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# Comparative Histopathological Diagnosis of Cutaneous Melanoma by H&E, Special Staining and Immunohistochemical Methods against Cutaneous Squamous Cell Carcinoma in Horse and Bovine

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## Abstract

**Background:** The aim of this study was to compare the role of special staining and to test melanoma associated antigens against paraffin embedded bovine and equine melanomas in order to assess their ability and utility as diagnostic tools for diagnosis and confirmation of the melanomas.

**Methods:** From 1991-2012, 13 cases were reviewed histologically, and each case was found to contain a large focus of melanoma and squamous cell carcinoma (SCC) on the hematoxylin and eosin-stained tissue section. All cases had documented cutaneous melanoma and SCC. Serial 5 µm-thick sections were cut from routine formalin-fixed paraffin blocks of 4 case cutaneous SCC (including 2 cases from horses and 2 cases from cattle) and 9 case cutaneous melanomas (including 6 horses cases and 3 cattle cases) from the files of the Department of Pathology have been diagnosed by H&E staining method. For diagnosis confirmation, Fontana-masson silver method, melanin bleaching method and immunohistochemical staining for S-100 protein and HMB45 antigen were applied. Majority of cases encompassed intradermal histologic pattern.

**Results:** In 6 cases out of 9 tumors, both S-100 protein and HMB45 staining methods were positive, whereas there were suspicious results for 3 cases. In all cases, results of Fontana-masson silver and melanin bleaching methods were positive. In 4 cases of squamous cell carcinoma, results of 3 tests were negative. Diagnosis of suspicious melanoma tumor cases must be confirmed or reconfirmed by special staining methods and/or immunohistochemical method.

**Conclusions:** The standard of practice in diagnosing melanoma is to use a panel of antibodies consisting primarily of S-100 protein and HMB-45. For spindle cell and desmoplastic melanomas, however, S-100 protein and HMB-45 should remain within antibody panel since S-100 protein is most sensitive marker for these entities and evidence is lacking that either Melan-A or Mitf are better markers than HMB-45 for recognizing spindle cell melanocytic lesions.

**Keywords:** Melanoma; Squamous Cell Carcinoma; HMB45 antigen; S-100 protein; Immunohistochemical

## Introduction

Melanomas are devastating neoplasms frequently encountered within both veterinary and human medicine. Since the terminology for this disease is not consistent in human and veterinary literature, it has become usual to use the term “melanoma” for all malignant melanocytic tumors, whereas “melanocytoma” refers to the benign forms [1]. These neoplasms are common in dogs and in gray or white horses; the incidence is occasional in the duroc and Sinclair miniature swine by genetic predisposition, and they are less frequent in cats and sheep. Although the congenital form is well known, melanomas are also common in Suffolk sheep and angora goats of different ages [1]. Cutaneous melanomas are the most frequently occurring tumors in horses, particularly in gray-skinned Camargo breeds in France. In these horses, it is known that the ratio of horsed bearing melanomas was in the magnitude of 80% at ages-15 years [2], melanocytic tumors are rare in cattle [1,3,4] and usually account for 5% to 6% of all tumors in this species [1,3] especially in the Aberdeen angus breeds [5,6]. Some melanocytic tumors are congenital [1,6,7] or occur in cattle younger than two years old [6-8] especially those of red, gray or black skin [1,9]. The tumors may also be found on the jaw [8,10] maxilla [11], trunk, limbs [11] and less frequently in the interdigital regions [4] and in the eyes [11]. Equine skin tumors are melanocytic in up to 15% of all skin tumors in horses. The vast majority appear in grey or white horses, at

or before the age of 5, corresponding to the period in their lives when their coat changes color [12-14]. Cutaneous squamous cell carcinoma (SCC) is the second most common form of skin cancer, after basal cell carcinoma (BCC). the incidence of SCC has increased considerably over the past 20 years, and epidemiological studies predict that it will increase even further in the coming decade [13,15]. Furthermore, the S-100 protein family contains a large number of related calcium-binding proteins, and are largely found in skin melanocytes and immunoreactivity for such proteins have been carried out as a diagnostic criterion for melanoma in human, dog, rat, mouse, guinea pig [16]. However, S-100 protein immunoreactivity in mammalian tissues is not restricted to melanocytes; hence various tissues such as nervous, lymphoid, endocrine and epithelial may also be S-100

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positive [17,18]. Intracytoplasmic hmb45 ag is an embryonic protein into the immature melanosome [19]. Additionally, the S-100 protein and hmb-45 ag tests confirmed the positive reactions of melanocytic tumors [20,21]. Assessment of both S-100 and Melan-A (MART1) gene expression is beneficial to confirm the diagnosis of canine melanoma, and Melan-A (MART1) gene may be particularly informative regarding biologic behavior of these tumors.

The aim of this study was to compare the role of special staining and to test melanoma associated antigens against paraffin embedded bovine and equine melanomas in order to assess their ability and utility as diagnostic tools for diagnosis and confirmation of the melanomas.

## Materials and Methods

### Animal data

The cases were retrieved from archives for the period 1961 to 2002 of the Research Unit, Department of Pathology, and Collage Veterinary Medicine. All cases were reviewed histologically, and each case was found to contain a large focus of melanoma and SCC on the hematoxylin and eosin-stained tissue section. All cases had documented cutaneous melanoma and SCC. Serial 5  $\mu$ m-thick sections were cut from routine formalin-fixed paraffin blocks of 4 case cutaneous SCC (including 2 cases from horses and 2 cases from cattle) and 9 case cutaneous melanomas (including 6 cases from horses and 3 cases from cattle) from the files of the Department of Pathology at the Collage Veterinary Medicine of Tehran University, Tehran, Iran, have been diagnosed by H&E staining method. For diagnosis confirmation, Fontana-masson silver method, melanin bleaching method and immunohistochemical staining for S-100 protein and HMB45 antigen were applied.

### The FMS Procedure

Tissue sections were deparaffinized and hydrated with the conventional protocol. Then they were incubated in an FM working solution (see solution 1 below for composition) in a microwave for 3 cycles of 50 sec at maximum power. Each of the successive steps was followed by three washes in distilled water. The sections were differentiated in 2% gold chloride for 5 min (ref: 481130 from Sigma-Aldrich, Steinheim, Germany), and the fixation of silver was carried out with 2% sodium thiosulfate (ref: HT1005; Sigma-Aldrich) for 1 min. The FM staining (see solution 2 below for composition) was performed for 30 min at room temperature [22]. The nuclear contrast was performed with Harris hematoxylin (ref: 253 949, from Panreac, Barcelona, Spain) for 3 min. The sections were dehydrated, cleared in xylol, and mounted in hydrophobic medium. Solution Fontana-Masson, Stock solution: 1)10% silver nitrate (ref: 131459; Panreac) 95 ml. Add ammonium hydroxide (ref: 131130; Panreac) drop by drop, until the solution FM and clears again. 2)10% silver nitrate 1 ml. The solution will become slightly cloudy; allow undisturbed for 4 to 24 hr. Work solution: 1)Stock solution 2.5 ml. 2)Distilled water 47.5 m

### Histologic and immunohistochemical examination

Tissues were fixed in formalin, embedded in paraffin, and 5- $\mu$ m thick sections were applied to glass microscopic slides and stained with hematoxylin and eosin. For melanin bleaching, sections were deparaffinized, hydrated, and incubated in 0.25% potassium permanganate (Sigma) for 30 min. After washing in water, sections were treated with 5% oxalic acid (Sigma) for 2-5 min until sections appeared clear, then washed again with water.

Immunohistochemical techniques were performed by Yu

et al. [23] as described previously. Sections were subjected to immunohistochemical analyses with antibodies to S-100 (rabbit anti bovine S-100 (S-100a, N1519 and S-100 Z0311; Dako), HMB-45 (Ag used Melanosome clone HMB-45, code: M0634 mouse (Dako). Immunocytochemical analysis was performed using standard protocols. Briefly, 5- $\mu$ m sections were cut, placed on electrostatically charged glass slides, and deparaffinized. Staining for S-100 protein, HMB-45 antigen antibodies was performed using the Ventana ES automated immunohistochemistry system and the Ventana DAB Detection Kit (Ventana Medical Systems, Tucson, AZ). Both 2 antibodies were prediluted and incubated with the tissue section for 1 hour at room temperature. Counterstaining was performed with hematoxylin. Appropriate positive and negative controls were used; the latter was achieved by omitting the antibodies. All cases were examined independently and blindly by 2 observers (D.C.C.; S-100 protein and HMB-45 by C.C.D., H.Y). Moreover, slides were rinsed with tris-buffered saline and incubated with normal saline 1% serum in 0.5% bovine serum albumin in TBS for 20 minutes at room temperature. The primary antibody (rabbit anti bovine S-100 (S-100a, N1519 and S-100 Z0311; Dako), was diluted 1:300 in 0.5% bovine serum albumin in TBS and applied to sections for half an hour incubation at room temperature, then were washed in TBS (for 10 minutes) and incubated with biotinylated link Ab (15 minutes Dako LSAB 676), washed in TBS and incubated with alkaline phosphatase labeled streptavidin (10 minutes). Applied substrate-chromogen solution (newfucsin), For HMB-45 Ag used Melanosome clone HMB-45, code: M0634 mouse (Dako). The sections were counterstained with hematoxylin.

For statistical analysis, t-test was performed to analyze the significant differences between groups and a statistically significant difference was considered at the level of  $P < 0.05$ .

## Results

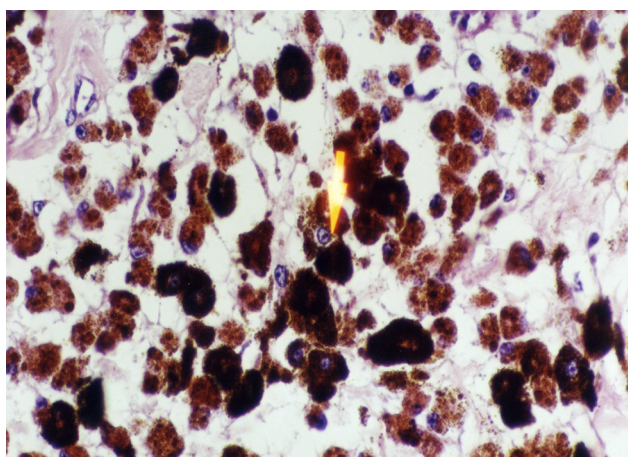
Results are summarized in Table 1. Macroscopically, tumor masses were polypoid, sessile nodules or lobulated with the range of 0.5-12 cm in gray or brown to black colors. In histological features, the cells were small spindle and epithelioid shape with intra cytoplasmic melanin granules and variable amount of collagen fibers were present. There were little nuclear or cellular pleomorphism (Figures 1 and 2). The horses were between 1.5 to 14 years of age and the cattle between 2 to 10 years of age. Regarding the sites of tumors were more visible on legs, trunk, perineum and tail. The majority of cases developed intradermal histologic patterns. Both S-100 protein and HMB45 staining's were positive for 6 cases of 9 tumors (Figures 3 and 4), but in 3 horses (3, 4 and 6 numbers), there were suspicious results. In all 9 cases, the results of Fontana-masson silver and melanin bleaching methods were positive. In 4 cases of squamous cell carcinoma, the results of 3 tests were negative (Table 1).

## Discussion

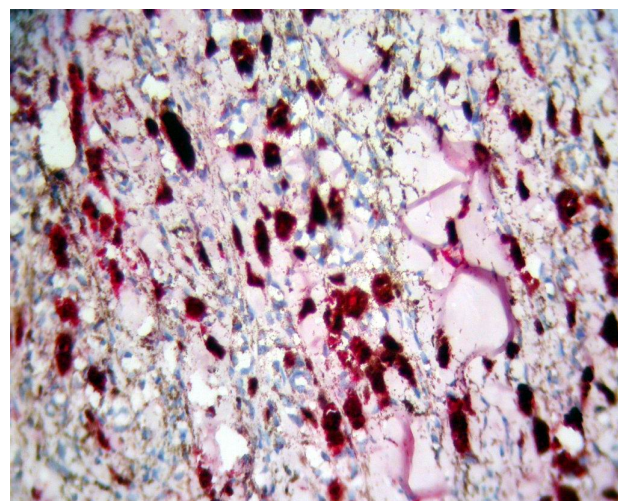
Obviously, up to 15% of all equine skin tumors are melanocytic. Initially, more than 90% of these tumors are benign, whereas approximately two-thirds are thought to progress toward malignancy and metastasis [1]. Cattle develop melanocytoma infrequently [24]. The last two cases in Table 1 were observed from 2 cows during a three-month period in which bottle shaped tumors were about 12 cm in diameter. In 98% of malignant melanomas, expression of S-100 protein takes place. On the other hand, S-100 protein has a remarkable role in melanocytic tumors diagnosis, especially for differential diagnosis of melanomas, and other tissues do not react with such protein. The 2 antibodies used most frequently in the clinical setting to confirm the

Case No	Species	H&E	Fontana-Masson	Bleaching method	S-100 protein	HMB-45	Tumor location	Benign/Malignant
1	Horse	Melanocytoma	+	+	+	+	Dermal	Benign
2	Horse	Melanocytoma	+	+	+	+	Dermal	Benign
3	Horse	Melanocytoma	+	+	±	±	Dermal	Benign
4	Horse	Melanocytoma	+	+	±	±	Dermal	Benign
5	Horse	Melanocytoma	+	+	+	+	Dermal	Benign
6	Horse	Melanoma	+	+	±	±	±	Malignant
7	Cattle	Melanocytoma	+	+	+	+	Dermal	Benign
8	Cattle	Melanocytoma	+	+	+	+	Dermal	Benign
9	Cattle	Melanocytoma	+	+	+	+	Dermal	Benign
10	Horse	SCC	-	-	-	-	-	Malignant
11	Horse	SCC	-	-	-	-	-	Malignant
12	Cattle	SCC	-	-	-	-	-	Malignant
13	Cattle	SCC	-	-	-	-	-	Malignant

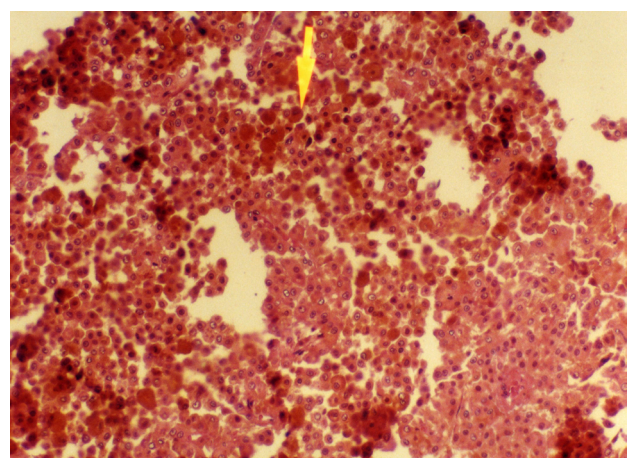
**Table 1:** Comparative results of melanocytic tumors by H&E, special staining and Immunohistochemical methods.



**Figure 1:** Melanocytoma, melanocytes contained different amount of melanin pigments. Cattle ( H&E ,400X).



**Figure 3:** Positive nuclear and cytoplasmic immunoreactivity for S-100 protein in melanocytoma (LSAB-Alkaline Phosphatase) (400X).

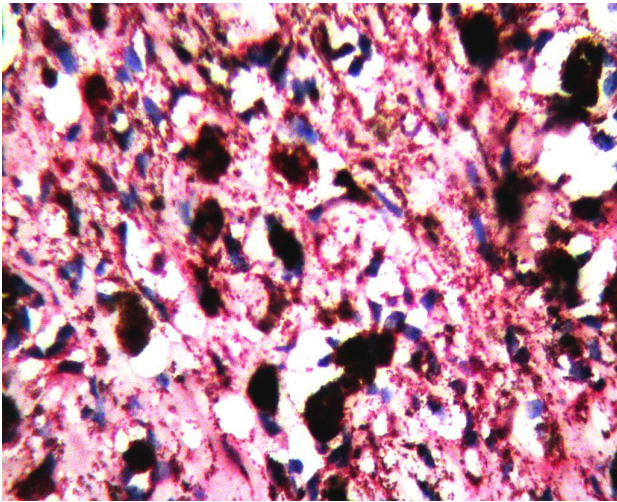


**Figure 2:** Round shaped cells with round nucleus and low melanin pigments in melanocytoma (arrow). Horse ( H&E 400X).

anti-S-100 protein often reacts with a host of non-melanocytic tumors that may share cytomorphologic features with malignant melanomas. [25,26].

HMB-45 is a more specific marker for melanoma than S-100 protein. Our experience supports this contention; the specificity of HMB-45 for detecting melanoma was 96.9% and, thus, considerably higher than that of S-100 protein. However, HMB-45 is detectable in only 50% to 75% of all melanomas [27,28]. Therefore, it is less sensitive as a marker for melanoma than S-100 protein. In addition, spindle cell and desmoplastic melanomas tend to be nonreactive with anti-HMB-45 [29,30]. One diagnostic challenge is to differentiate spindle cell and desmoplastic melanomas from other spindle cell lesions. The former frequently exhibits positive staining with S-100 protein but tends to be negative for HMB-45 [29,30]. However, as previously noted, positive S-100 protein immunostaining is of limited value because many non-melanocytic spindle cell lesions, including Schwannomas, neurofibromas, and malignant peripheral nerve sheath tumors, also may be immunoreactive [17,30]. The tumor masses contained different amounts of melanin pigment, from low to high degree. The reason for choosing SCC tumor as control sample was that the tumor is one of the differential diagnosis lesions for melanoma. Approximately, the

cytological diagnosis of melanoma are those directed against epitopes on S-100 protein and HMB-45. S-100 protein is a sensitive marker for melanomas, including amelanotic and spindle cell variants. However,



**Figure 4:** Positive cytoplasmic immunoreactivity for HMB-45 Ag in melanocytoma (LSAB –Alkaline Phosphatase) (400X).

same as other researcher's findings all cases were diagnosed as benign tumors [31-33].

Most of tumors histologically represented intradermal pattern with no epidermal component. Dermal melanocytomas might be less cellular. Neoplastic cells are often small spindle cells with melanin granules. Melanin granules are often difficult to identify within the cytoplasm of large round cell of melanocytoma, but will stain positive with the Fontana-Masson staining for melanin [24]. In addition, 3 melanocytoma cases indicated doubtful results in immunohistochemical method. The expression of S-100 protein and HMB-45, maybe low and immunoreactivity will be weakened due to Ag degradation in long time storage of the paraffin blocks. However, improper formalin fixation [34] and poor tissue processing, may affect the test results. Thus, DOPA (dihydroxyphenylalanine) test was not used since it requires fresh tissue and is more applicable to non-pigmented melanomas [1]. Overall, it is essential to mention that HMB-45, a monoclonal Ab, is capable of immunohistochemically staining that routinely fixed human melanomas, and may provide an efficient immunohistochemical staining for improved diagnosis of equine and bovine melanocytic tumors [34] and other animals [35-37].

The standard of practice in diagnosing melanoma is to use a panel of antibodies consisting primarily of S-100 protein and HMB-45. For spindle cell and desmoplastic melanomas, however, S-100 protein and HMB-45 should remain within the antibody panel since S-100 protein is the most sensitive marker for these entities and evidence is lacking that either Melan-A or Mitf are better markers than HMB-45 for recognizing spindle cell melanocytic lesions.

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