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Clinical Article

PATHOMORPHOLOGICAL AND IMMUNOHISTOCHEMICAL STUDIES ON OSTEOSARCOMA IN A DOG

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SUMMARY

An eight years old, male German shepherd dog was presented with history of inappetence, pain while chewing, unable to open the oral cavity completely and presence of black, irregular, firm hard growth in the left lower jaw around gums. Histopathologically, it revealed neoplastic mass comprised of osteoid tissue surrounded by osteoblasts characterized by pleomorphic nuclei, scanty to moderate cytoplasm, numerous multinucleated giant cells and infrequent mitotic figures. It was diagnosed as osteosarcoma on the basis of pathomorphological features. The intracytoplasmic immunopositive reaction for vimentin and no immunoreactivity for pancytokeratin in neoplasia confirmed their mesenchymal origin. Proliferating osteoblast and giant cells showed nuclear immunoreactivity for proliferating cell nuclear antigen.

Keywords: Dog, Osteosarcoma, Oral cavity, Proliferating cell nuclear antigen, Vimentin

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Osteosarcoma is a malignant tumour of bones. Among various types of primary bone tumours, it is the most common histological subtype reported in humans and dogs. This tumour is generally reported in middle to older aged dogs, giant and large breeds of dogs. This tumour is reported 3-4 times more commonly in the appendicular skeleton as compared to axial skeleton (Thompson and Dittmer, 2017). In dogs, mandibular or maxillary osteosarcoma is manifested as tumour of the oral cavity. In domestic animals, depending upon the matrix appearance, this can be classified into various types such as fibroblastic, chondroblastic, osteoblastic, telangiectatic and giant cell types (Thompson and Dittmer, 2017). The giant cell rich type of osteosarcoma is rare in the dogs (Oryan *et al.*, 2015). Histopathology is considered as gold standard for diagnosis and differentiation between benign and malignant tumours. However, in undifferentiated tumours or tumours of uncertain origin, histopathology is not always satisfactory for confirmatory diagnosis and predicting true biological behaviour of tumours. Therefore, other technique such as immunohistochemistry (IHC) by using tumour markers is employed for ascertaining the origin and proliferative activity of the neoplastic cells and aggressiveness of tumour. Keeping above in view, the present study was carried out with an objective to describe the pathomorphological features and immunohistochemical expression of pancytokeratin (PCK), vimentin and proliferating cell nuclear antigen (PCNA) in osteosarcoma of oral cavity in dog.

The present study was conducted on tumour tissue biopsy collected from an eight years old German shepherd dog presented to Department of Veterinary Clinical Complex, College of Veterinary Sciences (COVS), Lala Lajpat Rai University of Veterinary and Animal Sciences (LUVAS), Hisar for diagnosis and further treatment. Dog was presented with growth in the oral cavity with history of inappetance, pain while chewing and unable to open the oral cavity completely. Incisional biopsy was collected from the growth, fixed in 10% neutral buffered formalin for histopathology and immunohistochemistry (IHC). After fixation, it was processed routinely by paraffin embedding technique following the standard protocol (Luna, 1968). Sections of 3 µm thickness were cut using Histocore Multicut Leica Microtome System and then stained with haematoxylin and eosin (H&E) stain (Luna, 1968). The mitotic count was evaluated as per the procedure described by Meuten *et al.* (2017). For it, region at the periphery of tumour with maximum mitotic activity was selected and mitotic figures were counted in the 10 continuous, no overlapping high-power fields (HPFs) in H&E-stained slides. Areas with poor cellularity were avoided. Average value of mitotic figures was calculated and expressed as mitotic count. For IHC, tissue sections were taken on glass slides coated with 2% 3-Aminopropyl-triethoxysilane. The IHC was performed as per the standard procedure (Ramos-Vara, 2005) with minor modifications for expression of PCK, vimentin and PCNA. Microwave oven method with citrate buffer (pH-6.0) was used for antigen retrieval. Activity of endogenous peroxidase was blocked by 3% hydrogen peroxide solution. 5% normal goat serum was used for blocking of non-specific sites. The mouse monoclonal primary antibodies (Sigma Aldrich) *viz.*, anti-pancytokeratin (PCK26), anti-vimentin (V9) and anti-PCNA (PC10) were used at 1:200, 1:400 and 1:400 dilutions in 1% bovine

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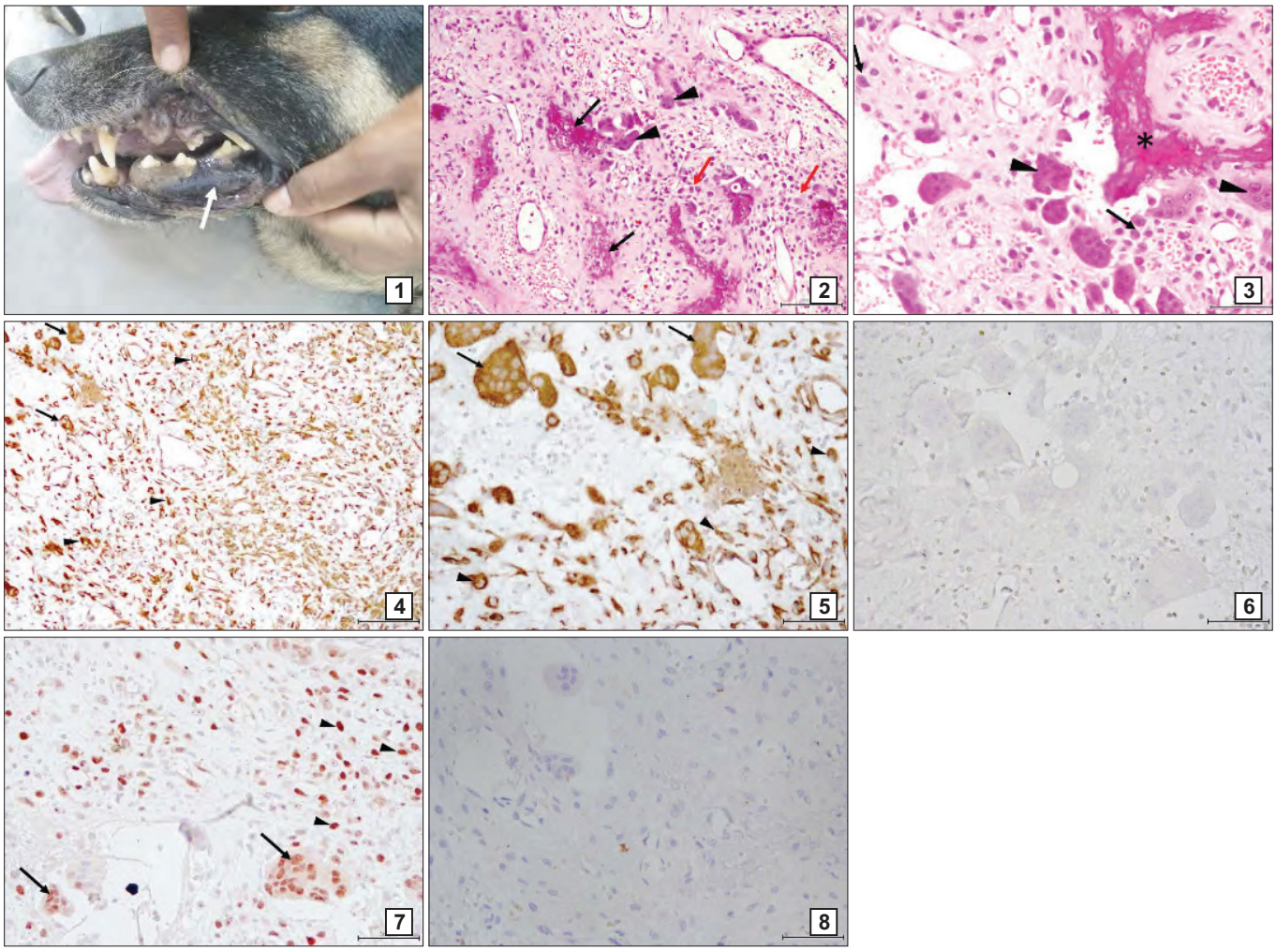

Figures 1 to 8. **(1)** A black, irregular growth in the left lower jaw around gums (arrow) extending from first premolar to last molar teeth; **(2)** Osteosarcoma showing osteoid tissue (black arrows) surrounded by a population of neoplastic osteoblast cells (red arrows) and numerous multinucleated giant cells (arrow heads). H&E×200; **(3)** Osteosarcoma exhibiting osteoid tissue (asterisk) surrounded by neoplastic osteoblast cells characterized pleomorphic nuclei with single prominent nucleoli, scanty to moderate eosinophilic cytoplasm (arrows) and numerous multinucleated giant cells (arrow heads). H&E×400; **(4)** Osteosarcoma showing brick red to brown coloured intracytoplasmic immunoreactivity for vimentin in neoplastic osteoblasts (arrow heads) and multinucleated giant cells (arrows). IHC×200; **(5)** Osteosarcoma showing brick red to brown coloured intracytoplasmic immunoreactivity for vimentin in neoplastic osteoblasts (arrow heads) and multinucleated giant cells (arrows). IHC×400; **(6)** Osteosarcoma showing no immunoreactivity in negative control for vimentin. IHC×400; **(7)** Osteosarcoma showing mild to strong, brick red to brown nuclear immunoreactivity for proliferating cell nuclear antigen in proliferating osteoblasts (arrow heads) and giant cells (arrows). IHC×400; **(8)** Osteosarcoma showing no immunoreactivity in negative control for proliferating cell nuclear antigen. IHC×400

serum albumin (BSA) solution prepared in phosphate buffered saline (PBS, pH = 7.2-7.4) for expression of PCK, vimentin and PCNA, respectively. Secondary antibody and Extravidin peroxidase (Sigma Aldrich) were used at 1:20 dilution in 1% BSA solution prepared in PBS (pH = 7.2-7.4). 3-Amino-9-ethyl-carbazole (AEC; Sigma Aldrich) was used as staining substrate for the colour development and the sections were counterstained with Gill's haematoxylin (Sigma Aldrich). For negative control, similar procedure was used except that the sections were incubated with 1% BSA (primary antibody omitted). For PCK and vimentin, brick red to brown coloured cytoplasmic staining in neoplastic cells was considered as positive. However, for PCNA brick red to brown coloured nuclear staining in neoplastic cells was considered as positive. PCNA index was calculated by counting at least 1000 neoplastic cells in 5-7 HPFs (40× objective) and expressed as percentage by calculating the fraction of immunopositive neoplastic cells out of the total neoplastic cells taken into account.

Physical examination of oral cavity revealed black, irregular growth in the left lower jaw around gums extending from first premolar to last molar teeth (Fig. 1). Growth was firm and hard in consistency. Similarly, more incidence of osteosarcoma has been reported in the dogs of middle to old age (median age of 8.8 years) and large to giant breeds (Coyle *et al.*, 2015). Histopathologically, the present case was diagnosed as osteosarcoma. Microscopically, the growth comprised of multifocal osteoid tissue surrounded by a population of osteoblasts characterized by presence of

pleomorphic nuclei with single prominent nucleoli, scanty to moderate eosinophilic cytoplasm and numerous multinucleated giant cells (Figs. 2, 3). Mitotic figures were infrequent (0.5/HPFs). Haemorrhages were also evident at some places. These features were similar to findings of Mallick *et al.* (2020) who reported a case of giant cell-rich osteosarcoma in a 52-year-old male human patient with involvement of mandible. Contrary to present study, no mitotic figures were observed by Mc Calla *et al.* (1989) in a multilobular osteosarcoma of mandible and orbit in a dog, but they noted metastasis in lungs and orbital space during necropsy of the said animal. However, Mallick *et al.* (2020) reported the presence of numerous atypical mitotic figures. In the present study, neoplastic osteoblasts and multinucleated giant cells revealed the intracytoplasmic brick red to brown immunoreactivity for vimentin (Figs. 4, 5), but, no immunoreactivity for PCK was noticed. No immunoreactivity was noticed in negative control for vimentin (Fig. 6). Immunopositive reaction for vimentin and no staining for PCK confirmed the mesenchymal origin of the neoplastic cells. Similar to present findings, Amaral *et al.* (2018) also observed immunoreactivity for vimentin in canine primary bone tumours. They noticed higher intensity of vimentin expression (+++) as compared to osteonectin (++) and osteocalcin (+) in primary bone tumours of dogs. They suggested that besides diagnosis, vimentin expression may be helpful prognosis of these tumours. Present finding supported the report of Sun *et al.* (2015) who noted diffuse positive immunostaining for vimentin in osteoclast-like giant cells in a giant cell rich osteosarcoma of mandible in a human patient. In present study, brick red to brown nuclear immunoreactivity for PCNA with mild to strong staining intensity was observed in proliferating osteoblasts and giant cells (Fig. 7) with PCNA index of 40.2%. However, no immunoreactivity was noticed in negative control for PCNA (Fig. 8). PCNA is a nuclear protein and it is used as cell proliferating cancer biomarkers. It is synthesized in G_1 phase of cell cycle, reaches to its peak level in S phase and then significantly decreases in G_2 and M phases (Ye *et al.*, 2020). PCNA expression indicated the proliferating activity neoplastic cells. Similar to present study, Junior *et al.* (2003) reported variable immunoreactivity for PCNA in osteosarcomas of the head and neck region. They observed positive reaction for PCNA in 92% of cases with strong positivity in 64% cases. Fattahian *et al.* (2008) also reported immunoreactivity for PCNA (25%) in the giant cell osteosarcoma in a dog. In conclusion, on the basis of pathomorphological features, the present case with growth in oral cavity of a dog was diagnosed as osteosarcoma (giant cell type). Besides, the expression of vimentin confirmed the mesenchymal origin of neoplastic cells. The proliferative activity of neoplastic cells was assessed by immunoreactivity for PCNA with mild to strong staining intensity.

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