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Original article

Immunohistochemical evaluation of cellular activities in canine osteoblastic osteosarcoma

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Abstract

Osteosarcoma is the most frequently occurring malignant bone tumor in animals and humans. A better understanding of the etiopathogenetic mechanisms of osteosarcoma, especially biomolecular ones, is fundamental to improving diagnosis and prognosis of the disease. Autophagy is a self-degradative process that removes dysfunctional cellular components, eliminates intracellular pathogens, and promotes cellular senescence. In neoplastic cells, autophagy suppresses tumorigenesis by inhibiting cancer cell survival mechanisms and promoting cell death. The aim of this study was to use immunohistochemistry to identify alterations in the expression of several apoptotic and proliferative biomarkers in multiple cases of canine osteosarcoma. Bcl-2, an intracellular membrane protein, inhibits cell death by blocking the p53-mediated pathway of apoptosis. While Bcl-2 overexpression has been described in many different premalignant and malignant lesions, it has yet to be analyzed in canine osteosarcoma. Our group investigated 10 primary canine osteoblastic osteosarcoma cases from the University of Perugia Department of Veterinary Medicine Teaching Hospital. Immunohistochemical analyses of proteins Bcl-2, Ki-67, and p53 have revealed interesting results as described in this paper. Expression of Bcl-2 was increased in all cases investigated while expression of p53 and Ki-67 was variable and no statistical association was observed between the expression patterns of p53, Bcl-2 and Ki-67.

Keywords

osteosarcoma, dog, autophagy, Bcl-2, Ki-67, p53

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Introduction

Osteosarcoma is the most common primary malignant bone tumor in dogs characterized by the formation of osteoid by neoplastic mesenchymal cells [1]. Osteosarcoma represents 80-85% of the primary bone tumors affecting dogs and is defined as a highly aggressive and invasive tumor with a tendency to metastasize [1, 2]. Osteosarcoma usually affects middle-aged to older medium and giant-breed dogs, with a median age range of 7-8 years deferring from humans where it usually involves younger patients [3, 5]. In all species, osteosarcoma predominantly arises within the metaphyseal region of long bones with forelimbs being affected twice as often as hindlimbs. The two most frequently affected regions are the distal radius (35% of cases) and the proximal humerus (18% of cases) [4, 5]. Known etiologic causes of osteosarcoma include irradiation, viral infections, immunodeficiency disorders, environmental and specific genetic mutations such as alterations of pRb genes [5, 6]. The development of osteosarcoma also involves alterations of cell cycle regulators which allows for uncontrolled growth [5]. Several studies on tumorigenesis have hypothesized that molecular mechanisms such as apoptosis and autophagy may be involved in the development of malignancy [7]. Autophagy is a stress-responsive process that regulates the degradation and recycling of cellular components. Autophagy also plays a housekeeping role by removing misfolded or aggregated proteins, clearing damaged organelles, and eliminating intracellular pathogens. In response to certain stimuli, autophagy can induce programmed cell death [8].

Bcl-2 family proteins control cell death through either inhibiting or inducing apoptosis. Bcl-2, the founding member of the Bcl-2 family proteins, regulates cell death through blocking or delaying the mitochondrial apoptosis pathway. Bcl-2 obtained its name from its discovery in B cell leukemia/lymphoma 2. Initially, Bcl-2 was classified as a factor involved in apoptosis and it was only after the discovery of its involvement in autophagy that the scientific community began studying the protein's involvement in numerous biomolecular processes [9]. Bcl-2 is overexpressed in multiple tumors which supports its role as a potent oncogene. In addition, recent studies have demonstrated an upregulated expression of Bcl-2 in multiple cases of human lung cancer supporting its potential involvement in the pathogenesis of these tumors [10].

The p53 protein known as the "guardian of the genome" [11] acts to transmit a variety of stress- responsive signals to multiple anti-proliferative cellular responses. Stimuli such as DNA damage, oncogene activation, and hypoxia, trigger the expression of p53. When activated, p53 initiates several

biological processes including apoptosis, cell-cycle arrest, senescence, or autophagy [12-14]. Interactions between Bcl-2 and p53 have been described in several tumors [15]. Multiple studies have demonstrated that apoptosis is tightly regulated and influenced by a series of quantitative and qualitative events that alter p53 activation [16]. P53 can activate the Damage-Regulated Autophagy Modulator (DRAM) gene leading to the production lysosomal proteins and the induction of autophagy [17]. P53 is a potential inducer of autophagy (inhibitor of Bcl-2) its altered activity seems to be responsible for the increase of autophagic activity in many tissues and pathological processes [18].

Ki-67 is a nuclear protein associated with cellular proliferation. Detailed cell cycle analyses revealed that Ki-67 is expressed in the nuclei of cells in S, G1, G2, and M phases of cell division. Quiescent cells in G0 phase lack expression of this protein [19]. Multiple studies have demonstrated that the expression of Ki-67 is strictly associated with cellular proliferation. During mitosis, this nuclear protein relocates to the surface of chromosomes [20]. Given that Ki-67 is expressed by all proliferating cells, evaluating the intracellular presence of Ki-67 may be an excellent indicator of cell growth across several tissues.

Our studies focused on the expression patterns of Bcl-2, p53 and Ki-67 in 10 cases of spontaneous canine osteosarcoma. The goal of the study was to evaluate the potential involvement of these proteins in the development of malignant spontaneous canine osteosarcoma.

Materials and Methods

10 cases of spontaneous canine osteoblastic primary and non-metastatic osteosarcoma were selected to investigate the immunohistochemical expression of Bcl-2, p53, and Ki-67. Tumor samples were fixed in 10% neutral buffered formalin, processed, and embedded in paraffin using routine methods. Hematoxylin and eosin (H&E) staining was performed on 3-5 μ m sections. Immunohistochemistry (IHC) was performed using the Avidin Biotin Complex (ABCL-2) method. Paraffin was removed with xylene and slides were dehydrated in sequentially diluted ethanol then rinsed in distilled water. To inhibit endogenous peroxidase activity, the tissue sections were treated with 3% hydrogen peroxide in tris phosphate-buffered saline (PBS). Samples were rinsed in normal goat serum for 30 minutes to block non-specific reactions. Immunohistochemistry was performed on serial 3-5 μ m sections using mouse monoclonal anti-p53 antibodies (clone DO-7, dilution 1:50, Dako), anti-Bcl-2 antibodies (clone 124, dilution 1:100, Dako), and anti-Ki67 antibodies (clone MIB-1, dilution 1:150, Dako). Negative controls for immunohistochemistry were processed identically to test

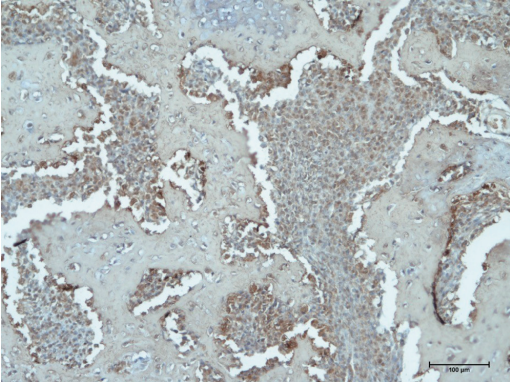


Fig. 1. Osteoblastic osteosarcoma. Immunohistochemical cytoplasmatic reactivity for Bcl-2 in osteoblastic malignant cells (20 \times).

slides, but the primary antibodies were omitted. Samples of canine fibrosarcoma were used as a positive control for antibodies against p53 and Ki-67 and unaffected canine tonsils were used as a positive control for antibodies against Bcl-2.

Results

Histologically, all tumors examined were highly cellular with polyhedral cells, sometimes round to spindle, hyperchromatic large nuclei, and prominent nucleoli. Tumors also exhibited frequent and numerous mitoses with varying amounts of osteoid production. Immunohistochemistry revealed an increased expression of Bcl-2 in all cases when compared to normal bone samples. The expression of Bcl-2 was rarely correlated with the expression of p53, and Ki-67 proteins and no statistical association was observed between the expression of p53, Bcl-2, and Ki-67 proteins. Bcl-2 is an anti-apoptotic protein that protects cells from a variety of apoptotic stimuli, including cytotoxic drugs, irradiation, heat, and/or growth factor withdrawal.

The overexpression of Bcl-2 has been identified in numerous types of human cancers, including breast, colon, ovarian, and prostate cancer, however, it has yet to be described in osteoblastic osteosarcoma. Although Bcl-2 confers resistance to malignant cells, expression is not always correlated with a poor prognosis [21]. Our results revealed a diffuse overexpression of Bcl-2 in all osteoblastic tumor cells in investigated canine osteosarcoma cases (Figure 1 and 2). The increased expression of Bcl-2 in cases of osteosarcoma suggests that tumor cells upregulate Bcl-2 as a mechanism of inhibiting programmed cell death allowing for the survival and proliferation of neoplastic cells.

The protein p53 is a cell cycle regulator that is often mutated in neoplastic cells. Expression of p53 was mainly detected within the nucleus of tumor osteoblasts, however, in

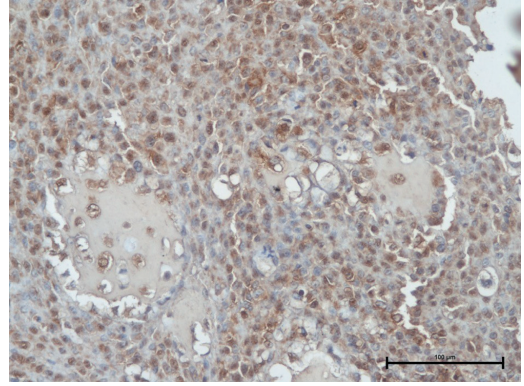


Fig. 2. Osteoblastic osteosarcoma. Immunohistochemical cytoplasmatic reactivity for Bcl-2 in osteoblastic malignant cells (40 \times).

most samples (80% of cases), p53 was also detected within the cytoplasm. The percentage of positive cells in each tumor examined ranged from a few (10-20%) scattered cells with positive nuclei to approximately 90% of the tumor population indicating that the expression of p53 is highly variable. Overall, samples of spontaneous canine osteosarcoma demonstrated significant cytoplasmic expression of p53 in osteoblastic cells (Figure 3). Studies conducted on the role of p53 in autophagy in different human tumors have shown that the cytoplasmic localization of this marker is associated with the inhibition of autophagy. Our results suggest that there is a correlation between the cytoplasmic localization of p53 and the overexpression of Bcl-2 [23].

Ki-67, a nuclear protein, and a marker of cellular proliferation has been upregulated in several forms of human cancer [20]. All osteoblastic canine osteosarcoma samples evaluated in our preliminary study demonstrated strong uniform nuclear expression of Ki-67 suggesting that osteoblastic tumor cells were highly proliferative (Figure 4).

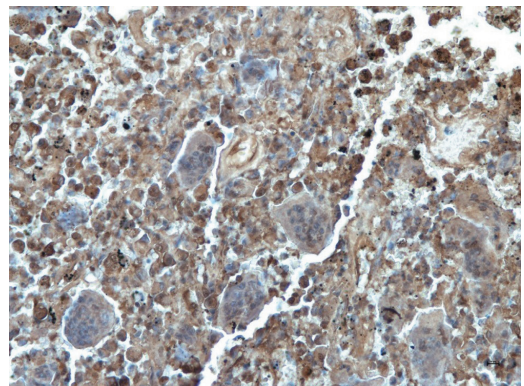


Fig. 3. Osteoblastic osteosarcoma. Immunohistochemical reactivity for p53 in osteoblastic tumor cells (40 \times).

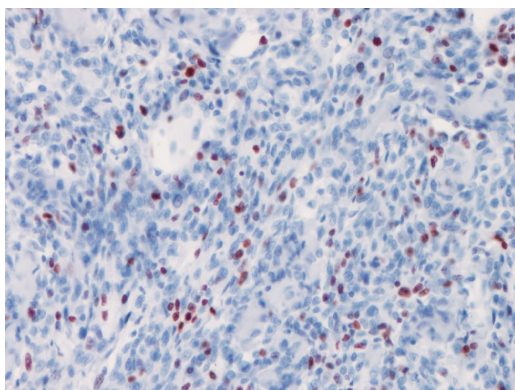


Fig. 4. Osteoblastic osteosarcoma. Immunohistochemical reactivity for Ki-67. Nuclear staining with hematoxylin (20×).

Discussion

The focus of this study was to analyze the mechanisms of cellular proliferation, apoptosis, and autophagy in cases of spontaneous canine osteoblastic osteosarcoma through the evaluation of immunohistochemical expressions of Bcl-2, p53, and Ki-67 proteins. Increased expression of p53 and Ki-67 in all cases of canine osteoblastic osteosarcoma indisputably indicates modifications of the cell cycle in neoplastic cells. We suggest that the increased cytoplasmic expression of p53 plays a role in the inhibition of autophagy and ultimately in the development of malignancy of osteosarcoma [24]. The upregulated nuclear expression of Ki-67 in all neoplastic cells supports aberrant proliferation of neoplastic cells. Bcl-2 is a potent inhibitor of apoptosis and autophagy; however, little is known about the role of Bcl-2 in the development of osteosarcoma. All 10 cases of canine osteoblastic osteosarcoma demonstrated an increased expression of Bcl-2 when compared to normal bone samples. Our results suggest that Bcl-2 acts to inhibit apoptosis in neoplastic osteosarcoma cells which may play a role in proliferation and malignancy of the tumor. Increased expression of Bcl-2 may support the diagnosis of osteosarcoma; however, further studies are required to establish the role of Bcl-2 as a prognostic indicator.

In conclusion, our studies have allowed us to establish alterations in cell cycle regulators, particularly Bcl-2, p53, and Ki-67, in canine osteoblastic osteosarcoma. Increased expression of Bcl-2, p53, and Ki-67 in all cases supports their involvement in the development of osteosarcoma. Our results revealed no statistical significance between the expression of all three proteins. Further investigations are necessary to evaluate the correlation and biomolecular activity between Bcl-2, p53, and Ki-67 proteins in canine osteoblastic osteosarcoma.

6. References

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