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Expression of E-Cadherin, β-Catenin and Ki-67 Antigen and their Reciprocal Correlations in Fibrosarcomas of Soft Tissues in Dogs

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Abstract. *Background: This study aimed at immunocytochemical demonstration of E-cadherin, β-catenin and Ki-67 expressions and the examination of correlation between these markers in primary fibrosarcomas in dogs. Materials and Methods: Material for the study was sampled in the course of surgery from 24 mongrel dogs aged 5 to 16 years. The neoplastic tumors were subjected to histopathological verification and immunohistochemical reactions were performed to detect the studied markers. Microphotographs of the preparations were subjected to computer-assisted image analysis using the MultiScaneBase V 14.02 software. Results: Expression of β-catenin was detected in all tumours examined while E-cadherin was expressed in only 8.2%. Expression of the Ki-67 proliferation-associated antigen was noted in over 33% of the tumours. Conclusion: The lack of correlation (r=–0.1035) between expression of Ki-67 and that of β-catenin detected here in contrast to the high values of Ki-67 antigen found as a prognostic factor in many other studies allowed us to conclude that the presence of β-catenin in cells of soft tissue fibrosarcoma in dogs manifested no unequivocal relationship to augmented proliferative potential of neoplastic cells, although it did not exclude participation of the protein in the development of this neoplasia in dogs.*

Soft tissue sarcomas (STS) are malignant tumours developing mainly from the mesoderm. They constitute around 1% of all malignant tumours in humans. Regrettably, their incompletely recognised etiopathogenesis and relatively ineffective therapy cause this group of tumours to pose a significant problem in oncology (1). In Poland, it is estimated that around 800 new cases of the disease develop annually (2). In veterinary medicine, tumours classified as soft tissue sarcomas form over 14% of all diagnosed malignant lesions in dogs, or in other words almost every seventh malignant tumour belongs to this group (3). In dogs, the dominating group of soft tissue sarcomas involves fibrosarcomas. The remaining tumours of the group, *i.e.* myxosarcomas, liposarcomas and leiomyosarcomatous myomas, account for no more than 3% of all mesenchymal tumours of the skin and subcutaneous tissue (4, 5). The problem, not only in veterinary medicine but also in medicine in general, involves the relatively late detection of tumours in the group resulting in the fact that patients report to the doctor with already highly advanced neoplasms. One of the reasons for the delayed diagnosis involves the hesitance of the patient to consult a doctor since the tumour as a rule induces no symptoms so its development does not bother the patient (2). In such a situation, accurate evaluation of histological malignancy (grading, G) represents an important element of histological diagnosis, allowing prognosis as to course of the disease. However, it is frequently insufficient to base evaluation of the G grade on a subjective estimation of cellular pleomorphism, mitotic activity or spread of necrotic foci in the tumour. In such a situation, attempts are made to take advantage of new markers of cell proliferation and metastatic activity (*e.g.* E-cadherin and β-catenin) which might allow the prognosis to be defined more precisely.

Cadherins belong to the transmembrane, calcium ion-dependent protein group. Experimental elimination of the calcium ion from cellular environment results in proteolytic destruction of the adherence molecule and in loss of its function (6, 7). The family of cadherins includes three types

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Table I. *Semi-quantitative immunoreactive score (IRS) taking into account both the percentage of positively stained cells (A) and the intensity of reaction product (B) in which the final results correspond to the product of the two variables (AxB).*

Point score	A	B
0	No cells with positive reaction	No colour reaction
1	≤10% Cells with positive reaction	Low intensity of colour reaction
2	11-50% Cells with positive reaction	Average intensity of colour reaction
3	51-80% Cells with positive reaction	Intense colour reaction
4	>80% Cells with positive reaction	

of molecules: E-cadherin (participating in epithelial cell adherence), N-cadherin (involved in adhesion of nerve cells) and P-cadherin (mediating adhesion of placental cells). Cadherins link neighbouring cells by interacting with other cadherins, forming homophilic bonds through the *N*-terminal amino acid sequence, His-Ala-Val (HAV). The cytoplasmic domain of E-cadherin interacts with the group of reciprocally linked proteins termed catenins (α, β and γ). Both β and γ-catenins compete with each other for a direct binding to E-cadherin, while α-catenin links E-cadherin to actin F and α-actinin, forming the cell cytoskeleton (8). Disturbed function of the cadherin-catenin complex, resulting from dysfunction or deficiency of catenins or cadherin, clearly decreases the ability of cells to adhere, disturbs their differentiation and augments the invasive potential of a tumour (8). Such disturbances in expression of both cadherin and catenins may reflect mutations (*e.g. null* type of the E-cadherin gene on chromosome 16q or of the β-catenin gene on chromosome 3p21). In humans such mutations may give rise to the invasive phenotype of cells (7). On the other hand, some authors point to epigenetic lesions as a cause of disturbances in the E-cadherin–β-catenin system, resulting in a reduced expression of E-cadherin and, thus, in reduced cellular adhesion, promoting release of potentially metastatic cells from a tumour (6). The direct proof of the high significance of the E-cadherin expression level for the development of the tumour cell malignant phenotype was provided by experiments conducted on cell lines of mouse mammary carcinoma. Following transfection of E-cadherin cDNA, the authors demonstrated a significantly reduced invasiveness of the cancer cells (9). A similar dependence was demonstrated in a group of sarcomas. Sun *et al.* (10) examined 72 cases of sarcoma in humans and noted marked elongation of survival in patients with preserved expression of E-cadherin.

Thus, the E-cadherin–β-catenin molecular system not only participates in adhesion mechanisms but also plays an important role in cell growth and transformation processes, while any disturbances of the axis may promote neoplastic transformation.

Our study aimed at the demonstration of E-cadherin and β-catenin expression as compared to the expression of the proliferation-associated antigen Ki-67 in soft tissue fibrosarcomas of dogs.

Materials and Methods

Material for the studies was sampled during surgery in 24 dogs of various breeds, aged 5 to 16 years. The tumours were verified as fibrosarcomas by histopathology.

Formalin-fixed, paraffin-embedded tissue was freshly cut (4 μm). The sections were mounted on Superfrost slides (Menzel Gläser, Germany), dewaxed with xylene and gradually hydrated. Activity of endogenous peroxidise was blocked by 5 min exposure to 3% H_2O_2. Detection of E-cadherin, β-catenin and Ki-67 antigen expression was preceded by 15 min exposure of the sections in a microwave oven to boiling Antigen Retrieval Solution (DakoCytomation, Denmark) at 250 W. For demonstration of E-cadherin, β-catenin and Ki-67 antigen expression in the paraffin sections, mouse monoclonal antibodies were used in the following concentrations: clone NCH-38 (1:150) (DakoCytomation, Denmark); clone β-catenin-1 (1:200) (DakoCytomation); clone MIB-1 (1:100) (DakoCytomation). The antibodies were diluted in the Background Reducing Antibody Diluent (DakoCytomation). The sections were incubated with an antibody for 1 h at room temperature. Subsequently, incubations were performed with biotinylated antibodies (15 min, room temperature) and with streptavidin-biotinylated peroxidise complex (15 min, room temperature) (LSAB2, HRP; DakoCytomation). DAB (DakoCytomation) was used as a chromogen (7 min, room temperature). All the sections were counterstained with Meyer's haematoxylin. In every case, controls were included in which specific antibody was substituted by the Primary Negative Control (DakoCytomation).

Microphotographs of the obtained preparations were subjected to computer-assisted image analysis *via* a computer coupled to Axiophot optical microscope (Carl Zeiss, Germany). The set had the potential to record images and to perform their digital analysis. The measurements took advantage of the MultiScanBase V 14.02 software (Computer Scanning Systems, Warszawa, Poland).

Expression of E-cadherin and β-catenin was appraised using the modified semiquantitative IRS scale according to Remmele (Table I) (11). The method takes into account both the proportion of positively stained cells and the intensity of the reaction colour, while its final result represents the product of the parameters, with values ranging from 0 to 12 points: no reaction = 0 points (–); weak reaction = 1-2 points (+), moderate reaction = 3-4 points (++), intense reaction = 6-12 points (+++). Expression of Ki-67 was evaluated quantitatively by estimation of the percentage of positive cells: (0-5% = no reaction (–), 6-25% = weak reaction

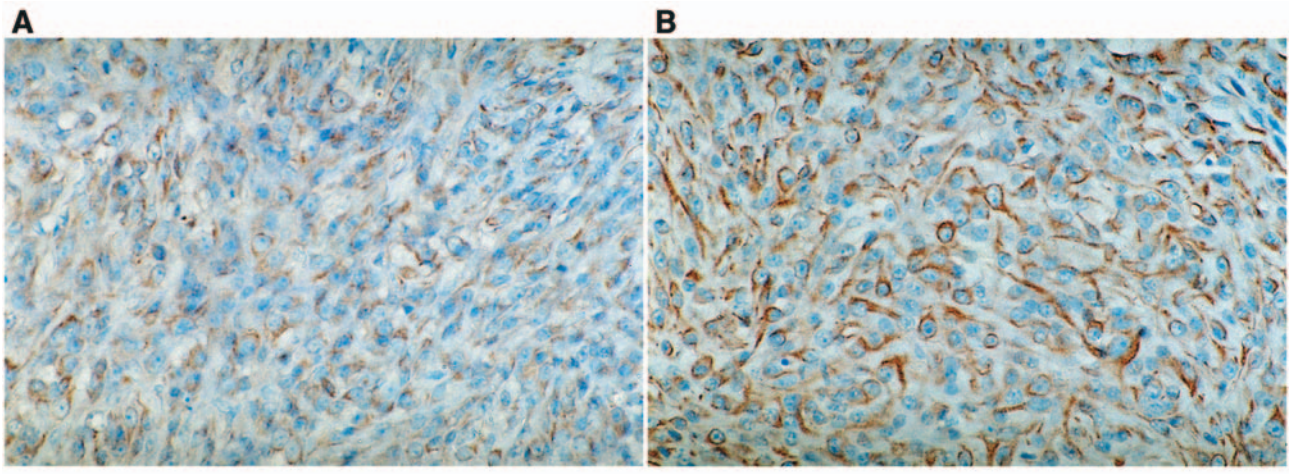

Figure 1. *Expression of E-cadherin in cells of canine fibrosarcoma. A) Low expression, B) high expression. (x400, counterstained with haematoxylin).*

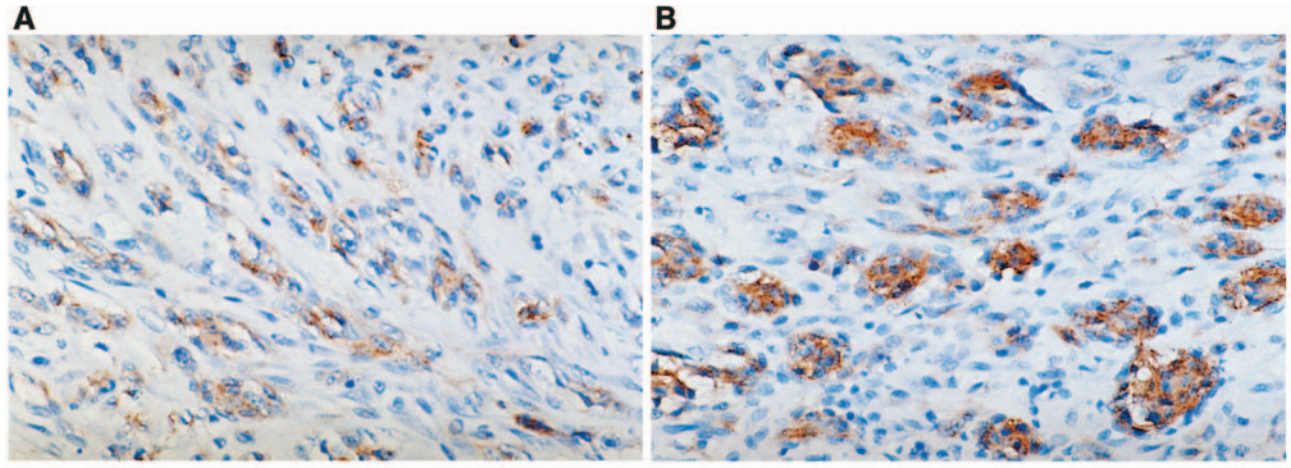

Figure 2. *Expression of β-catenin in cells of canine fibrosarcoma. A) Low expression, B) high expression. (x400, counterstained with haematoxylin).*

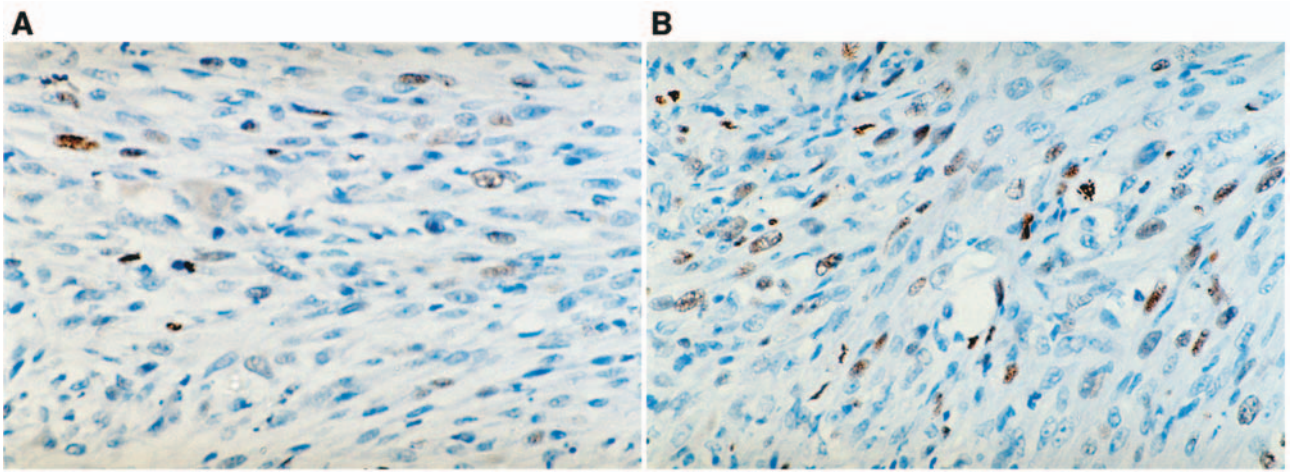

Figure 3. *Expression of Ki-67 in cells of canine fibrosarcoma. A) Low expression, B) high expression. (x400, counterstained with haematoxylin).*

(+), 26-50% = moderate reaction (++), above 50% = intense reaction (+++). The results were subjected to statistical analysis using Statistica PL software (STATSoft, Krakow, Poland) employing Spearman's correlation analysis.

Results

In soft tissue fibrosarcomas of dogs, expression was demonstrated of Ki-67 antigen, E-cadherin and β-catenin (Figures 1-3). The immunocytochemical technique is one of the most frequently employed ways of detecting E-cadherin and β-catenin in both normal and neoplastic cells. It permits evaluation under light microscopy of the expression of the proteins, their localisation in the cell (cell nucleus, cytoplasm) and the intensity of the reaction. Expression of β-catenin was noted in all tumours examined while E-cadherin was detected in only 8.2% tumours, *i.e.* the adhesion protein was not expressed in over 91% tumours.

Evident differences were noted in intensity of the protein expression in our results. In 25% tumours β-catenin expression was noted at + intensity, in over 20% at ++, and in over 54% at +++ intensity. In the case of E-cadherin, over 4% of the tumours examined showed expression of the protein at the + level and over 4% at the +++ level. It should be mentioned that expression of Ki-67 proliferation-associated antigen was noted at the + or ++ level in over 33% of tumours examined; in none of the samples did the expression reach +++ intensity. In over 66% of the tumours examined, no expression of this protein could be detected. Among the tumours which showed no expression of Ki-67, over 56% manifested intense (6-12 points), 25% moderate (3-4 points) and almost 19% a weak (1-2 points) expression of β-catenin. It should be added that in over 66% tumours with strong expression of Ki-67, an intense (6-12 points) or moderate (3-4 points) expression of β-catenin was detected. Expression of E-cadherin manifested a slightly different pattern since all tumours with expression of this molecule demonstrated absence or weak expression (+) of the Ki-67 antigen. Tumours which exhibited expression of E-cadherin presented expression of β-catenin at a similar level.

The statistical analysis conducted on the tumours using Spearman's rank correlation demonstrated an absence of a relationship between expressions of β-catenin and Ki-67 antigen (correlation coefficient r=–0.1035) (Figure 4). Analysis of potential correlations between levels of E-cadherin expression and the remaining markers could not be performed due to the very low number of cases manifesting expression of the protein.

Discussion

β-Catenin exhibits a binary involvement in processes of neoplastic transformation. On one hand, formation of its complex with E-cadherin represents an indispensable element of normal intercellular adhesion, the weakening of which promotes development of metastases. On the other hand, involvement of β-catenin in the Wnt (Wingless-type murine mammary tumor virus integration site)-dependent signalling pathway exerts a basic influence on cell proliferation and differentiation and is important for neoplastic transformation. Cytoplasmic levels of β-catenin are controlled by the activity of glycogen kinase3-β synthase (GSK-3β). The phosphorylated β-catenin binds to ubiquitin, which is followed by its degradation in a proteasome (12). When activity of GSK-3β is blocked by *e.g.* ligands of Wnt (glycoproteins of weak oncogenic properties, transforming *e.g.* Wnt-1, -3A, -8 and 8B (l3)), which may develop in neoplastically transformed cells, it accumulates in the cytoplasm and it is translocated to the cell nucleus. The β-catenin molecules imported to the cell nucleus form complexes with the transcription factors TCF/LEF, which results in the induction of the transcription of genes participating in the control of the cell cycle, apoptosis, proliferation and progression of the tumour, *i.e. cyclin D, c-myc, tcf-1* (7, 12-15).

In evaluation of the aggressiveness of tumours, including soft tissue sarcomas, examination of cell proliferative activity by appraisal of Ki-67 antigen expression is of principal value (16-19). The antigen belongs to the group of non-histone proteins present in the cell nucleus (20). Expression of Ki-67 is detected as easly as the G_1-phase of the cell cycle. It evidently increases in S- and G_2-phase, reaching a peak in the M-phase and manifesting an abrupt disappearance in G_0. Therefore, the protein can be detected only in proliferating cells (21). The ratio of cells manifesting Ki-67 expression to those which manifest no such expression used to be termed the proliferation index, which reflects the mitotic activity of the cells (22).

In our studies on soft tissue fibrosarcomas in dogs, expression of the Ki-67 antigen was demonstrated in 33% tumours, expression of E-cadherin in only 8.2% tumours and that of β-catenin was noted in all tumour samples examined. Very similar results of E-cadherin expression were obtained by Okamoto *et al.* (23), who failed to detect expression of the adhesion molecule in 90% of human sarcomas examined. Our results are also similar to those obtained by Sun *et al.* (10), who examined 72 cases of sarcoma and detected evidently reduced expression of E-cadherin in almost 78% of cases. The authors also showed that reduced expression of E-cadherin increased the potential for relapse, the development of metastases and also clearly deteriorated prognosis. Yoo *et al.* (24) examined 91 cases of soft tissue sarcomas and detected expression of E-cadherin in just 12% of tumours. Moreover, the authors detected lack of correlation between the expression of E-cadherin and that of p53 protein.

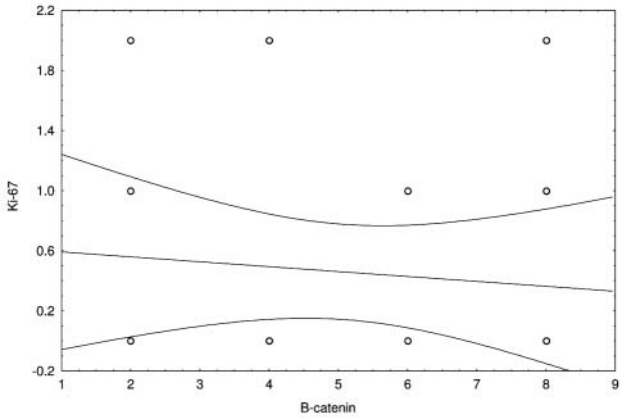

Figure 4. *Graph of the correlation between expressions of β-catenin and Ki-67 antigen in canine fibrosarcoma. Correlation coefficient r=–0.1035; p<0.05.*

Statistical analysis conducted using Spearman's rank correlation test detected no correlation between the expression of β-catenin and that of Ki-67 antigen. The results might suggest that accumulation of β-catenin in canine soft tissue sarcomas exhibits no unequivocal relationship with the intensity of tumour cell proliferation. However, this does not exclude an involvement of the protein in the development of neoplastic transformation in dogs. Our results are consistent with the observations of other authors, *e.g.* those of Sakamoto *et al.* (25), who noted in a few cases only a correlation between the expression of β-catenin and the proliferation of cells. On the other hand, Saito *et al.* (26) examined 72 cases of sarcoma and documented a relationship between the expression of β-catenin and the proliferative potential (expression of Ki-67 antigen).

In summary, it should be stressed that in view of certain divergencies related to expression and co-expression of studied proteins in human sarcomas and the very restricted knowledge on alterations of such markers in dogs, further studies on these topics are required. In view of the significant and confirmed involvement of Wnt/β-catenin signalling pathway in carcinogenesis, the search continues for effective drugs directly influencing the proteins which control this process. Much hope has been placed on inhibitors of the Wnt pathway, currently at the stage of testing (13, 27). Finally, it may be added that a similar behaviour of the studied proteins in soft tissue sarcomas of humans and animals may permit use of the animal model in studies on tumour development in humans.

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