Case Report

Spontaneous extraskeletal osteosarcoma with various histological growth patterns in the abdominal wall of an ICR mouse

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Abstract: Extraskeletal osteosarcoma is extremely rare in mice. This case report demonstrates a spontaneous murine extraskeletal osteosarcoma that exhibited various histological growth patterns in an ICR mouse. At necropsy, the tumor mass was located in the abdominal wall and was 45 × 30 × 25 mm in size. Histopathologically, the tumor showed the following four growth patterns: a solid pattern of polygonal cells embedded in an osteoid eosinophilic matrix with calcification, an irregular sheet pattern of short spindle cells accompanying some eosinophilic multinucleated cells, a fascicular pattern of spindle cells and a cystic pattern lined by short spindle cells. Immunohistochemically, most of the tumor cells were positive for vimentin, proliferating cell nuclear antigen and osterix. The multinucleated cells mentioned above were desmin positive and were regarded as regenerative striated muscles but not tumor cells. Since no clear continuity with normal bone tissues was observed, the tumor was diagnosed as an "extraskeletal osteosarcoma." (DOI: 10.1293/tox.2015-0046; J Toxicol Pathol 2016; 29: 39–43)

Key words: abdominal wall, extraskeletal osteosarcoma, mouse, osterix, spontaneous tumor

Extraskeletal osteosarcoma (EO) is defined as a malignant mesenchymal tumor of soft tissue composed of neoplastic cells that recapitulate the osteoblast phenotype and synthesize bone without another mesenchymal differentiation[1]. EO is rare as compared with skeletal osteosarcoma in both humans and animals. In animals, the occurrence of spontaneous EO has been reported in rats, hamsters, dogs, a rabbit, a goat and a maned wolf[2–10], but not in mice according to a search of the relevant literature. In the online National Toxicology Program (NTP) historical control database for B6C3F1 mice, only 1 of 700 males developed EO in the skin, and none of 700 females developed EO in any organs[11]. In mouse skeletal osteosarcoma, more variable histologic features and a greater range of anaplasia than any other bone tumor, such as osteochondroma, osteoma or osteofibroma, can be assumed, and multiple subtypes have been recognized[12, 13]; they include eburnating (osteoplastic), chondroblastic, osteoclastic, anaplastic, osteoblastic, fibroblastic, telangiectatic (vascular), and compound (mixed). This case report demonstrates an extremely rare spontaneous murine EO that exhibited various histological growth patterns.

The animal was a female ICR [Crlj:CD1(ICR)] mouse (Charles River Laboratories Japan Inc., Kanagawa, Japan) allocated to a low-dose group in a feeding carcinogenicity study for a period of 18 months. In the study, no similar tumors, including skeletal osteosarcoma, were observed in any other mice, and the present tumor was considered to have occurred spontaneously. The mouse was housed in a barrier-sustained animal room controlled at a temperature of 22 ± 2°C and humidity of 50 ± 20% with ventilation 10 times or more per hour (all-fresh-air basis) and illumination 12 hours per day (light on at 7:00 a.m. and off at 7:00 p.m.). Diet and tap water were provided to the animal *ad libitum*. A certified diet MF Mash (Oriental Yeast Co., Ltd., Tokyo, Japan) was used as the basal diet. The study was conducted in accordance with the "Act on Welfare and Management of Animals" (Act No. 105, 1973), "Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain" (Notice No. 88 of the Ministry of Environment, 2006) and "Basic Guidelines for Animal Experimentation in the Research Laboratories under the Jurisdiction of the Ministry of Agriculture, Forestry and Fisheries" (18-Nou-kai-No.307, 2006).

In the female mouse, a right caudal abdominal mass was palpated clinically at 68 weeks of treatment (73 weeks of age). The mass gradually increased in size to 30 × 15 × 10 mm until 72 weeks of treatment. Emaciation was observed from 70 weeks of treatment, and the mouse showed a moribund condition, including soiled fur in the external genital region, pale-colored skin of the whole body, bradypnea, decreased spontaneous motor activity, and dark-colored eyes,

from 71 weeks of treatment. At 72 weeks of treatment (77 weeks of age), abdominal distention caused by the mass was observed, and the mouse was deeply anesthetized with isoflurane and then euthanized by exsanguination.

At necropsy, the abdominal mass located in the right abdominal wall was white in color and 45 × 30 × 25 mm in size (Fig. 1A). No clear continuity with the ribs, vertebrae or right hind limb was observed. The cut surface after formalin fixation was composed mainly of firm white components and contained multiple cystic spaces (Fig. 1B). No other macroscopic lesions were observed in the mouse.

The mass and systemic organs were removed from the mouse and fixed in 10% neutral-buffered formalin. Then, they were embedded in paraffin wax and sectioned, and the paraffin sections were stained with hematoxylin and eosin (HE) by a routine method. Sections of the mass were also subjected to Kossa stain and immunohistochemistry. The primary antibodies used for immunohistochemistry were as follows: mouse anti-cytokeratin (AE1/AE3, monoclonal, prediluted, Dako, Glostrup, Denmark), anti-vimentin (V9, monoclonal, 1:50, Dako), anti-smooth muscle actin (SMA) (1A4, monoclonal, 1:100, Dako) and anti-proliferating cell nuclear antigen (PCNA) (PC10, monoclonal, 1:200, Dako), and rabbit anti-osterix (polyclonal, 1:200, Abcam, Cambridge, UK), anti-desmin (polyclonal, prediluted, Dako), anti-S100 (polyclonal, 1:400, Dako), anti-glial fibrillary acidic protein (GFAP) (polyclonal, prediluted, Dako), anti-factor VIII-related antigen (polyclonal, prediluted, BioGenex Laboratories, Fremont, CA, USA) and anti-ionized calcium binding adaptor molecule 1 (Iba1) (polyclonal, 1:500, Wako Pure Chemical Industries, Osaka, Japan). Antigen retrieval was performed in 0.1 M citrate buffer (pH 6.0) using an autoclave at 121°C for 10 min except for the anti-factor VIII-related antigen antibody. For this antibody, antigen retrieval was performed by 0.4 mg/ml proteinase K (Dako) digestion at room temperature for 15 min. Following a treatment with 4% Block Ace (DS Pharma Biomedical, Osaka, Japan) at room temperature for 20 min, sections were incubated with the primary antibodies at 4°C overnight, and this was followed by secondary antibody reactions at 37°C for 30 min using EnVision+ System-HRP anti-mouse or anti-rabbit (Dako). Finally, the positive reactions were visualized with 3,3-diaminobenzidine (DAB) solution consisting of one DAB tablet (Wako Pure Chemical Industries) in 20 ml PBS and 10 μl 30% hydrogen peroxide and counterstaining with Gill's hematoxylin. Frozen sections from the formalin-fixed samples of the mass were also prepared and stained with oil red O.

In histopathological examination of the abdominal mass, a relatively well-circumscribed tumor was observed in the subcutis that developed beyond the abdominal wall to the abdominal cavity. The tumor cells showed four growth patterns in the following four regions, as shown in Fig. 2 (regions A to D). Region A was composed of polygonal cells with abundant cytoplasm that grew in a solid pattern (Fig. 2A). In the region, abundant osteoid-like eosinophilic matrix was observed, and the matrix was slightly positive
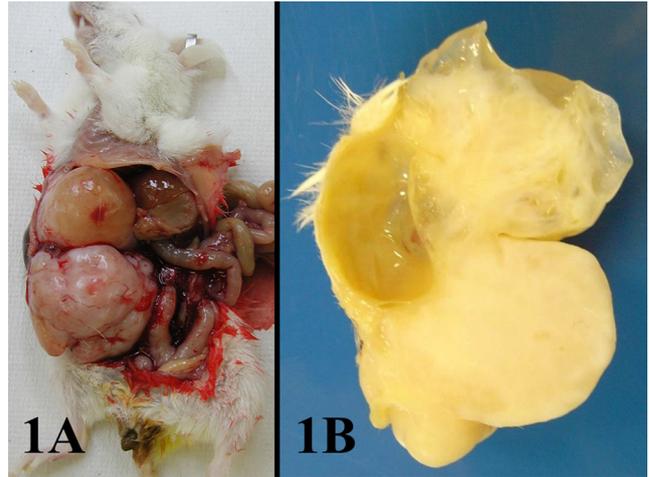
Fig. 1. A: Abdominal mass in the right abdominal wall at necropsy. It was white in color and 45 × 30 × 25 mm in size. B: Cut surface of the abdominal mass after formalin fixation. It was composed of mainly firm white components and contained multiple cystic spaces.

for Kossa stain. Some of the polygonal cells contained small vacuoles that were negative for oil red O stain. Region B was composed of irregular sheets of short spindle cells accompanying eosinophilic multinucleated cells (Fig. 2B). In the region, normal striated muscle cells were sporadically observed and associated with the eosinophilic multinucleated cells. Region C was composed of spindle cells that grew in bundles (Fig. 2C). Region D was composed of small and large cysts that were observed macroscopically (Fig. 2D). The cysts were lined by short spindle tumor cells and contained pale eosinophilic materials, erythrocytes and macrophages. The boundaries of each region were ambiguous. Marked nuclear pleomorphism and slight infiltration of inflammatory cells, such as macrophages, lymphocytes and neutrophils, were observed in all the regions. No histopathological lesions considered to be directly related to the tumor were observed in any other organs. In immunohistochemistry, the staining properties of the tumor cells in regions A to D were the same, and most of the polygonal cells in region A, short spindle cells in regions B and D and spindle cells in region C were positive for vimentin (Fig. 3A), PCNA (Fig. 3B) and osterix (Fig. 3C and 3D) and weakly positive for SMA (Fig. 3E and Table 1). These tumor cells were negative for all other antibodies examined. However, the eosinophilic multinucleated cells in region B were positive for desmin (Fig. 3F) and weakly positive for vimentin. The specificities of all antibodies were confirmed in appropriate sites of surrounding normal tissues, such as the epidermis, vascular smooth muscles and endothelial cells, peripheral nerve fibers, macrophages and cutaneous muscles, and in samples of mouse skeletal osteosarcoma.

The abdominal mass in the present case was considered to be a malignant tumor because the tumor cells showed marked pleomorphism and invasiveness into the abdominal
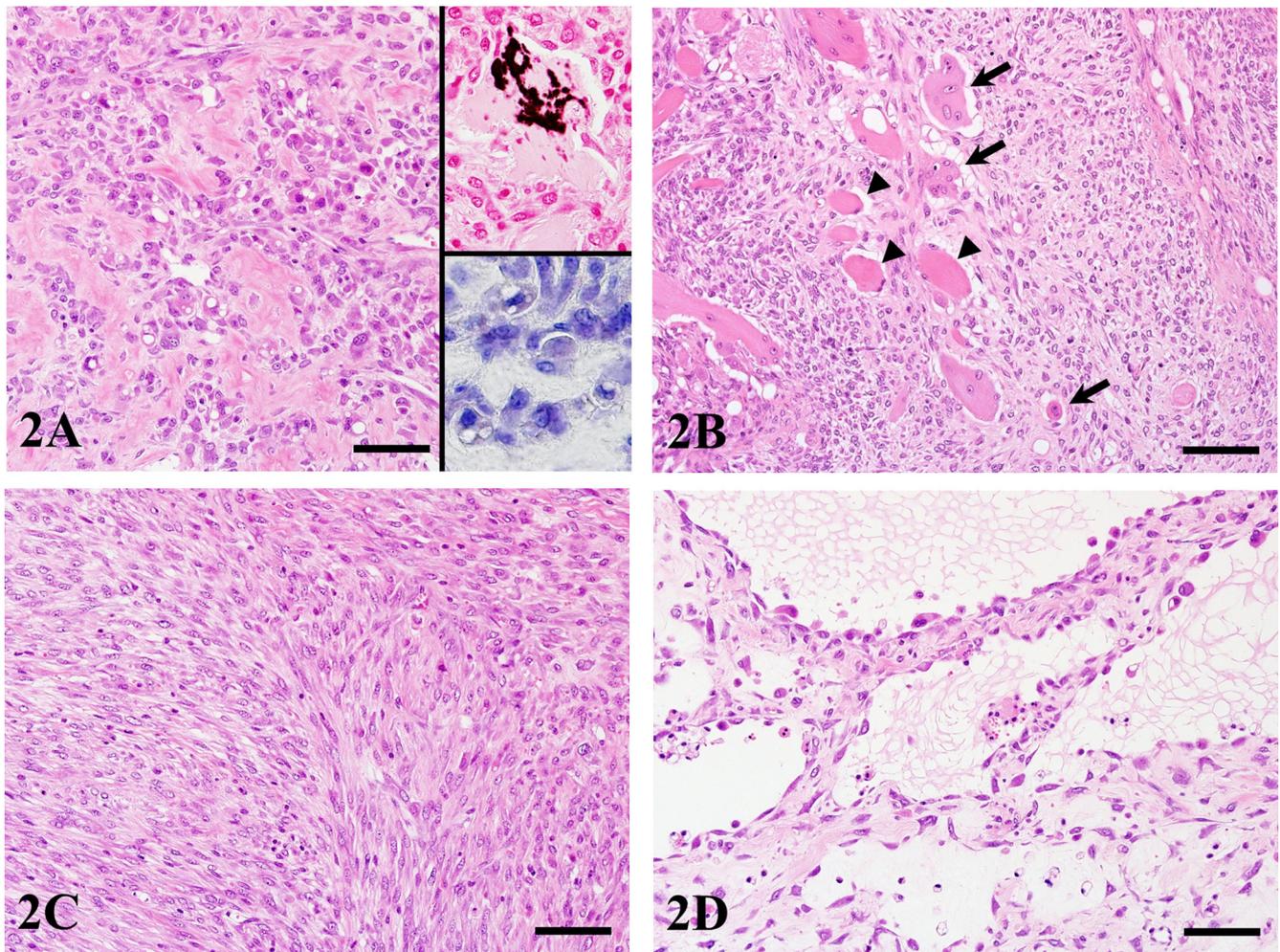
Fig. 2. A: Region A was composed of polygonal tumor cells with abundant cytoplasm and osteoid eosinophilic matrix. The eosinophilic matrix was slightly positive for Kossa stain (upper right inset). The vacuoles in the polygonal tumor cells were negative for oil red O stain (lower right inset). B: Region B was composed of irregular sheets of short spindle cells accompanying eosinophilic multinucleated cells (arrows). Normal striated muscle cells were also observed (arrowheads). C: Region C was composed of spindle cells that grew in bundles. D: Region D was composed of small and large cysts lined by short spindle cells. HE. Bar = 50 μm.

cavity, and had high growth activity as revealed by PCNA immunoreactivity. Although the tumor cells showed various histological growth patterns, the results of immunohisto-chemistry revealed that almost all tumor cells were positive for vimentin and osterix, which is a marker of osteoblasts. In addition, osteoid eosinophilic matrix with slight calcification was noted in region A. These findings strongly suggested that the tumor cells had developed osseous tissue. However, it was clinically first palpated as a caudal abdominal mass, and no clear continuity with normal bone in the ribs, vertebrae or right hind limb was observed in either macroscopic or microscopic examinations. Therefore, the tumor was diagnosed as an "extraskeletal osteosarcoma."

Differential diagnosis from other mesenchymal tumors is discussed below. Immunohistochemically, the tumor cells were weakly positive for SMA. Since the tumor cells, except those in region C, were not morphologically similar to neo-plastic smooth muscles and all the tumor cells were immu-nonegative for desmin, leiomyosarcoma was excluded from the diagnosis. SMA-positive tumor cells in skeletal osteosar-coma were previously reported in human cases[14–16]. It was proposed that EO might arise from multipotent mesenchy-mal cells and perivascular mesenchymal stem cells in hu-man and rabbit case reports, respectively[10, 17]. In the present case, it was speculated that the origin of the tumor cells was pericyte which expresses SMA and its immunohistochemi-cal properties remained because pericytes were considered to be multipotent mesenchymal stem cells and were report-ed to have the potential to differentiate into osteoblasts[18–20]. On the other hand, osteoblastic tumor cells with numerous myofilaments have been observed in low-grade intraosseous osteosarcoma by electron microscopic examination[15]. These tumor cells were described as myofibroblast-like cells in a recent report[16]. Immunophenotypically, myofibroblast can be positive for SMA and negative for desmin[21]. Therefore, it was also speculated that myofibroblastic differentiation had
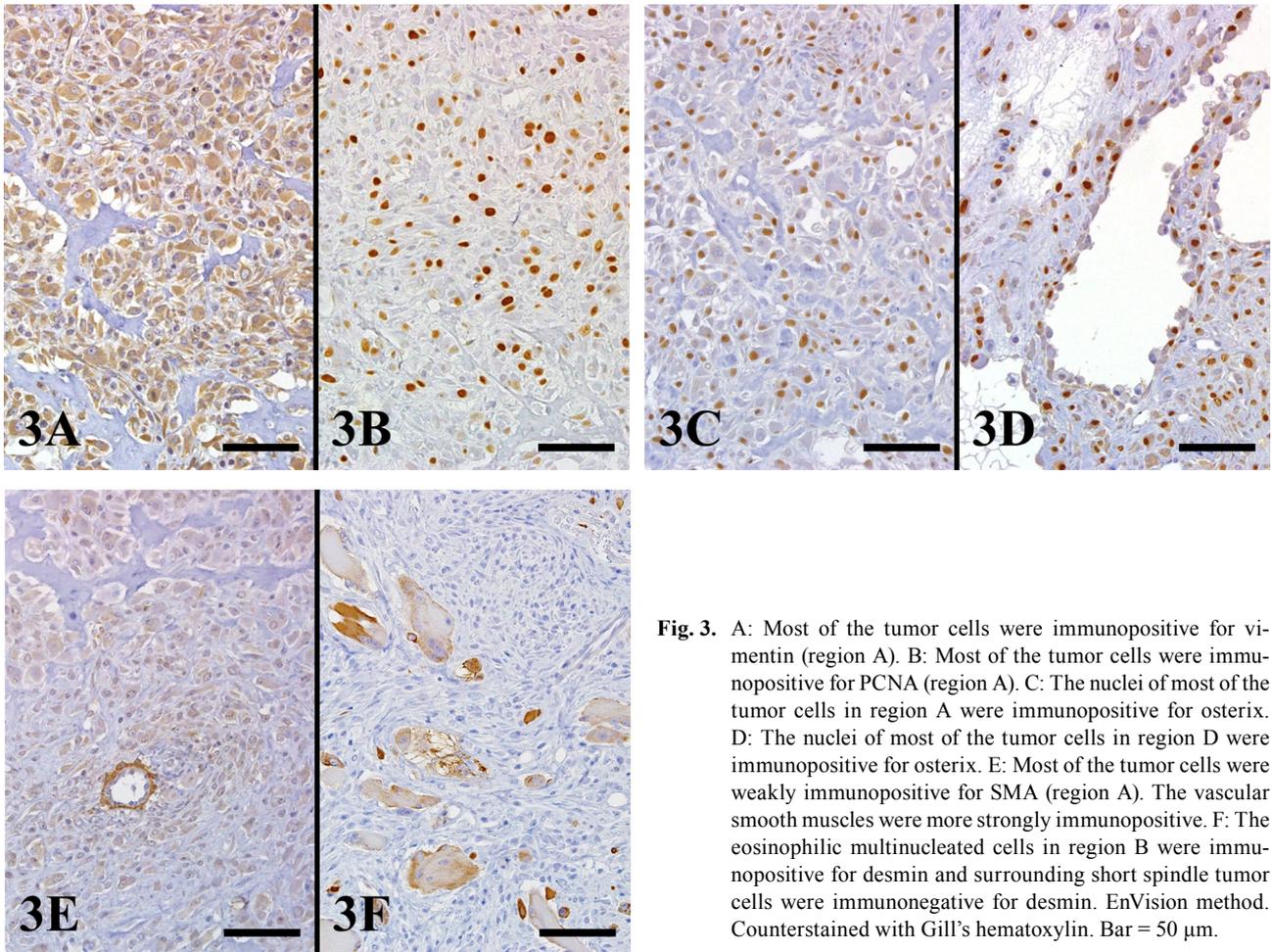
Fig. 3. A: Most of the tumor cells were immunopositive for vimentin (region A). B: Most of the tumor cells were immunopositive for PCNA (region A). C: The nuclei of most of the tumor cells in region A were immunopositive for osterix. D: The nuclei of most of the tumor cells in region D were immunopositive for osterix. E: Most of the tumor cells were weakly immunopositive for SMA (region A). The vascular smooth muscles were more strongly immunopositive. F: The eosinophilic multinucleated cells in region B were immunopositive for desmin and surrounding short spindle tumor cells were immunonegative for desmin. EnVision method. Counterstained with Gill's hematoxylin. Bar = 50 μm.

Table 1. Results of Immunohistochemical Examination of the Tumor Cells

Markers	Region of the tumor			
	A	B	C	D
Vimentin	+	+	+	+
Osterix	+	+	+	+
PCNA	+	+	+	+
SMA	±	±	±	±
Cytokeratin	−	−	−	−
Desmin	−	−	−	−
S100	−	−	−	−
GFAP	−	−	−	−
Factor VIII	−	−	−	−
Iba1	−	−	−	−

Criteria for grading: +, positive; ±, weakly positive; and −, negative.

occurred in the present case. Since the small vacuolations observed in some of the tumor cells in region A were negative for oil red O stain, liposarcoma was excluded from the diagnosis. The eosinophilic multinucleated cells observed in region B were considered not to be neoplastic striated muscle cells but instead to be regenerative ones because the cells were positive for desmin, weakly positive for vimentin and negative for osterix and surrounding short spindle tumor cells showed different immunoprofiles, such as being positive for osterix and negative for desmin. Therefore, the tumor presumably did not arise from normal striated muscle tissue but invaded into them. Our immunohistochemical examination excluded other suspected tumors such as fibrosarcoma, malignant schwannoma and hemangiosarcoma.

Spontaneous EO is extremely rare in mice[11], and the hallmark of the present tumor was various histological growth patterns observed in the regions A to D. Regarding mouse skeletal osteosarcoma, multiple subtypes have been recognized, and they include eburnating (osteoplastic), chondroblastic, osteoclastic, anaplastic, osteoblastic, fibroblastic, telangiectatic (vascular), and compound (mixed)[12, 13]. The present tumor may be subtyped as compound (mixed) because the growth patterns of region A, B, C and D resembled those of the osteoplastic, osteoblastic, fibroblastic and telangiectatic subtypes, respectively. In spite of the various histological growth patterns in the present case, most of the tumor cells were positive for osterix, and osterix has been used as a marker of EO in a rat and rabbit[9, 10]. Therefore, osterix is one of the most useful markers for the diagnosis of osteosarcoma. Since osteoid matrix, which is one of the hallmarks of osteosarcoma, was seen only in region A in

the present case, histopathological examination of all the tumor regions is likely essential for precise diagnosis of EO in mice.

Disclosure of Potential Conflicts of Interest: The authors declare that they have no conflicts of interest.

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