antigens. If Melan-A/MART-1 were important for pigment production, its absence from cells that lack melanin would be predictable. Additionally, melanin production can be reinduced in canine melanoma cell lines that have lost melanin expression by growing the cells on soft agar, but promotion of Melan A/MART-1 expression under these same conditions has not been examined. Because Melan A/MART-1 is an immunodominant antigen, loss of its expression could be a way for melanomas to evade the immune system. (Koenig.,et al., 2001).

The Melan-A stain distributions in this study were observed in all melanic tumoural cases that were positive immunostaining, between moderate to high stain marker (fig 2.3, diagram 2), that weren't conform with other study that indicate to existence this marker in all intensity levels of staining (Ramos-vara, et al., 2000), whereas the Melan-A distributions in this study indicate to capable this marker to stain dog melanic tumors.

Melan-A has also been stained epithelioid and mixed epithelioid and spindle melanomas more intensely and consistently than spindle variants, (table.2, fig.1.8.9,diagram.3), that conformed with other studies (Busam, et al., 1998-Jungbluth, et al., 1998), but this differential staining was not evident in the other study (Koenig.,et al., 2001).

In this study amelanotic and melanotic melanic tumors had a different sensitive for Melan-A from absent until high stain, with a little tendency to stain capable in amelanotic melanic tumors, (fig.3), but in other study, Melan A was reported to have a higher sensitivity for human amelanotic melanomas (Kaufmann, et al., 1998).

The percentage of Melan-A immunostain of metastatic melanoma was 100% in one case but the number of metastatic cases wasn't sufficient for give a accurate result, but in other study, all metastatic melanoma negative primary site were negative immunostaining and all metastatic melanoma positive primary site were positive immunostaining (Ramos-Vara et al., 2000).

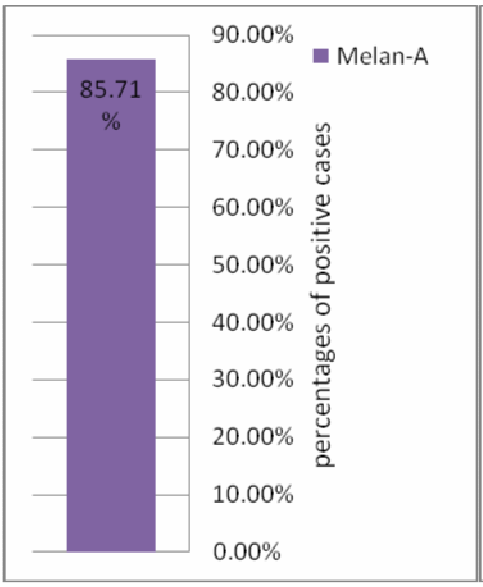

Fig. 1: The range of percentage of positive melanic tumors with Melan-A marker.

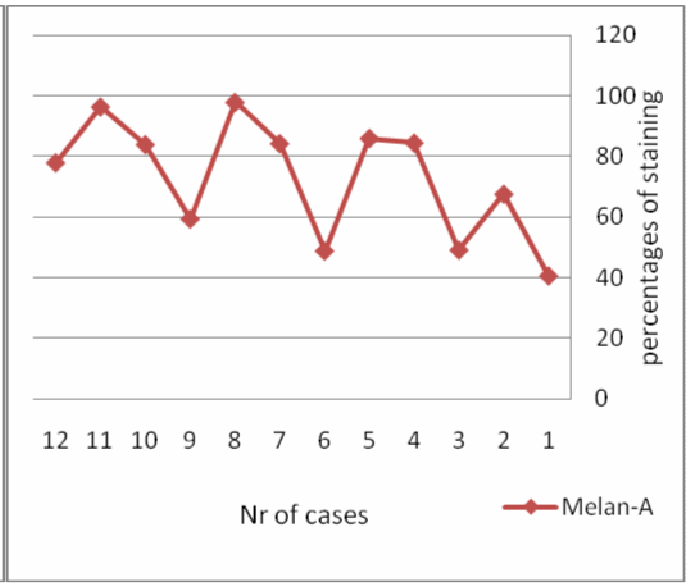

Fig. 2: The percentage of positive melanic tumors with Melan-A marker.

In this study, melanoma a weak melanin had a high sensitive while metastatic melanoma had a moderate sensitive (59.27%), but in other studies, a higher percentage of immunoreactivity has also been demonstrated in melanoma metastases of both humans (81%) (Kaufmann, et al), and dogs (71%) (Ramos-Vara, et a., l., 2000),

Nuclear was staining visible in some cells and invisible in others in 8 cases but wasn't visible in 4 cases, (fig 1,3), that indicated to the Melan-A has a moderate capable to stain of nuclei, whereas these results weren't conform with specialist literature, Ramos-Vara who noticed: nuclear staining was not visible in any melanoma (Ramos-vara, et al., 2000).

The staining of cases was homogeneous (41.66%), while the staining was heterogeneous stain (58.33%), (fig 1,3) that indicate to the distribution of Melan-A protein in melanic cells tend to heterogeneous aspect, whereas $P < 0.05$ then there was a significant difference in distribution of marker with stain percentages (reject the null hypothesis), and these results conform with specialist literature, Ramos-Vara who noticed: The staining in the majority cases was heterogeneous (Ramos-Vara, et al., 2000).

In this study, all specimens were diffusely pattern, that indicate to distributed Melan-A protein in whole tumoural cytoplasm cell, but in other study Melan A was detected in the cytoplasm in three patterns: diffuse (67.4%), polar or punctuate (3.9%), and both diffuse and polar (20.1%) (Ramos-vara, et al., 2000).

There was no obvious relationship between tumoural type (malign & benign) and Melan-A.

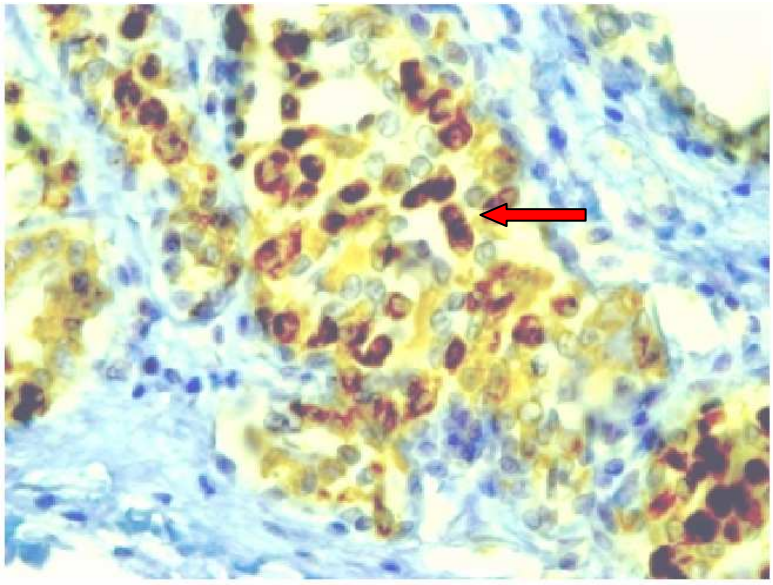

Fig. 1. Metastatic in intestine; Dog. Melanoma. Numerous epithelioid melanoma cells are positive for Melan-A with a diffusion pattern of staining, mixed visible and invisible stain nuclei with this marker. DAB stain with Mayer's Hematoxylin counterstain. (400x)

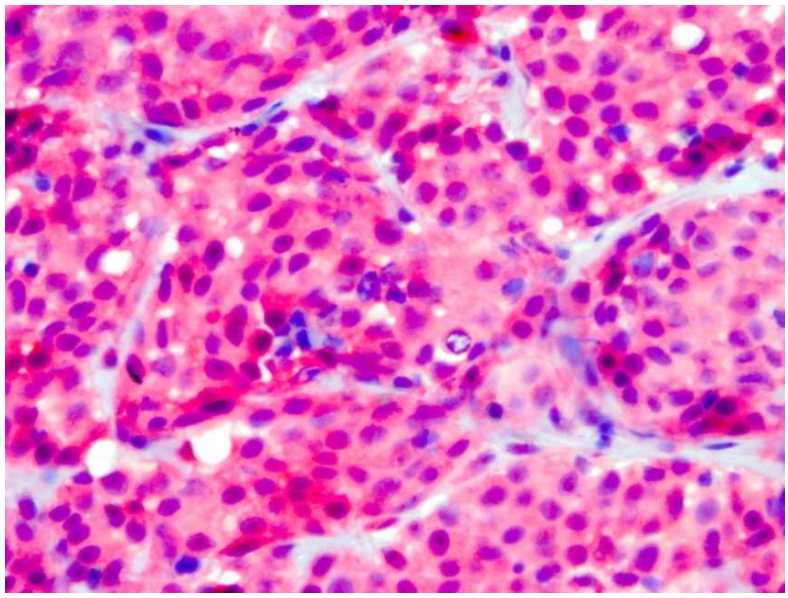

Fig. 2. Mandibular region; Dog. Melanoma. Numerous epithelioid melanoma cells are positive for Melan A with a diffusion pattern of staining, mixed visible and invisible stain nuclei with this marker. Alkaline phosphatase stain with Mayer's Hematoxylin counterstain. (400x)

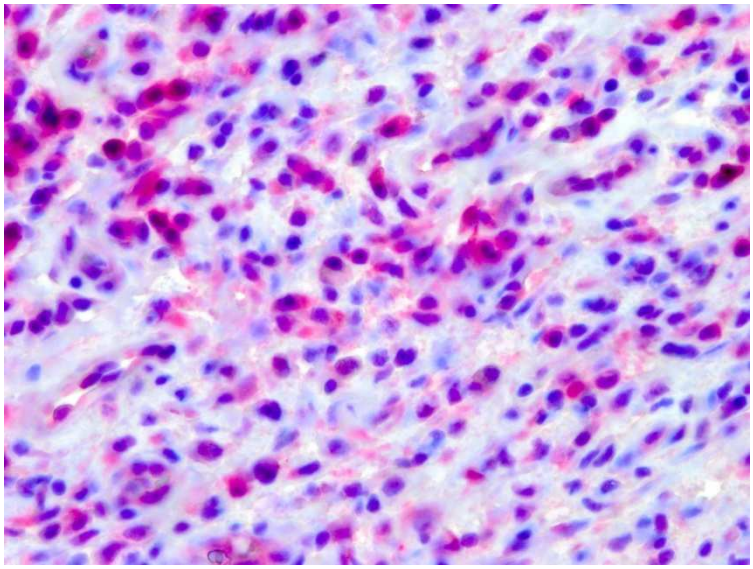

Fig. 3. Gingiva; Dog. Melanoma. Some spindle melanoma cells are positive for Melan-A with a diffusion pattern of staining, mixed visible and invisible stain nuclei with this marker. Alkaline phosphatase stain with Mayer's Hematoxylin counterstain. (400x)

CONCLUSIONS

- In the period 2001–2010, were diagnosed 12 dog cutaneous melanoma, 1 dog cutaneous melanocytoma and 1 metastatic melanoma in intestine.
- The percentages of marker staining were between (40.43% - 97.89%).
- Melan-A reactivity was in majority cases (85.71%).
- The stain was in cytoplasm in all cases with some cases had some staining of nuclei.
- The staining in the majority cases was heterogeneous.
- There was no obvious relationship between breed, sex, age, localization of tumors and diameter of tumors reactivity for Melan-A.
- All tumor cell types demonstrated reactivity for Melan-A.
- Epithelioid cells type were a tendency to intensity staining, mixed epithelioid and spindle cells type were a tendency to intensity staining, Spindle cells type were over moderate stain in (3/3) one of them is melanocytoma ,but balloon cells type was high stain with Melan-A.
- All cases tumoural cells were reacted diffusely in cytoplasm.
- There was no obvious relationship between tumoural type (malign & benign) and Melan-A.

ACKNOWLEDGMENTS

This study has been financed by the UEFISCSU as part of Contract no. 1106 / 2009.

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