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RESEARCH ARTICLE

Comparison of Cell Markers Expression in Canine and Human Spontaneous Osteosarcomas and Established Osteosarcoma Cell Lines

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ABSTRACT

The aim of the study was to demonstrate and analyze the expression of the following proteins, β-catenin, E-cadherin, vimentin, Ki-67, mini-chromosome maintenance 3 (MCM3), mini-chromosome maintenance 7 (MCM7), osteocalcin, cyclooxygenase-2 (COX-2), and microsomal PGE2 synthase-1 (mPGES-1) in the spontaneous osteosarcomas isolated in dogs and in humans, and to compare the results with these obtained in the established canine (D-17) and human (U-2 OS) cell lines. Immunohistochemical tests were conducted on 4 μm -thick paraffin sections and cells from established canine and human osteosarcoma cell lines. The sections and cells from cell lines were overlayed with primary antibodies. The immunocytochemical reaction was induced by 3,3 diaminobenzidinetetrahydrochloride (DAB) solution. In a number of cases, the expression of the investigated cell markers was of comparable intensity in the cell lines and spontaneous osteosarcomas, except β-catenin and mPGES-1 in dogs. There were also no significant differences in the expression of the investigated cell markers between the group of humans and dogs. It is also worth noting that the obtained results open a perspective for developing an appropriate animal model for the study of oncogenesis in humans.

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INTRODUCTION

Osteosarcoma is a malignant mesenchymatic tumor, originating from osseous tissue and reckoned to represent one of primary osseous tumors. It is the most frequently diagnosed osseous tumor both in dogs and in humans. In dogs aged between 2 and 15 years, the tumor comprises 80-85% of all bone tumors (Gârjoabă *et al*., 2009). Nevertheless, it is relatively rare as compared to the incidence of all canine tumors: in the years 2009-2011, in the area of Lower Silesia (Poland) it accounted for 0.6% of all tumors isolated in dogs (Ciaputa *et al*., 2013). In humans, it is usually diagnosed in childhood and pubescence, with peak incidence at the age of 16, which is linked to an accelerated osseous turnover in this period. Another increase in the incidence is observed in the senescence (Ta *et al*., 2009)

Osteosarcoma is frequently diagnosed in dogs of large and giant breeds, such as Dobermann pinscher, great dane, sighthound, St. Bernard dog and Rottweiler (Ru *et al*., 1998). It is manifested more frequently in the sterilized than non-sterilized animals (Ru *et al.,* 1998). Both in dogs and in humans the most common tumor location involves long bones that are the most exposed to overloading and ensuing microtraumas, particularly frequent in tall humans and dogs of large and giant breed (Mirabello *et al.,* 2011; Rankin *et al*., 2012). Osteosarcoma is less frequently encountered in flat bones. Etiopathogenesis of the tumor development in dogs and in humans has been incompletely recognized (Gârjoabă *et al*., 2009).

The tumor belongs to the group of particularly aggressive proliferative lesions, resulting in osseous destruction evident in radiological patterns and manifested by a pronounced pain in the affected region. It spreads

rapidly to the surrounding soft tissues and it frequently metastasizes to the regional lymph nodes and to the lungs (Mullins *et al*., 2004)

This study aimed at a demonstration and analysis of the expression of such proteins as β-catenin, E-cadherin, vimentin, Ki-67, mini-chromosome maintenance 3 (MCM3), mini-chromosome maintenance 7 (MCM7), osteocalcin, cyclooxygenase-2 (COX-2) and microsomal PGE2 synthase-1 (mPGES-1) in the neoplastic tumors isolated in dogs and in humans, and a comparison of the results with these obtained in the established cell lines of canine (D-17) and human (U-2 OS) osteosarcomas.

MATERIALS AND METHODS

Spontaneous tumors: The study was conducted using paraffin sections of osteosarcomas originating from 10 dogs and 5 humans diagnosed with the tumor in long bones. The material was sampled during an amputation of the neoplastically altered extremities. The tumors were histopathologically verified in line with the World Health Organization (WHO), divides these changes according to several criteria, i.a.: a type of lesion, a type of matrix dominant in their construction, location and histologic grade of malignancy – "grading" (Dorfman *et al*., 2002).

All examined spontaneous tumors, which derived from both humans and animals, were primary tumors, the structure of which was osteoblastic and high grade. Such a selection was in order to standardize a research group. The preparations of human osteosarcomas originated from the archival collection of "HistMed", while the preparations of canine osteosarcomas originated from the archival collection of our Faculty.

The isolated fragments of canine osteosarcomas were fixed in 7% buffered formalin (Chempur, Poland) and embedded in paraffin blocks. Immunohistochemical tests were conducted on 4 μm-thick paraffin sections. Antigens of the tissues fixed in formalin (Chempur, Poland) and of the cells fixed in a mixture of acetone and methanol (Stanlab, Poland) were retrieved in EnVision™ FLEX Target Retrieval Solution (DAKO, Denmark). Endogenous peroxidase was blocked in 3% solution of EnVision™ FLEX Peroxidase Blocking Reagent (DAKO, Denmark). Subsequently, the sections were overlaid with primary antibodies, including Mouse Monoclonal Anti-Human E-Cadherin, clone 36 (Biogenex, USA), Mouse Monoclonal Anti-Human Ki-67, clone MIB-1 (DAKO, Denmark), Rabbit Monoclonal Anti-Human MCM3, clone EP202 (BioSB, USA), Mouse Monoclonal Anti-Human MCM7, clone 101 (DAKO, Denmark), Mouse Monoclonal Anti-Human Beta-Catenin, clone β-catenin-1 (DAKO, Denmark), Mouse Monoclonal Anti-Vimentin, clone V9 (DAKO, Denmark), Rabbit Monoclonal Anti-Human COX-2, clone RBT-COX2 (BioSB, USA), Rabbit Polyclonal Anti-Human Osteocalcin (AbDSerotec, UK), Rabbit Polyclonal Anti-Human mPEGS-1 (Acris Antibodies, Germany). This was followed by washing in EnVision™ FLEX Wash Buffer (DAKO, Denmark) and application of the visualization system, EnVision™ FLEX/HRSM802 (DAKO, Denmark). The immune-cytochemical reaction was induced by 3,3-diaminobenzidinetetrahydrochloride (DAB) solution, EnVision™ FLEX DAB+ Chromogen (DAKO, Denmark).

For each cell marker, a proper positive and negative control was performed. Positive controls for individual markers were selected from the samples obtained from the pathology laboratory, and their positivity was confirmed by comparison with other samples. In the negative control the primary antibodies were omitted.

Cell culture: For the evaluation of selected marker expression, adherent established cell lines of canine (D-17) and human (U-2 OS) osteosarcoma were used. The canine osteosarcoma cell line was cultured in Eagle's Minimal Essential Medium (ATCC, USA) and the human osteosarcoma cell line in McCoy's 5A (ATCC, USA), both media were supplemented with 10% fetal calf serum (Sigma-Aldrich, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich, USA).

The cells from human and canine osteosarcoma cell lines were adjusted to the concentration of $2x10^4$/40 μl of respective medium and dispensed to 10 well hydrophobic culture glasses (Thermo Scientific, USA). The preparations were overlaid with a cold 1:1 mixture of methanol (Stanlab, Poland) and acetone (Stanlab, Poland) in order to attach the cells to the glass and to permeabilize their membranes. The preparations were subjected to immunohistochemical staining using the same antibodies and a scheme analogous to that applied for the material originating from the spontaneous tumors.

Expression of E-cadherin, vimentin, β-catenin, osteocalcin, mPGES-1, COX-2 was appraised using a modified semi-quantitative IRS scale according to Remmele (Brouckaert *et al*., 2013). Expression of Ki-67, MCM3 and MCM7 was evaluated quantitatively by estimating the percentage of positive cells.

The results were subjected to a statistical analysis using Statistica PL software (StatSoft, Poland) and Mann-Whitney U analysis. The statistical significance was set at P=0.05.

RESULTS AND DISCUSSION

The results of immunohistochemical studies concerning the markers mentioned above, carried out on established cell lines of human and canine osteosarcoma, as well as on osteosarcoma spontaneous tumors, are presented in Fig. 1 to show the best comparison between the selected protein expression. Immunohistochemical reactions are shown in the Fig. 2. Statistical analysis of the results using Mann-Whitney's U test demonstrated no significant differences in the expression of the investigated cell markers between the spontaneous tumors and cell culture in every cell marker in humans and in every cell marker except β-catenin and mPGES-1 in dogs. There were also no significant differences in the expression of the investigated cell markers between the group of humans and dogs.

An increasing incidence of osteosarcoma diagnosis in dogs prompted us to design and carry out a study aimed at comparing the expression of selected proteins in the cells of the established cell lines and the cells originating from the tumors spontaneously developing in humans and dogs. Showing similarities or differences in the expression of the studied markers in the two species might be helpful in the interpretation of our results obtained in *in vitro* studies concerning cytotoxic activity of selected cytostatic drugs, non-steroid anti-inflammatory drugs and bisphosphates,

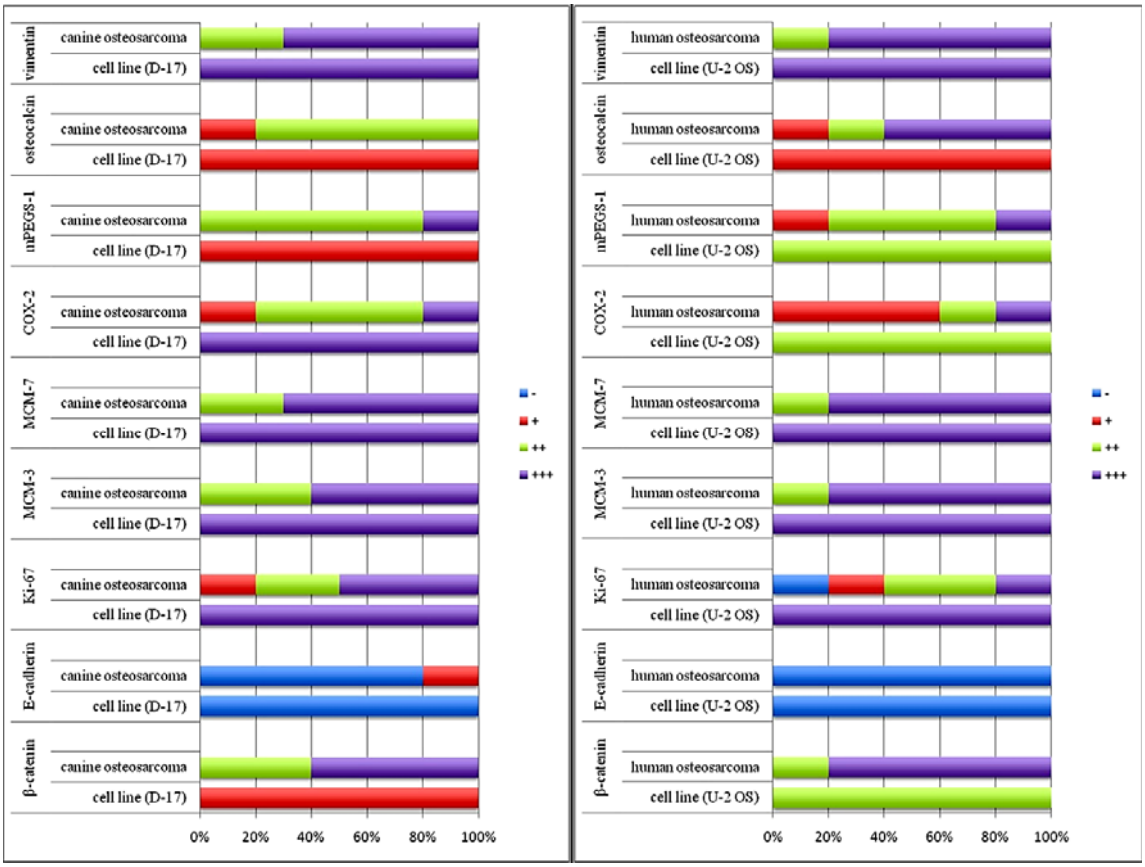

Fig. 1: Percentage distribution of the reaction strength of each antibody in the canine and human spontaneous osteosarcomas and established osteosarcoma cell lines.

alone or in combinations, targeted at human and canine established cell lines that will be investigated in the future.

In order to define the osteosarcoma ability to metastasize, the analysis involved β-catenin and E-cadherin, the proteins participating in anchoring and organization of cytoskeleton and securing cell-to-cell adhesion (Iwaya *et al*., 2003). A presence of β-catenin in the normal cells is restricted to the cell membrane, in which it is present in small amounts (Iwaya *et al*., 2003), while in the neoplastic cells it is found in the cytoplasm or, less frequently, in the nucleus (Haydon *et al.,* 2002). The results of our study have confirmed the presence of β-catenin in the tumor cell cytoplasm, its pronounced expression in the spontaneous human and canine tumors, and lower expression in the cells of the established cell lines. The differences might have reflected the absence of the tumor sublayer in the cellular cultures and the absence of intercellular links. It was demonstrated in both human tumors (Haydon *et al.,* 2002) and canine tumors (Bongiovanni *et al.,* 2012) that β-catenin expression within the cytoplasm and/or cell nucleus was similar to that documented in our study. β-catenin, together with E-cadherin, the Ca++-dependent transmembrane protein, belongs to cadherin family, important for the formation of intercellular junctions. In normal osteoblasts, the expression of E-cadherin is restricted to the cell membrane (Kashima *et al.,* 1999). An increased cytoplasmic and/or nuclear expression and reduced membrane expression of β-catenin, accompanied by lacking or poor membranous reaction for E-cadherin was found to be linked to an increased metastasizing ability of the tumor and worse prognosis for the patient. In our study, we were unable to detect E-cadherin expression in the canine or human osteosarcoma cell cultures or in the spontaneous human osteosarcomas. However, in the canine osteosarcomas, E-cadherin expression was manifested in a small percentage of cells and it was restricted to the cytoplasm. Such a situation may indicate high malignancy of the tumor, since an absence or low expression of E-cadherin and high level of β-catenin expression in cytoplasm and/or cell nucleus might correlate with higher risk of metastasis development (Iwaya *et al*., 2003; Kashima *et al.,* 1999).

The presence of vimentin, a protein forming intermediate filaments in the mesenchymal cells, might have indicated a non-epithelial origin of the studied tumor (Herrmann and Aebi, 2000). Our studies documented a pronounced cytoplasmic expression of the protein, both in the cell lines and in the tumors. Löning *et al.* (1985) and Barger *et al.* (2005), demonstrated the expression of vimentin in the spontaneous human and canine osteosarcomas, and Loukopoulos *et al.* (2004) confirmed the expression of vimentin in the canine osteosarcoma cell line.

Ki-67, MCM3 and MCM7 are proteins playing an important role in the cell cycle, and thus they represent recognized and reliable markers of the cellular proliferation. They show a nuclear staining reaction. In our study, a pronounced and stable expression of Ki-67 was

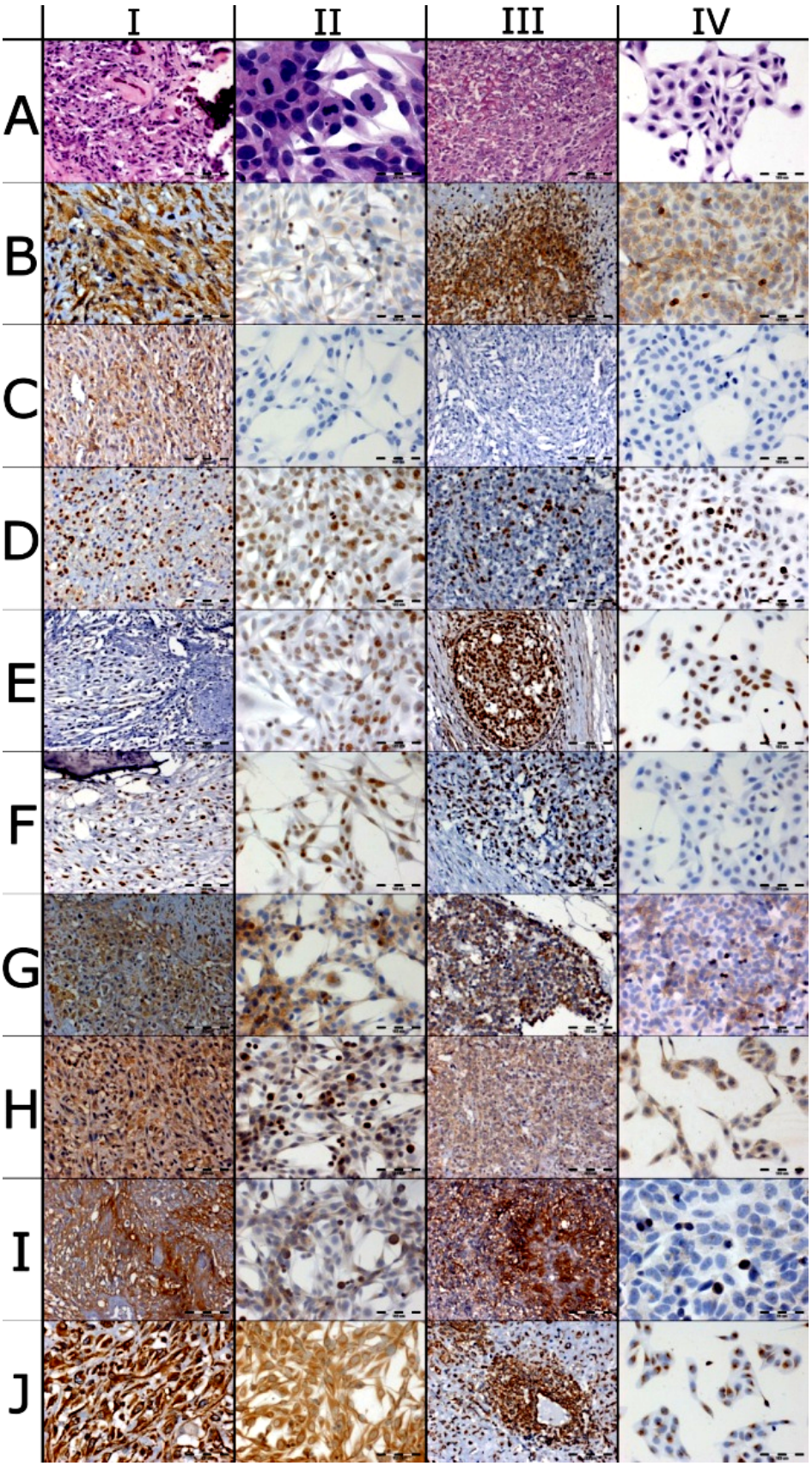

Fig. 2: Immunohistochemical reaction of the selected cell markers in: I) Canine osteosarcoma, II) Canine osteosarcoma cell line (D-17), III) Human osteosarcoma and IV) Human osteosarcoma cell line (U-2 OS). Staining: A) H&E; Immunohistochemical reaction for: B) β-catenin, C) E-cadherin, D) Ki-67, E) MCM-3, F) MCM-7, G) COX-2, H) mPEGS-1, I) osteocalcin and J) vimentin. Magnification: Scale bars = in I) 100μm (A, C to I), 50μm (B, J); II) 100μm (B to J), 50μm (A); III) 100μm (A, B and D to J), 50μm (C) and IV) 100μm (A to H and J), 50μm (I).

demonstrated in the cell lines of both canine and human origin, but it was highly variable in spontaneous tumors. In the case of MCM-3 and MCM-7, a pronounced expression was demonstrated in both cell lines and it was also rather stable in neoplastic tumors. This may indicate that the cells in the established cell lines divide continuously in the manner depending on of the culture conditions. In the spontaneous neoplastic tumors, the proliferation pattern is more variable, and thus the expression of proliferation markers may be more variable. Park *et al.* (1995) and Scotlandi *et al.* (1995) demonstrated a variable expression level of Ki-67 in the spontaneous human osteosarcoma.

Osteocalcin is synthesized by osteoblasts, odontoblasts and some chondrocytes. It binds to hydroxyapatite, securing an appropriate mineralization of bone matrix and it controls the rate of bone formation and bone resorption. Osteocalcin presence in the studied material provided evidence of its osseous origin and of secretory activity of the neoplastically transformed osteoblasts (Cloos and Christgau., 2004; Park *et al*., 1995; Singer and Eyre, 2008). Analyzing the results obtained in the neoplastic tumors with various levels of osteocalcin expression, we found them similar to the results of Park *et al.* (1995) and El-Badawi *et al.* (2012). On the other hand, a decreased expression of osteocalcin might be explained by inability of the cell cultures to form tissue structures through a restriction of the mineralization process, which may suggest a reduced secretory activity as compared to that of the cells in the spontaneous osteosarcoma tumors. Lajeunesse *et al.* (1990) reported that osteocalcin secretion in the human osteosarcoma cell line was very low in the unstimulated cultures, which might confirm the observed reduced expression of this protein in the cells originating from the cell culture. Pautke *et al.* (2004), examined three different cell lines of human osteosarcoma, including U-2 OS, and detected a very low or none expression of the protein, thus confirming our results.

COX-2 and mPGES-1 participate in the biosynthesis of prostaglandin E (Ricciotti and Fitzgerald, 2011; Murakami *et al.,* 2002). Prostaglandin E (PGE) stimulates the proliferation of tumor cells, reduces their apoptosis rate, suppresses immune responses in the body and is involved in the angiogenesis within a neoplastic tumor (Takahashi *et al.,* 2014; Kamata *et al.,* 2011; Mohammed *et al*., 2002). In the examined tumors, COX-2 expression was variable, similarly to the findings of other authors related to the canine or human osteosarcomas (El- Badawi *et al.*, 2012; Millanta *et al.,* 2012; Mullins *et al.,* 2004).This might be linked to variable intensity of the inflammation in the neoplastic tumors. In the cell lines, the expression of both proteins was high, and this was confirmed for the canine cell line by Wolfesberger *et al.* (2006). We obtained similar results for mPGES-1, but its expression was much less pronounced in the canine cell line than in the human cell line.

Conclusions: It can be concluded that the expression of most of the investigated cell markers in dogs and humans was at a similar level both in the cell lines and in the spontaneous osteosarcomas. This may indicate that the analyzed markers in the cell line can be a useful experimental model reflecting the biological behavior of the cells in the spontaneous osteosarcoma. Moreover, as humans and dogs share almost identical environmental conditions and the expression level of numerous markers is very similar in these two species, canine spontaneous osteosarcoma seems to be a promising model for studying the mechanism of the development of the osteosarcoma in humans.

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