

# Analysis of the expression of N-cadherin and survivin in an established D-17 cell line and in canine cells of spontaneous osteosarcoma

RAFAŁ CIAPUTA, DOMINIK PORADOWSKI\*, IZABELA JANUS, MARCIN NOWAK, BOŻENA OBMIŃSKA-MRUKOWICZ\*, JANUSZ A. MADEJ, MAŁGORZATA KANDEFER-GOLA

Department of Pathology, \*Department of Biochemistry, Pharmacology and Toxicology, Faculty of Veterinary Medicine, Wrocław University of Environmental and Life Sciences, C. K. Norwida 31, 50-375 Wrocław

Received 20.01.2015

Accepted 17.03.2015

Ciaputa R., Poradowski D., Janus I., Nowak M., Obmińska-Mrukowicz B., Madej J. A., Kandefefer-Gola M.

## Analysis of the expression of N-cadherin and survivin in an established D-17 cell line and in canine cells of spontaneous osteosarcoma

### Summary

The aim of the study was to demonstrate and evaluate the expression of N-cadherin and survivin in spontaneous osteosarcoma tumours in dogs compared to the expression of these proteins in an established (D-17) cell line of canine osteosarcoma. Osteosarcoma samples were taken from the limbs of 15 dogs and fixed in 7% buffered formalin. An adherent canine osteosarcoma cell line (D-17) was also used for the study. The cytoplasmic expression of N-cadherin and survivin was shown in spontaneous osteosarcoma, whereas the cytoplasmic and nuclear expression of N-cadherin and survivin was observed in cells derived from the D-17 cell line. The results indicate that the higher the cytoplasmic expression of N-cadherin, the higher the expression of survivin. By analyzing the strength of intercellular adhesion, which is based on the type and strength of N-cadherin expression, as well as the degree of apoptosis inhibition, one may conclude that these markers may be used as supplementary to routine tests to evaluate the degree of the tumour's malignancy and the patient's prognosis.

**Keywords:** immunohistochemistry, N-cadherin, survivin, canine osteosarcoma

Neuronal cadherin (N-cadherin) belongs to the superfamily of cell adhesion molecules (CAM), which also includes epithelial E-cadherin, placental P-cadherin, and vascular endothelial VE-cadherin (3, 5, 10). These are single-chain transmembrane glycoproteins whose function is calcium-dependent (17). Together with the  $\beta$ -catenin molecule, this protein plays an important role in the formation of intercellular connections, which maintain tissue integrity (3, 6). Moreover, it participates in the differentiation, proliferation, migration, and apoptosis of cells (10). Often, there is a disruption of intercellular communication between tumour cells, which leads to a weakening of intercellular adhesion and an increased possibility of tumour cells metastasising (8). Survivin, on the other hand, belongs to the inhibitors of apoptosis protein (IAP) family and takes part in the control of the cell cycle (9). An increased expression of survivin is noted in dysplastic lesions and cells of almost all tumour types. The expression level of this protein correlates with the degree of malignancy of the tumour: the stronger the expression, the more malignant the tumour. Physiologically, survivin is

expressed during embryonic development. The protein is also present in trace concentrations in normal cells of adults, in tissues with a high proliferation potential, which are subject to continuous renewal (such as cells of the placenta, thymic cells, CD34<sup>+</sup> hematopoietic stem cells, or epithelial cells of the large intestine) (6, 12).

Osteosarcoma is a malignant mesenchymal tumour derived from bone, which usually occurs in dogs aged 2-15 years. It is frequently diagnosed in long bones of giant and large breed dogs (2, 4, 5, 14, 15, 20). It leads to bone damage visible on X-ray and causes severe pain at the lesion site. The tumour infiltrates the surrounding soft tissue and often metastasizes to the regional lymph nodes and lungs (11, 16, 20).

The D-17 cell line, created by Nelson-Rees in 1969, is an experimental model of spontaneous osteosarcoma in the dog. This line is used in numerous research centers to conduct studies on the mechanisms of bone carcinogenesis in companion animals.

The aim of the study was to demonstrate and evaluate the expression of N-cadherin and survivin in spontaneous osteosarcoma tumours in dogs compared to the

expression of these proteins in an established (D-17) cell line of canine osteosarcoma.

## Material and methods

**Spontaneous tumours.** Osteosarcoma samples, which constituted the diagnostic material of the Department of Pathology of the Wrocław University of Environmental and Life Sciences, were taken from the limbs of 15 dogs. They were fixed in 7% buffered formalin for 2 days and then decalcified in a mixture of hydrochloric and formic acid. Subsequently, they were embedded in paraffin blocks and cut into 4 µm thick sections. The slides were stained with hematoxylin and eosin and evaluated histopathologically according to the WHO Classification of Tumors.

Immunohistochemistry was carried out on 4 µm thick paraffin blocks. They were placed on silanized glass slides (DAKO, Denmark), deparaffinized in xylene, and passed through a series of decreasing alcohol concentrations to water. The antigens of tissues fixed in formalin and cells fixed in an acetone and methanol solution were retrieved with the EnVision™ FLEX Target Retrieval Solution pH 6.0 (DAKO, Denmark) by heating in a water bath at 96°C for 20 minutes.

Endogenous peroxidase was blocked with a 3% solution of EnVision™ FLEX Peroxidase-Blocking Reagent for 5 minutes. Next, primary Monoclonal Mouse Anti-Human N-Cadherin – clone 6G11 (DAKO, Denmark) and Monoclonal Mouse Anti-Human Survivin – clone 12C4 (DAKO, Denmark) antibodies, both diluted to a 1: 50 ratio, were applied. They were incubated at room temperature for 30 minutes.

The sections were then washed 20 times in EnVision™ FLEX Wash Buffer, overlaid with an EnVision™ FLEX/HR SM802 visual system, and incubated at room temperature for 30 minutes. A 3,3'-diaminobenzidine tetrahydrochloride (DAB) EnVision™ FLEX DAB+ Chromogen DAKO solution elicited the immunohistochemical reaction. The sections were washed in distilled water, counterstained with hematoxylin, and dehydrated by being passed through a series of alcohol concentrations. The sample was made translucent with xylene and sealed.

**Cell culture.** An adherent canine osteosarcoma cell line (D-17), derived from the American Type Culture Collection-ATCC (Rockville, MD, USA), was used to assess the expression of the selected markers. The canine osteosarcoma line was grown in the RPMI-1640 (IITD PAN, Wrocław) culture medium with an addition of 10% bovine serum (Sigma, USA), 4 nM of L-glutamine (Sigma, United Kingdom), 100 U/ml of penicillin, and 100 µg/ml of streptomycin (Sigma, Germany). Next, an (0.25%) EDTA and (0.02%) trypsin solution was added to the cell culture to detach it from the culture bottle. The cells of the osteosarcoma cell line were concentrated to  $2 \times 10^5$  cells/800 µl of the culture medium (RPMI-1640). The canine osteosarcoma tumour cells, at a concentration of  $2 \times 10^4$ /40 µl of the culture medium, were placed on 10-well hydrophobic slides (Thermo Scientific, USA). The cells were incubated on the slides for 24 hours. Subsequently, the culture medium was removed, and the cells were rinsed in PBS (IITD Academy of Sciences, Wrocław). The sections were dried, and a cold

solution of acetone and methanol at a 1 : 1 ratio was applied in order to fix the cells on the slide and to permeabilize the cell membrane (they were incubated for 15 mins). The samples underwent immunohistochemical staining with the same antibodies and according to the same regimen as the samples derived from spontaneous tumours.

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

Photos of the tested sections, both those derived from spontaneous tumours and those from cell cultures, were subjected to computer-assisted image analysis by a computer coupled with an Olympus BX53 (Olympus, Japan) optical microscope. A morphometric analysis was carried out by means of the CellS software (Olympus Soft Imaging Solution GmbH, Germany).

The expression of N-cadherin and survivin was evaluated using the modified semiquantitative IRS scale according to Remmele (1, 19). The method takes into account both the proportion of positively stained cells and the intensity of the reaction color, and its final result represents both parameters, with values ranging from 0 to 12 points. Protein expression for each slide was assessed by counting a mean value from 5 high power fields (no reaction = 0 points (-); weak reaction = 1-2 points (+), moderate reaction = 3-5 points (++) , intense reaction = 6-12 points (+++)). Additionally, the percentage of cells showing membrane and cytoplasmic expression was calculated.

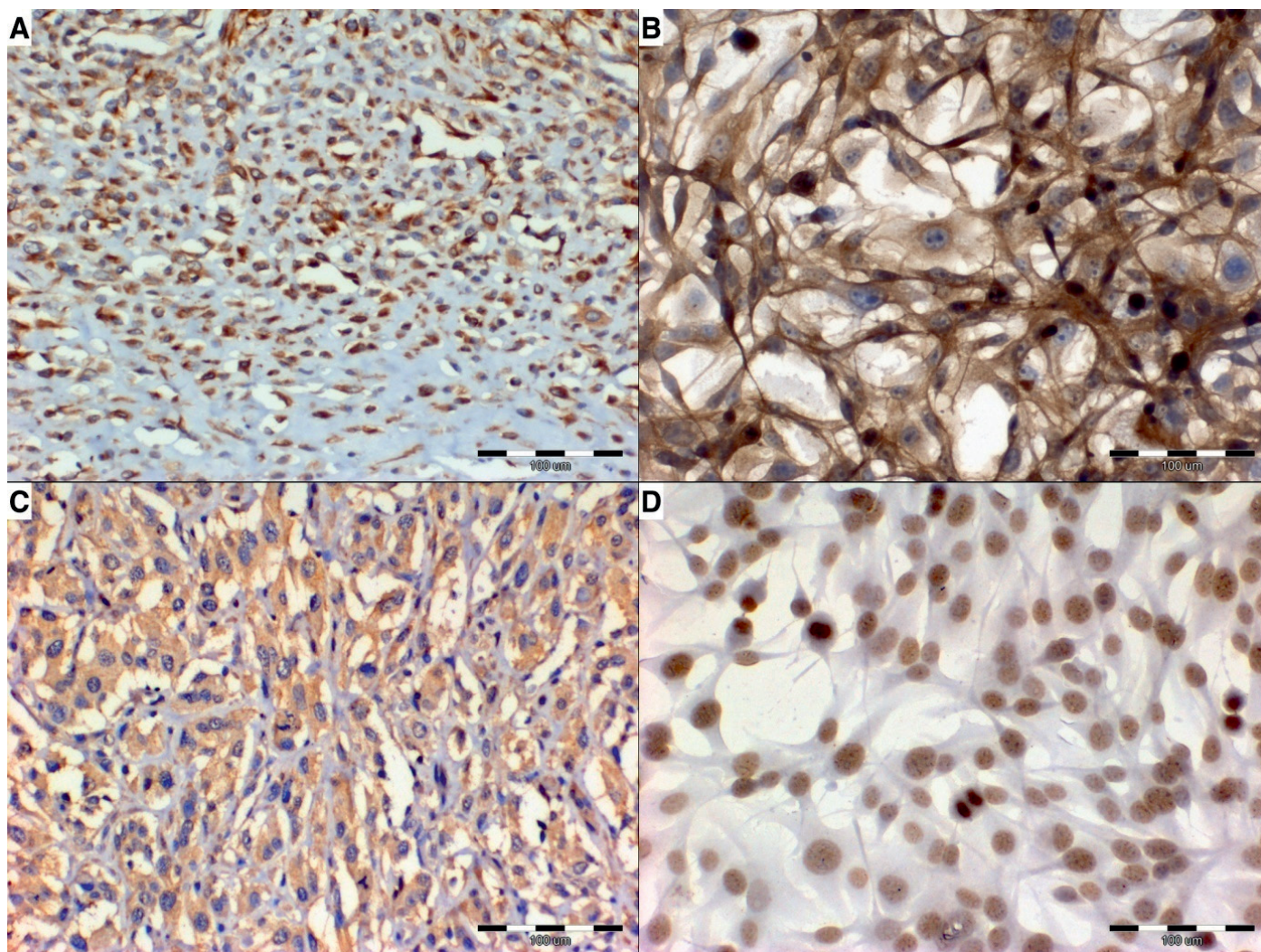
Statistical analysis was carried out using the StatisticaPL software (StatSoft, Poland). The Mann-Whitney U analysis, Wilcoxon test, and Spearman's correlation analysis were performed, and significance was set at  $p < 0.05$ .

## Results and discussion

The expression of N-cadherin in spontaneous canine osteosarcoma was given four points in 66.5% of cases and three points in 33.5% of cases. The cytoplasmic expression of survivin was given three points in 66.5% of osteosarcoma cases, two points in 13.5% of cases, and one point in 20% of cases (Fig. 1).

In the osteosarcoma cell line, the expression of N-cadherin was given twelve points and the nuclear expression of survivin was given five points (Fig. 1). There was a clear shift in the expression of N-cadherin from the cell membrane to the cytoplasm and nucleus, on average, in 86.7% of the spontaneous tumours and in all the cells of the established D-17 line (Fig. 1).

The statistical analysis revealed that there was a higher expression of the two proteins in the cell line compared to the spontaneous tumours ( $p < 0.01$ ; Fig. 2) and a greater expression of survivin than that of N-cadherin in all sections ( $p < 0.001$ ; Fig. 2). Furthermore, a strong positive correlation was found between N-cadherin and survivin ( $p < 0.05$ ;  $r = 0.89$ ) in all sections.



**Fig. 1.** The expression of the analyzed proteins in the spontaneous tumours and the established D-17 osteosarcoma cell line. A – membrane and cytoplasmic expression of N-cadherin in the spontaneous tumours, B – strong cytoplasmic expression of N-cadherin in the D-17 cell line, C – cytoplasmic expression of survivin in cells from the spontaneous tumours, D – nuclear expression of survivin in the D-17 cell line

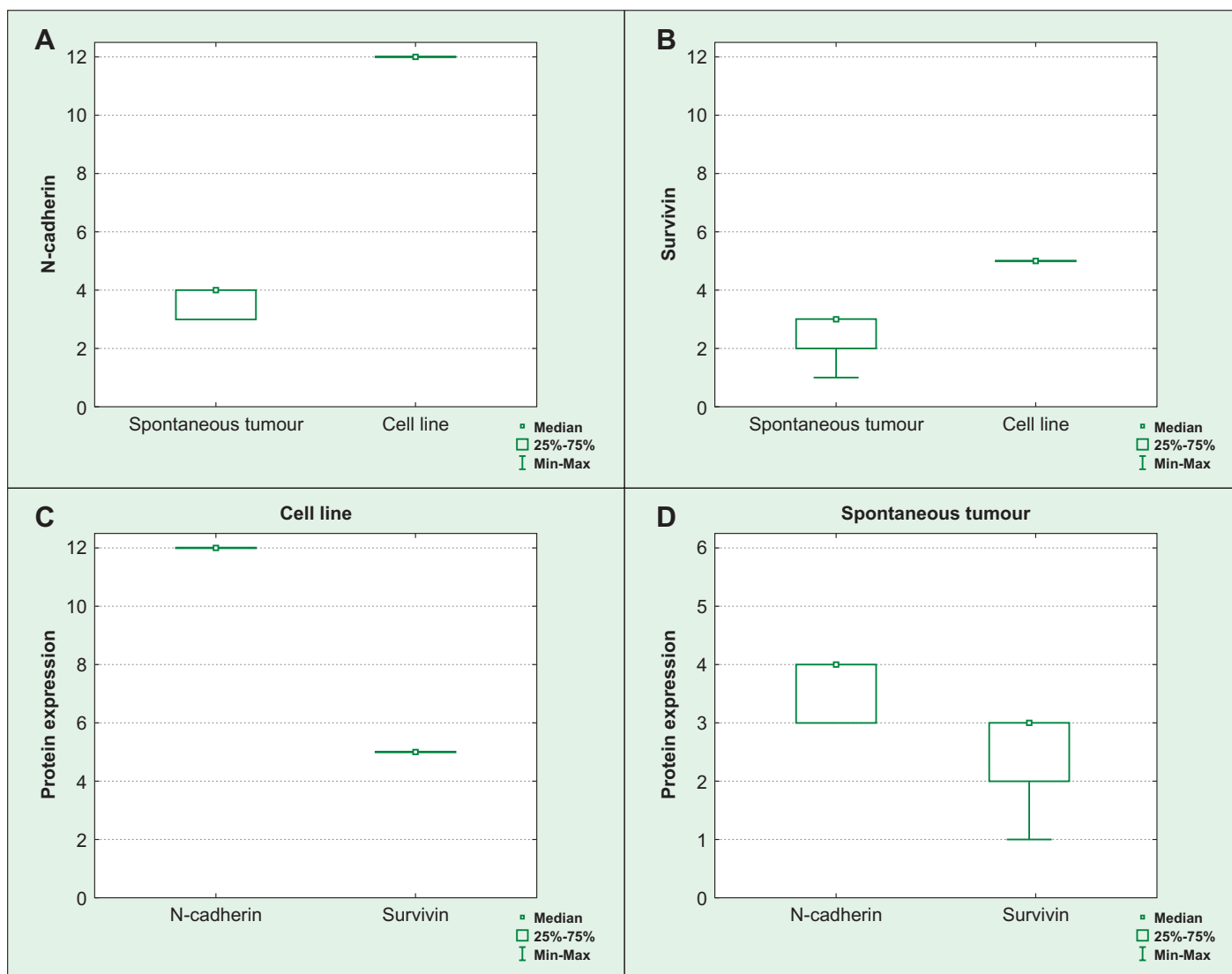
Much attention has been paid to understanding the biology of osteosarcoma, because of its incidence in the canine and human skeletal systems as well as its aggressive nature. The cell markers used in this study are applied to evaluate the adhesion, proliferation, and apoptosis of various types of tumours (7, 12). Therefore, determining the expression level of these proteins is helpful in assessing the patient's prognosis (19).

Cells from the D-17 line showed a stronger cytoplasmic expression of N-cadherin compared to cells of spontaneous osteosarcoma. In spontaneous tumours, there was also a membrane expression of N-cadherin, although to a lesser extent.

The higher expression of both N-cadherin and survivin in the cell line compared to cells from spontaneous tumours may have been caused by the fact that these cells were derived from an established (immortal) line. In such a line, cells are proliferated under optimal culture conditions, which leads to their having constant protein expression levels.

On the other hand, the development and proliferation of cells from spontaneous tumours are influenced by many factors. In addition, the lack of intercellular junctions in the D-17 cell line may explain the difference in the expression of N-cadherin compared to cells from spontaneous tumours, where these junctions were present to a varying degree.

It is clear that there was a higher expression of N-cadherin than that of survivin in both the cells from the established osteosarcoma line and those from the spontaneous tumours. Similar results were obtained by Iurlaro et al. (6), who used E-cadherin and survivin in their research on ovarian cancer in women. Since both E-cadherin and N-cadherin belong to the superfamily of proteins responsible for intercellular adhesion, this may explain the analogy in their expression. A similar tendency can be found in the expression of these proteins in the cells of the D-17 osteosarcoma cell line, where the difference in the expression levels of N-cadherin and survivin is more visible than in the case of spontaneous tumours (6). Iurlaro et al. (6) carried



**Fig. 2.** The expression of the analyzed proteins in the spontaneous tumours and the D-17 cell line. A – expression of N-cadherin in the spontaneous tumours and the D-17 cell line, B – expression of survivin in the spontaneous tumours and the D-17 cell line, C – expression of N-cadherin and survivin in the D-17 cell line, D – expression of N-cadherin and survivin in the spontaneous tumours

out their study on normal human cell lines (primary pancreatic cell and keratinocyte cultures) and neoplastic cells (five different lines of pancreatic tumours and two lines of neoplastic keratinocytes) and showed a similar correlation between the expression levels of the markers (6).

The strong positive correlation between the expression of survivin and cytoplasmic N-cadherin may be explained by the simultaneous increase in the immunoreactivity of both these proteins, which may, in turn, be associated with malignancy (18). The results obtained may be associated with the effect cadherins have on the synthesis of survivin in canine osteosarcoma, in both spontaneous tumours and in the cell line. This was confirmed by Iurlaro et al. (6) in ovarian and pancreatic cancer in humans.

In summary, these results indicate that the higher the cytoplasmic expression of N-cadherin, the higher the expression of survivin. This relationship is specific not only to neoplasms in humans, but also in animals,

as shown by the results of this study. By analyzing the strength of intercellular adhesion, which is based on the type and strength of N-cadherin expression, as well as the degree of apoptosis inhibition, one can conclude that these markers may be used as supplementary to routine tests to evaluate the degree of the tumour's malignancy and the patient's prognosis.

## References

- Brouckaert O., Paridaens R., Floris G., Rakha E., Osborne K., Neven P.: A critical review why assessment of steroid hormone receptors in breast cancer should be quantitative. *Ann. Oncol.* 2013, 24, 47-53.
- Burk R., Feeney A.: *Small Animal Radiology and Ultrasonography: a Diagnostic Atlas and Text.* Saunders, St. Louis 2003, p. 506.
- Ferrari S. L., Traianedes K., Thorne M., Lafage-Proust M. H., Genever P., Cecchini M. G., Behar V., Bisello A., Chorev M., Rosenblatt M., Suva L. J.: A role for N-cadherin in the development of the differentiated osteoblastic phenotype. *J. Bone Miner. Res.* 2000, 15, 198-208.
- Gârjoabă I., Tudor N., Soare T., Tănase A., Alistar A., Vlăgioiu C.: A study on the prevalence of skeletal osteosarcoma in dogs and cats, *Lucrări. Stiințifice. Medicină. Veterinară.* 2009, 42, 102-106.
- Holmberg B. J., Farese J. P., Taylor D., Uhl E. W.: Osteosarcoma of the humeral head associated with osteochondritis dissecans in a dog. *J. Am. Anim. Hosp. Assoc.* 2004, 40, 246-249.

6. Iurlaro M., Demontis F., Corada M., Zanetta L., Drake C., Gariboldi M., Peiro S., Cano A., Navarro P., Cattelino A., Tognin S., Marchisio P. C., Dejana E.: VE-cadherin expression and clustering maintain low levels of survivin in endothelial cells. *Am. J. Pathol.* 2004, 165, 181-189.
7. Kashima T., Nakamura K., Kawaguchi J., Takanashi M., Ishida T., Aburatani H., Kudo A., Fukayama M., Grigoriadis A. E.: Overexpression of cadherins suppresses pulmonary metastasis of osteosarcoma in vivo. *Int. J. Cancer* 2003, 104, 147-154.
8. Kashima T., Kawaguchi J., Takeshita S., Kuroda M., Takanashi M., Horiuchi H., Imamura T., Ishikawa Y., Ishida T., Mori S., Machinami R., Kudo A.: Anomalous cadherin expression in osteosarcoma. Possible relationships to metastasis and morphogenesis. *Am. J. Pathol.* 1999, 155, 1549-1555.
9. Liang X., Da M., Zhuang Z., Wu W., Wu Z., Wu Y., Shen H.: Effects of survivin on cell proliferation and apoptosis in MG-63 cells in vitro. *Cell. Biol. Int.* 2009, 33, 119-124.
10. Marie P. J.: Role of N-cadherin in bone formation. *J. Cell. Physiol.* 2002, 190, 297-305.
11. Mullins M. N., Lana S. E., Dernell W. S., Ogilvie G. K., Withrow S. J., Ehrhart E. J.: Cyclooxygenase-2 expression in canine appendicular osteosarcomas. *J. Vet. Intern. Med.* 2004, 18, 859-865.
12. Osaka E., Suzuki T., Osaka S., Yoshida Y., Sugita H., Asami S., Tabata K., Sugitani M., Nemoto N., Ryu J.: Survivin expression levels as independent predictors of survival for osteosarcoma patients. *J. Orthop. Res.* 2007, 25, 116-121.
13. Remmele W., Stegner H. E.: Recommendation for uniform definition of an immunoreactive score (IRS) for immunohistochemical estrogen receptor detection (ER-ICA) in breast cancer tissue. *Pathologie* 1987, 8, 138-140.
14. Rosenberger J. A., Pablo N. V., Crawford P. C.: Prevalence of and intrinsic risk factors for appendicular osteosarcoma in dogs: 179 cases (1996-2005). *J. Am. Vet. Med. Assoc.* 2007, 231, 1076-1080.
15. Selvarajaha G. T., Kirpensteijn J.: Prognostic and predictive biomarkers of canine osteosarcoma. *Veterinary Journal* 2010, 185, 28-35.
16. Spodnick G. J., Berg J., Rand W. M., Schelling S. H., Couto G., Harvey H. J., Henderson R. A., MacEwen G., Mauldin N., McCaw D. L., et al.: Prognosis for dogs with appendicular osteosarcoma treated by amputation alone: 162 cases (1978-1988). *J. Am. Vet. Med. Assoc.* 1992, 200, 995-999.
17. Takeichi M.: Cadherin cell adhesion receptors as a morphogenetic regulator. *Science* 1991, 25, 1451-1455.
18. Takeichi M.: Cadherin in cancer: implications for invasion and metastasis. *Curr. Opin. Cell. Biol.* 1993, 5, 806-811.
19. Trieb K., Lehner R., Stulnig T., Sulzbacher I., Shroyer K. R.: Survivin expression in human osteosarcoma is a marker for survival. *Eur. J. Surg. Oncol.* 2003, 29, 379-382.
20. Withrow S. J., Vail M. D.: Withrow and MacEwen's Small Animal Clinical Oncology, Saunders, St. Louis 2007, pp. 540-544.

**Corresponding author: lek. wet. Rafał Ciaputa, ul. C. K. Norwida 31, 50-375 Wrocław, Poland; e-mail: rafal.ciaputa@up.wroc.pl**