J Toxicol Pathol 2013; 26: 309–312

Case Report

Spontaneous Extraskeletal Osteosarcoma in a Rabbit (*Oryctolagus cuniculus*): Histopathological and Immunohistochemical Findings

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Abstract: A spontaneously occurring subcutaneous mass in the left forelimb of a nine-year-old rabbit (*Oryctolagus cuniculus*) was examined histopathologically and immunohistochemically. Clinically, edema and hemorrhage were seen around the mass. No connection of the tumor mass to the appendicular skeleton was found. The tumor was arranged in a solid growth pattern and irregular bundles, and neoplastic cells were polygonal to spindle-shape. Osteoid (positive for osteocalcin) and multinucleated giant cells were diffusely or focally seen. Neoplastic cells were positive for vimentin, osterix and Ki-67, indicating the nature of osteoblasts with proliferating activity, but negative for α-smooth muscle actin, desmin or CD204. Based on these findings, a diagnosis of extraskeletal osteosarcoma was made, a very rare tumor both in laboratory and pet rabbits. (DOI: 10.1293/tox.26.309; J Toxicol Pathol 2013; 26: 309–312)

Key words: rabbit, spontaneous tumor, extraskeletal osteosarcoma, immunohistochemistry

Osteosarcoma is the primary malignant tumor derived from bone tissue; therefore, the tumor is characterized by osteoid and immature bone formed by neoplastic osteoblasts[1]. Osteosarcomas vary greatly in the amount and quality of matrix and in histological patterns[1]. Canine osteosarcoma accounts for 85–98% of all canine bone tumors[2]. In humans, osteosarcoma is the most common primary solid bone tumor in childhood and adolescence[3,4]. Extraskeletal osteosarcoma (ESOS) is a rare malignant mesenchymal neoplasm, which is never attached to the skeleton, although the histopathological characteristics such as osteoid and bone formation are similar to those of bone-derived osteosarcomas[5,6]. ESOS has been reported in dogs, cats, hamsters, a rat, a hedgehog, a maned wolf and a goat as an infrequent tumor[7–12].

In aged rabbits, uterine adenocarcinoma is the most common spontaneous tumor followed by lymphomas[13,14]. A few cases of spontaneously occurring osteosarcomas have been reported in rabbits[13,15]; out of them, to our knowledge, only one case was diagnosed as an ESOS arising in the upper lip[16]. Because of the rarity of this tumor type and its necessity for pet and laboratory animals, we herein report the detailed histopathological and immunohistochemical characteristic of a case of rabbit ESOS.

A nine-year-old male rabbit (*Oryctolagus cuniculus)* (1.8 kg body weight, mixed, colored) was brought to a private animal hospital with a complaint of hemorrhage and edema around the dewclaw of the left forelimb. A subcutaneous solid mass, 3.5 cm × 1.5 cm × 1.5 cm, was surgically removed. The cut surface of the formalin-fixed mass appeared grayish white and pink color (Fig. 1A). The mass was freely mobile without attachment to the skeletal system. Clinical and radiological examinations also revealed no connection to the bone or abnormality in other organs. Two months after the surgical resection, the rabbit was readmitted with an ulcerative mass at the resection site. The recurred mass resembled the primary mass in terms of gross morphology.

The subcutaneous masses were fixed in 10% neutral buffered formalin, embedded in paraffin and sectioned at 3–5 μm. Besides hematoxylin and eosin (HE) staining, the Periodic acid-Schiff (PAS), von Kossa and azan-Mallory methods were performed for histopathology. Immunohistochemical labeling was performed with peroxidase conjugated secondary antibody (Histofine Simple Stain MAX PO®; Nichirei Inc., Tokyo, Japan). Primary antibodies used were osteocalcin (clone, OC4-30; 1:500; GeneTex, CA, USA), vimentin (clone, V9; 1:500; Dako, Denmark), desmin (clone, D33; 1:200; Dako, Denmark), CD204 (clone, SRA-E5; 1:200; TransGenic, Japan), Ki-67 (clone, MIB-1; 1:200; Dako, Denmark), *α*-smooth muscle actin (*α*-SMA) (clone, 1A4; 1:1000; Dako, Denmark), S-100 (1:200; Dako, Denmark), and osterix (1:200; Abcam, UK). Positive reac-

Received: 21 March 2013, Accepted: 4 April 2013
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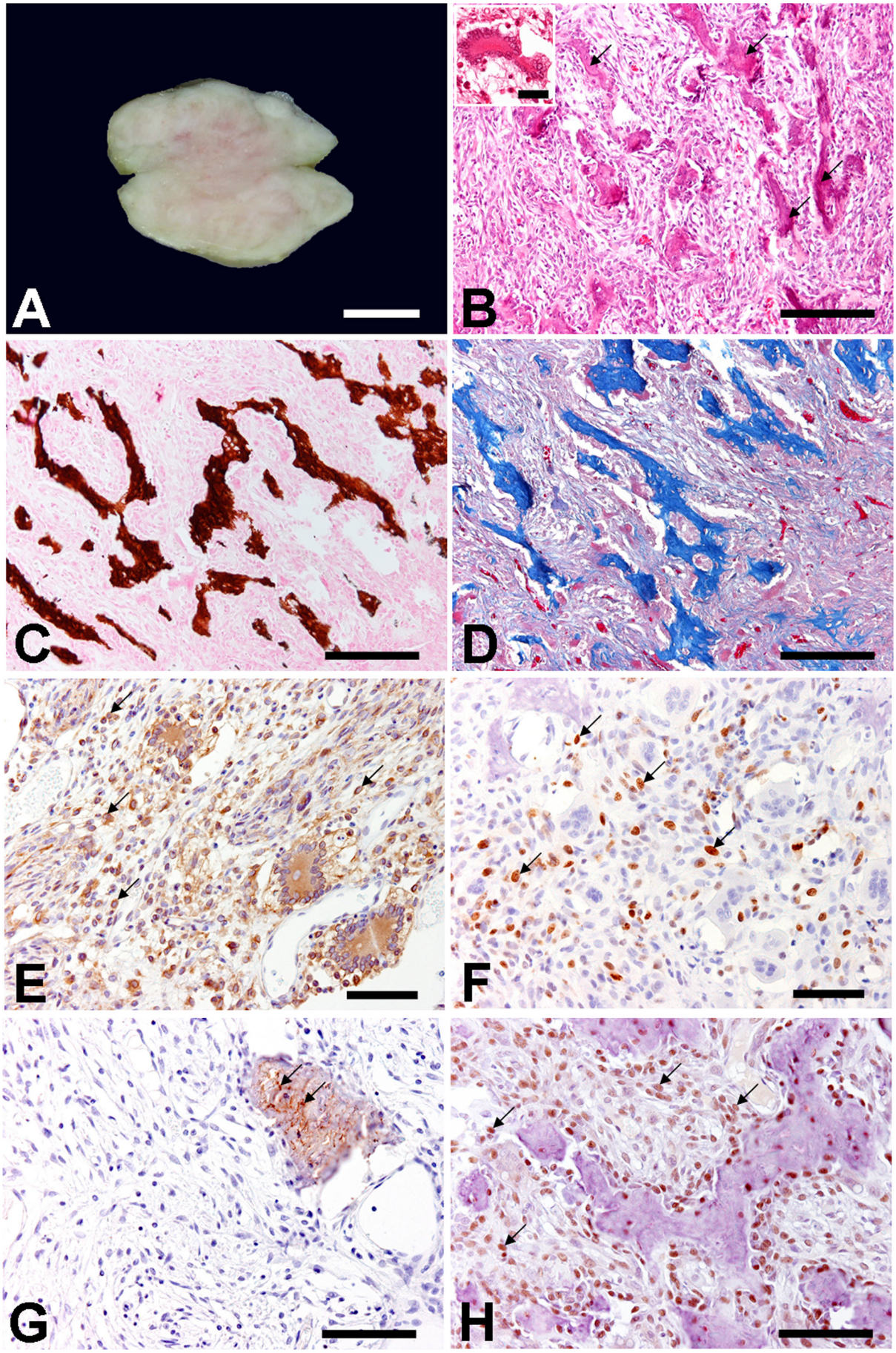
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Fig. 1. Gross, histopathological and immunohistochemical findings of an extraskeletal osteosarcoma in a rabbit. (A) Gross finding of the formalin-fixed subcutaneous tumor; the cut surface appears solid and grayish white to pink in color. Bar = 1 cm. (B) The tumor is composed of polygonal to spindle-shaped neoplastic cells with areas of osseous formation (arrows). Inset: representative multinucleated giant cells. HE stain. Bar = 200 μm. (C) The osseous matrix is stained black by the von Kossa stain, indicating the presence of calcium salt. Bar = 200 μm. (D) Osseous matrix is stained blue by the azan-Mallory stain. Bar = 200 μm. (E) Cytoplasm of neoplastic cells (arrows) and multinucleated giant cells is positive for vimentin. Immunohistochemistry, counterstained with hematoxylin. Bar = 200 μm. (F) Nuclei of many neoplastic cells (arrows) show strong positivity for Ki-67. Immunohistochemistry, counterstained with hematoxylin. Bar = 200 μm. (G) The osseous matrix (arrows) reacts positively with the anti-osteocalcin antibody. Immunohistochemistry, counterstained with hematoxylin. Bar = 200 μm. (H) Nuclei of neoplastic cells (arrows), particularly within osseous tissues, show a positive reaction to osterix, indicative of the nature of osteoblasts. Immunohistochemistry, counterstained with hematoxylin. Bar = 200 μm.

tions were visualized with 3,3'-diaminobenzidine (DAB; Vector Laboratories, Inc., Burlingame, CA, USA). Sections were lightly counterstained with hematoxylin. For negative controls, tissue sections were treated with mouse or rabbit nonimmune serum instead of the primary antibody. Some normal rabbit tissues were used as positive controls.

On histopathological examination, the tumor was composed of solid growth or irregular bundles of polygonal to spindle-shaped neoplastic cells including areas of osteoid and osseous formation (Fig. 1B). Tumor cells had oval to elongated hyperchromatic nuclei with marked pleomorphism. Mitotic figures were frequently present. Multinucleated giant cells were focally or sporadically observed (Fig. 1B; inset). The osseous matrix was stained black and blue by the von Kossa and azan-Mallory methods, respectively (Figs. 1C and 1D). The PAS reaction for glycogen and mucinous matrix showed negative results. Histopathological findings of the recurred mass were similar to those of the primary tumor.

Immunohistochemically, neoplastic cells and multinucleated cells showed a positive reaction for vimentin (Fig. 1E). Neoplastic cells were strongly positive for Ki-67 in the nuclei (Fig. 1F). There were no neoplastic cells reacting to α-SMA, desmin or S-100. The osseous matrix reacted strongly with osteocalcin antibody (Fig. 1G); osteocalcin, also known as bone gamma-carboxyglutamic acid-containing protein, is a non-collagenous protein found in bone and dentin[17], and acts as a regulating factor for osteogenesis that is produced by osteoblasts[18]. Many neoplastic cells, particularly within the osseous tissue, showed strong immunoreactivity with osterix antibody (Fig. 1H); osterix is known as a transcription factor for osteoblast differentiation, and the immunoreactivity was seen in the nuclei[5,19]. A few CD204-positive macrophages were scattered among the neoplastic cells indicative of infiltrating macrophages, whereas multinucleated giant cells did not react with the antibody.

Although osteosarcomas exhibit a variety of histological appearances, the most definitive diagnosis is based on the presence of osteoid and bone formed by neoplastic mesenchymal cells[1]. In our case, the tumor was composed mainly of polygonal and spindle-shaped vimentin-positive neoplastic cells with diffusely distributed islands of osseous material; the osseous tissues were clearly demonstrated by the von Kossa method for calcification and by osteocalcin immunohistochemistry[5]. Negative reactions for α-SMA/desmin and S-100 denied the presence of myogenic and neurogenic cells, respectively. In addition to the presence of osseous tissue, the positive reactions to osterix and Ki-67 indicated that the major component of this tumor was osteoblasts with proliferating activity[1]. Based on the clinical, gross, histopathological and immunohistochemical findings, we diagnosed this case as a spontaneous rabbit ESOS. Generally, osteosarcomas are classified histologically into poorly differentiated, osteoblastic, chondroblastic, fibroblastic, telangiectatic and giant cell types[1,20,21]. Based on the predominant findings such as spindle-shaped neoplastic cells and osterix-positive osteoblasts, the present case was regarded as an osteosarcoma with both osteoblastic and fibroblastic phenotypes, although it has been considered that the fibroblastic phenotype closely resembles the osteoblastic type except that many neoplastic cells appear polygonal or rounded in shape in the latter[20].

The occurrence of ESOS is extremely rare both in pet and laboratory rabbits. An ESOS, diagnosed as the fibroblastic phenotype, was reported on the left upper lip of a 4-year-rabbit[16]. ESOS is considered a rare occurrence in domestic animals as well; some cases of ESOSs have been recorded in dogs and cats. Usually, ESOS occur in aged dogs with a mean age of 10 to 11 years and have a poor prognosis[22,23]. In a previous case of rabbit ESOS, the tumor recurred within a week of primary surgical incision[16]. Fundamentally, ESOSs may show high recurrence and metastasis. In the present case, the tumor recurred at the same location after two months; one month later, the affected leg was amputated based on the owner's request and consent. The patient is alive without any metastasis. In human ESOSs, mechanical injury and irradiation have been considered as possible remote causes[24]. In dogs, ESOSs have been reported in association with parasitic infections and retained surgical sponge[25,26]. Because the mesenchymal stem cells (MSCs) have the potential to differentiate into the osteoblastic lineage[27], we speculate that perivascular MSCs may have contributed to the development of ESOS in this case. The etiology of ESOSs remains elusive.

In conclusion, because of the rarity in occurrence, a spontaneous ESOS, which was encountered in an aged pet rabbit, was characterized by histopathological and immunohistochemical methods. The findings could be useful for differential diagnosis of mesenchymal tumors without connection to bone in laboratory animals.

Acknowledgment: The authors would like to thank Dr. Mika Nagaike of Ishihara Sangyo Kaisha, Ltd., Japan, who kindly provided assistance in osterix immunohistochemistry.

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