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NOTE

Pathology

Cutaneous malignant melanoma in two rabbits (*Oryctolagus cuniculus*)

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ABSTRACT. Despite being rarely reported, improved diagnostic and prognostic indicators are necessary for treating malignant melanoma in rabbits. In this study, two cases of primary skin lesions, on the scrotum and on eyelid, with systemic metastases, were examined. The tumors formed intra-dermally by sheet-like proliferation of polymorphic cells, with anisocytosis and varying amount of melanin granules. Tumors had displaced almost 50% of the lung and liver tissue, and tumor metastasis was the cause of early death in both rabbits. Ki-67-positive population was high in both, and it was found to be useful in assessing the outcome and malignancy. In addition, Melan-A, HMB-45, PNL2 and S100 established a useful immunohistochemical panel for the diagnosis of melanocytic tumor in rabbits.

KEY WORDS: Ki-67, melanoma, rabbit, skin

The types of skin tumors reported in rabbits (*Oryctolagus cuniculus*) include myxomatosis, fibroma, fibrosarcoma, papilloma, basal cell carcinoma, squamous cell carcinoma, sebaceous gland carcinoma, and lymphoma; melanocytic tumors are extremely rare, with only 10 cases reported to date [7, 8, 17, 18]. In a previous analysis with 190 different tumors in rabbits [17], malignant melanoma accounted for only eight cases (4.2%). Out of the five cases of malignant melanoma, whose outcome information (including euthanasia) were available, metastasis was confirmed in two, indicating high malignancy of these tumors [7, 17, 18]. However, owing to the limited number of cases reported in rabbits, a comprehensive understanding of the occurrence site, pathological characteristics, immunological phenotype, mitotic index, and proportion of Ki-67-positive cells associated with clinical outcome is lacking. Moreover, there is no report on the investigation of multiple melanocyte markers, used in humans, with respect to rabbits. In the present study, we analyzed the clinical and pathological characteristics of two cases of rabbit cutaneous malignant melanoma, affecting different sites. Additionally, we investigated the efficacy of Ki-67 as a marker for predicting malignancy of melanoma, and the cross-reactivity of four human melanocyte markers in rabbits.

Case 1 was a mixed-breed, intact, four-year-old male rabbit weighing 1.2 kg. The rabbit was brought to our clinic with anorexia, associated with molar malocclusion, as the primary complaint. A general physical examination revealed molar malocclusion and an irregular black mass (4 × 3 × 2 cm) on the skin of scrotum, with ulceration and bleeding (Fig. 1A). Chest radiography revealed multiple radio-opaque lesions, suggestive of metastasis in the lungs. In accordance with the owner's approval, we resected the scrotal tumor including orchiectomy. The perioperative period concluded with no problems. Despite subsequent clinical progress, the rabbit died after two weeks.

Case 2 was a mixed-breed, intact, ten-year-old male rabbit weighing 1.4 kg. The rabbit was brought to our clinic with an enlarged mass (1 × 1 × 1 cm) on the right eyelid as the primary complaint; this was first observed approximately nine months earlier. A partially ulcerated mass was confirmed near the internal canthus of the right eye. Despite local swelling and bleeding from the ulcer, the rabbit was in good general health. Radiography did not reveal any marked abnormality; hence, we followed up without performing proactive treatment. However, the tumor eventually grew in size (to 2 × 2 × 1 cm) (Fig. 1B), and the rabbit died 11 months after the tumor was first identified by the owners, and two months after being brought to our clinic.

Grossly and microscopically, metastasis was observed in the lungs, liver, spleen, and kidneys in both cases (Fig. 1C). This was particularly prominent in the lungs and liver, and the tumor had displaced around 50% of the lung and liver tissue in both the rabbits (Fig. 1C). The primary cutaneous mass on the scrotum of case 1 was composed of two distinct neighboring regions below the ulcerated epidermis: one with a solid growth of giant polymorphic epithelioid tumor cells and another with a solid growth of spindle cells (Supplementary Fig. 1A). The tumor cells had highly atypical nuclei and an abundant eosinophilic cytoplasm; there were many mitotic figures (mitotic index: 20 cells/400×, 10 fields) (Fig. 1D), and multinucleated giant cells were also frequently observed (Supplementary Fig. 1B). Approximately 20% of the cells contained melanin granules. In addition, the tumor cells

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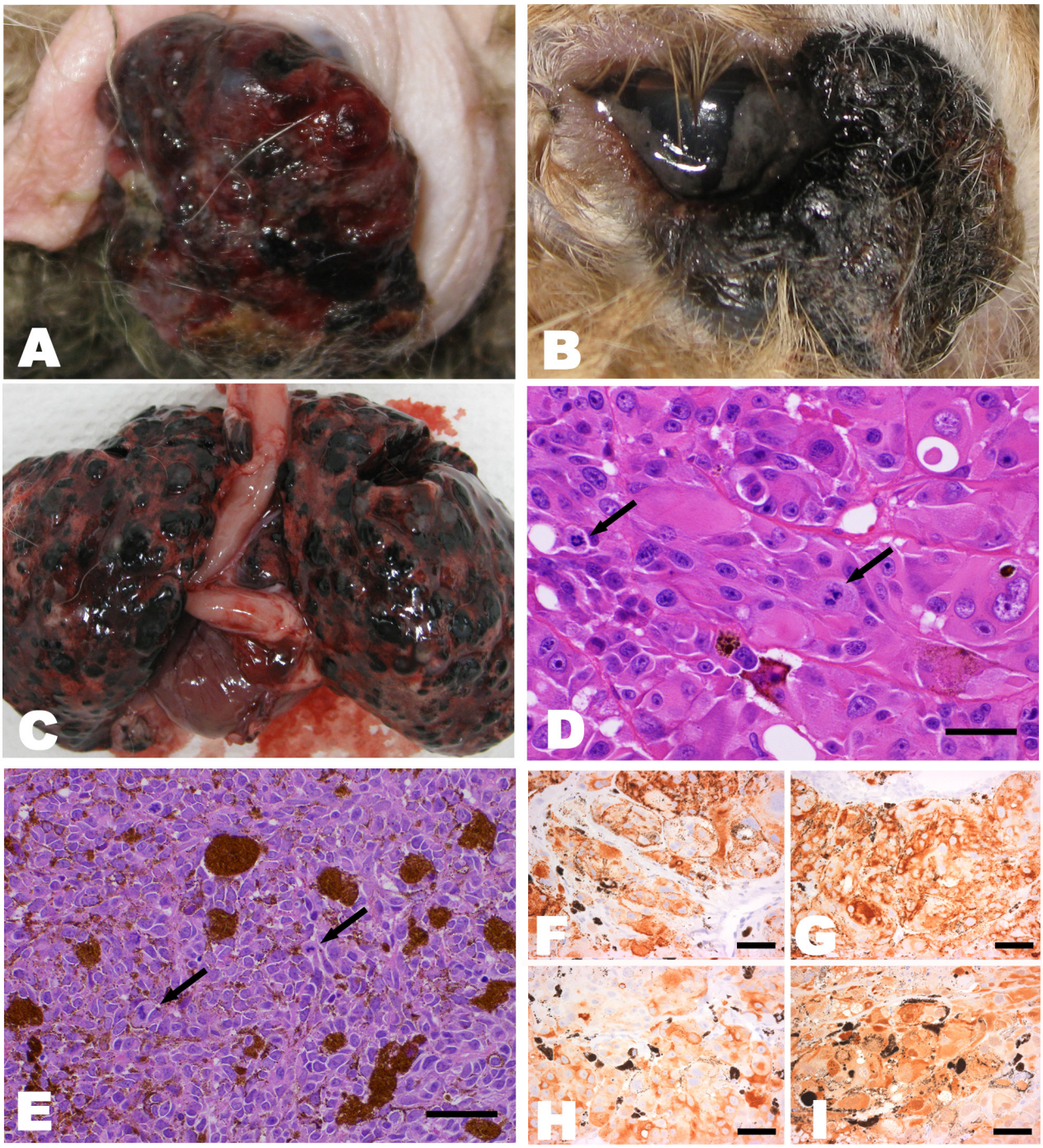

Fig. 1. (A) Case 1, scrotum: An irregular black mass with hemorrhage and ulceration is observed in the scrotal skin. (B) Case 2, eyelid: An irregular black mass with ulceration is seen in the medial angle of the right eyelid. (C) Case 1, lung: Multiple black nodular lesions are visible throughout the lungs. (D) Case 1, scrotum: Giant polymorphic epithelioid tumor cells show highly atypical nuclei of varying sizes and abundant eosinophilic cytoplasm. Some cells contain melanin granules. Mitotic figures are also seen (arrows) by hematoxylin and eosin staining. Bar, 50 μm. (E) Case 2, eyelid: Tumor cells have oval nuclei of varying sizes, small-to-moderate amounts of cytoplasm, and large amounts of melanin. Mitotic figures are also seen (arrows) by hematoxylin and eosin staining. Bar, 50 μm. (F) Case 1: Immunohistochemical staining for HMB-45. The cytoplasm of the tumor cells is stained light brown. The black granules are melanin. Bar, 50 μm. (G) Case 1: Immunohistochemical staining for PNL2. The cytoplasm of the tumor cells is stained light brown. The black granules are melanin. Bar, 50 μm. (H) Case 1: Immunohistochemical staining for MelanA. The cytoplasm of the tumor cells is stained light brown. The black granules are melanin. Bar, 50 μm. (I) Case 1: Immunohistochemical staining for S100. The cytoplasm of the tumor cells is stained light brown. The black granules are melanin. Bar, 50 μm.

frequently invaded blood vessels. The primary cutaneous mass on the eyelid of case 2 comprised of a solid proliferation of plump spindle-to-oval cells with mild-to-moderate anisocytosis and mild nuclear pleomorphism, along with scattered mitotic figures (12 cells/400×, 10 fields) (Fig. 1E). Approximately 80% of the cells contained melanin granules.

Immunohistochemical staining, using a labeled-polymer method, was carried out with N-Histofine MAX PO (M or R) (Nichirei Biosciences, Tokyo, Japan). The sections were deparaffinized in xylene and rehydrated through graded ethanol at room temperature. Rehydrated sections were immersed in citrate buffer (pH 6.0) and autoclaved to retrieve the antigens. Solutions and washes were prepared between the various steps, using 0.05 M Tris-buffered saline (TBS, pH 7.6) with 0.01% Tween 20 (TBST). Nonspecific endogenous peroxidase activity was blocked by exposure to 0.03% hydrogen peroxidase in 100% methanol for 5 min, and then masked with 5% goat serum albumin in phosphate-buffered saline for 5 min at room temperature. Incubation was carried out overnight at 4°C using the respective primary antibodies: anti-Melan A (1:50, mouse monoclonal antibody; DAKO, Glostrup, Denmark), anti-human melanosome HMB45 (1:10, mouse monoclonal antibody; DAKO), anti-PNL2 (ready for use, mouse monoclonal antibody; DAKO), anti-cow S100 (1:320, rabbit polyclonal antibody; DAKO), and anti-human Ki-67 (MIB-1) (1:1,100, mouse monoclonal antibody; DAKO) [5]. The Ki-67-positivity index was estimated as the percentage of Ki-67-labeled nuclei per 1,000 tumor cells. Rabbit skin melanocytes were used as positive control. As a negative control, mouse or rabbit isotype immunoglobulin, diluted to the same concentration, was substituted for the primary antibody. The tumor cells and normal melanocytes were positive for a number of melanocyte markers (HMB-45, PNL2, Melan A, and S100) (Fig. 1F). An appreciable proportion of cells (case 1, 60%; case 2, 40%) was found to be Ki-67 positive.

There are only 10 reported cases till date, of malignant melanoma in rabbits: seven with lymphatic vessel invasion and two with metastases to distant sites. Five cases resulted in malignant melanoma-related death (including euthanasia), suggesting that the melanomas in rabbits were highly malignant [7, 17, 18]. Consistent with these previous reports, both the rabbits in the present study died due to metastasis [7, 17, 18]. In veterinary medicine, malignant melanoma occurs frequently in dogs, most commonly in the oral cavity, followed by the skin [15]. The site of occurrence is considered a crucial determinant of the clinical outcome. Tumors in the oral cavity are highly malignant and frequently metastasize to the lymph nodes or lungs. On the contrary, tumors that occur in the skin of the extremities or limbs are considered to be less malignant [15]. In cats, malignant melanomas are most commonly detected in the eyes and are reported to metastasize frequently [10]. In rabbits, malignant melanomas tend to occur in the auricle, eyelid, head, extremities, or groin; metastasis and death are associated with tumors occurring on the head and groin [7, 17, 18]. In the present study, malignant melanomas were observed in the skin of the scrotum and eyelid; in both cases, the tumors metastasized, resulting in death. Based on the cases presented here and those in the previous reports, cutaneous malignant melanomas in rabbits are found to be much more malignant than those in dogs and cats.

The ratio of Ki-67-positive cells is frequently used as a marker to objectively differentiate malignancy in canine malignant melanoma. This ratio has higher values in highly malignant melanoma than in melanocytoma or in low-grade malignant melanoma [1, 9, 12, 13]. In canine melanoma, if Ki-67-positive ratio exceeds 12.35–19.5%, the one-year survival rate is significantly reduced [1, 9]. In both the cases reported here, this value was higher than the thresholds for diagnosing high-grade malignancy in dogs. Both the rabbits in the present study had systemic organ metastasis and died as a result of their tumors. The results of this study suggest that Ki-67 positivity may be an indicator of cutaneous melanoma malignancy in rabbits; however, it is necessary to continue investigations on more cases.

In the present study, it was found that the four melanocyte markers used in humans, Melan-A (MART-1), HMB-45, S100 and PNL2, can also be used in rabbits. Malignant melanoma is often difficult to distinguish from soft tissue sarcoma, when the pigmentation level is low. Definitive diagnoses have been earlier established using the Fontana–Masson stain for melanin and electron microscopy for melanosomes. However, the Fontana–Masson stain is sometimes unable to detect malignant melanomas [18], while an electron microscope is too inconvenient and expensive. To overcome these issues, immunostaining for the melanocyte markers Melan-A (MART-1), HMB-45, microphthalmia-associated transcription factor, tyrosinase, neuron-specific enolase, S100 and PNL2 is being widely used in humans, dogs, and cats [2–4, 6, 11, 13, 14, 16]. Amelanotic melanoma, which is not detectable using the Fontana–Masson stain, has also been confirmed in rabbits based on positive staining for S100 and MART-1 [18]. However, since none of these markers has 100% specificity, higher diagnostic accuracy would require the use of multiple markers. The use of these markers for the differential diagnosis of malignant melanoma may improve clinical diagnostic accuracy.

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