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Expression of E-cadherin, β-catenin and Ki-67 antigen and their reciprocal relationships in mammary adenocarcinomas in bitches

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Abstract: In progression of tumours, resulting from, *i.e.*, release of cells from the parental tumour and development of metastases, expression of cell adhesion molecules (CAM) plays a significant role. CAM, including E-cadherin and the linked to it β-catenin, determine the extent of adhesion between normal and neoplastically altered cells. Moreover, the unbound form of β-catenin in a cell nucleus may affect the rate of cell proliferation This study aimed at demonstrating intensity and localisation of E-cadherin and β-catenin expression as related to expression of the proliferation-associated antigen, Ki-67 in mammary adenocarcinomas of bitches. The study was performed on 35 cases of the above mentioned tumours. On paraffin sections immunohistochemical reactions were performed using monoclonal antibodies directed against E-cadherin, β-catenin and Ki-67 antigen. In the studies a membranous expression of E-cadherin, a cytoplasmic-nuclear expression of β-catenin and nuclear expression of Ki-67 antigen were demonstrated. Statistical calculations using Spearman's test demonstrated a pronounced positive correlation between expression of β-catenin and Ki-67 antigen and absence of correlation between expression of E-cadherin and Ki-67 antigen. No correlation could be detected between expression intensities of E-cadherin and β-catenin.

Key words: E-cadherin - β-catenin - Ki-67 - Adenocarcinoma - Dogs

Introduction

Mammary carcinoma is the most frequent malignant tumour in women worldwide. Despite application of increasingly effective diagnostic procedures and increasingly successful therapies mortality induced by the tumour remains very high. The most frequent cause of death despite the applied radical therapy involves appearance and development of distant metastases and of the local relapse. Development of metastatic potential requires that the neoplastic cells acquire the so called invasive phenotype. The phenotype allows the neoplastic cells to translocate, infiltrate surrounding tissues and to home in places distant from the parental tumour [1].

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Neoplastic progression, resulting from, *i.e.*, release of cells from the primary tumour and transgression of natural barriers (*e.g.*, basement membrane), expression of cell adhesion molecules (CEM) is of a high significance [2]. Four principal groups of such molecules are distinguished, including cadherins, selectins, immunoglobulin-resembling molecules and integrins. The molecules are important for development of links between cells and extracellular matrix (ECM). However, it should be added that the role of CAM in the body is not restricted to development and function of intercellular bonds: they also play crucial roles as effector factors in intracellular transmission of signals, which allows to control the extent of adhesion depending on intensity of phosphorylation processes in the cell [3].

Cadherins belong to the group of Ca^{2+}-dependent transmembrane proteins [3,4]. The family of cadherins includes three types of molecules, including E-cadherin (participating in adhesion of epithelial cells), N-cadherin (participating in adhesion of nerve cells) and P-cadherin (securing adhesion of placental cells).

Cytoplasmic domain of E-cadherin interacts with a group of reciprocally bound proteins, termed catenins (α, β and γ). β and γ-catenins compete with each other for a direct binding of E-cadherin, while β-catenin links the latter with F actin and α-actinin, participating in formation of cell cytoskeleton [5]. A disturbed function of cadherin-catenin complex, resulting from dysfunction or deficiency of catenins or cadherin, markedly restricts cellular potential to adhere, disturbs differentiation of the cells and augments invasive potential of a tumour [5]. A direct proof for a high significance of E-cadherin expression level for the development of malignant phenotype of tumour cells was provided by experiments on cell lines of mouse mammary carcinoma. Following transfection of E-cadherin cDNA, the authors detected a marked decrease in invasiveness of the cancer cells [6]. β-catenin level in cytoplasm is controlled by activity of glycogen synthase kinase-3 β (GSK-3β), which phosphorylates excess of such molecules. When activity of GSK-3 β is blocked by, *e.g.*, ligands of Wnts (glycoproteins of oncogenic/transforming properties), which may take place in neoplastically transformed cells, its accumulation develops in the cytoplasm with the subsequent translocation to cell nucleus. Molecules of β-catenin which penetrate cell nucleus induce conversion of TCF4 transcription factor from a repressor to an activator. This, in turn, stimulates activity of genes the products of which involve proteins responsible for, *i.e.*, control of cell cycle (*e.g.*, cyclin D1). Enhanced expression of type D cyclins results in augmented sensitivity of cells to action of growth agents, thus stimulating cell divisions and promoting neoplastic transformation of cells [3].

Hence, apart from its role in adhesion mechanisms, the E-cadherin-β-catenin molecular complex is involved in processes of cell growth and transformation and any disturbances in the system may promote neoplastic growth.

Our studies aimed at demonstrating intensity and localisation in expression of E-cadherin and β-catenin as related to expression of proliferation-associated antigen Ki-67 in mammary adenocarcinomas in bitches. They also aimed at comparing the obtained results with those of studies conducted on human tumours. Analogous results would provide rationale for using adenocarcinomas in bitches as an experimental system in studies on biology of human tumours.

Materials and methods

The material for studies was sampled in the course of surgery from 35 bitches of various races, aging 6 to 16 years, diagnosed with a mammary tumour. The tumours were verified histopathologically.

Tumour samples were fixed in 10% buffered formalin, dehydrated and embedded in paraffin. For detection of expression of E-cadherin, β-catenin and Ki-67 antigen in paraffin sections mouse monoclonal antibodies were used, involving, respectively, clone NCH-38 (1:150); clone β-catenin-1 (1:200); clone MIB-1 (1:100). Presence of the antigens was visualized using LSAB2+ kit of reagents and diaminobenzidine (DAB). The studied paraffin sections were subjected to microwave boiling in Antigen Retrieval Solution to unblock antigenic determinants. Every experiment was accompanied by a negative control using Primary Negative Control. All antibodies and reagents originated from DakoCytomation.

The obtained preparations were used for photographic documentation and the microphotographs were subjected to computer-assisted image analysis using a computer coupled to Axiophot microscope (Carl Zeiss). The system had the potential to document images and to perform their digital analysis. The measurements took advantage of MultiScaneBase V 14.02 software working in the Windows environment.

Expression of E-cadherin and of β-catenin took advantage of the modified semi-quantitative IRS scale according to Remmele (Table 1) [7]. The method took into account both percentage of positive cells and intensity of the reaction colour, and the final score represented a product of the values, ranging from 0 to 12 points (no reaction 0 pts. [-], weak reaction 1-2 pts. [+], moderate reaction 3-4 pts. [++], intense reaction 6-12 pts. [+++]). Expression of Ki-67 was also evaluated semi-quantitatively, percent of immunopositive cells (0-5% - no reaction [-], 6- 25% weak reaction [+], 26-50%, moderate reaction [++], above 50% intense reaction [+++]). The results were subjected to statistical analysis using Statistica PL software (StatSoft, Poland) and Spearman's correlation analysis.

Results

In studies performed on mammary gland adenocarcinomas of bitches expression of both E-cadherin (a membraneous reaction pattern) and β-catenin (a cytoplasmic reaction mainly, in few cells accompanied by a nuclear reaction pattern) (Fig. 1-4). Moderate or high levels of E-cadherin expression were detected in over 65% and of β-catenin in almost 49% of examined tumours. Evident differences were noted in expression intensity of the examined proteins (Fig. 6). In over 25% tumours E-cadherin expression was appraised at +, in over 31% tumours at ++, and in over 34% tumours at +++. In the case of β-catenin, expression of the protein at the level of + was detected in over 51% of examined tumours, at the level of ++ in almost 26% tumours, at +++ in almost 23% tumours. It should be noted that expression of Ki-67 proliferation associated antigen (a nuclear reaction pattern) at the level of at least + was detected in almost 54% tumours, including slightly below 3% tumours at the level of +++ (Fig. 5 and 6). Over 45% of examined tumours manifested no expression of the protein. Out of the Ki-67 negative tumours, 75% manifested in parallel a moderate (3-4 pts.) or intense (6-12 pts.) expression of E-cadherin. Expression of β-catenin presented a slightly different pattern since almost 88% tumours which showed no Ki-67 presence demonstrated in parallel catenin axpression at the very low level of 1-2 pts. Among tumours with strong expression of E-cadherin (6-12 pts.), 50 % demonstrated moderate (3-4 pts.) to intense (6-12 pts.) expression of β-catenin. Slightly lower proportion (slightly less than 38% tumours with high

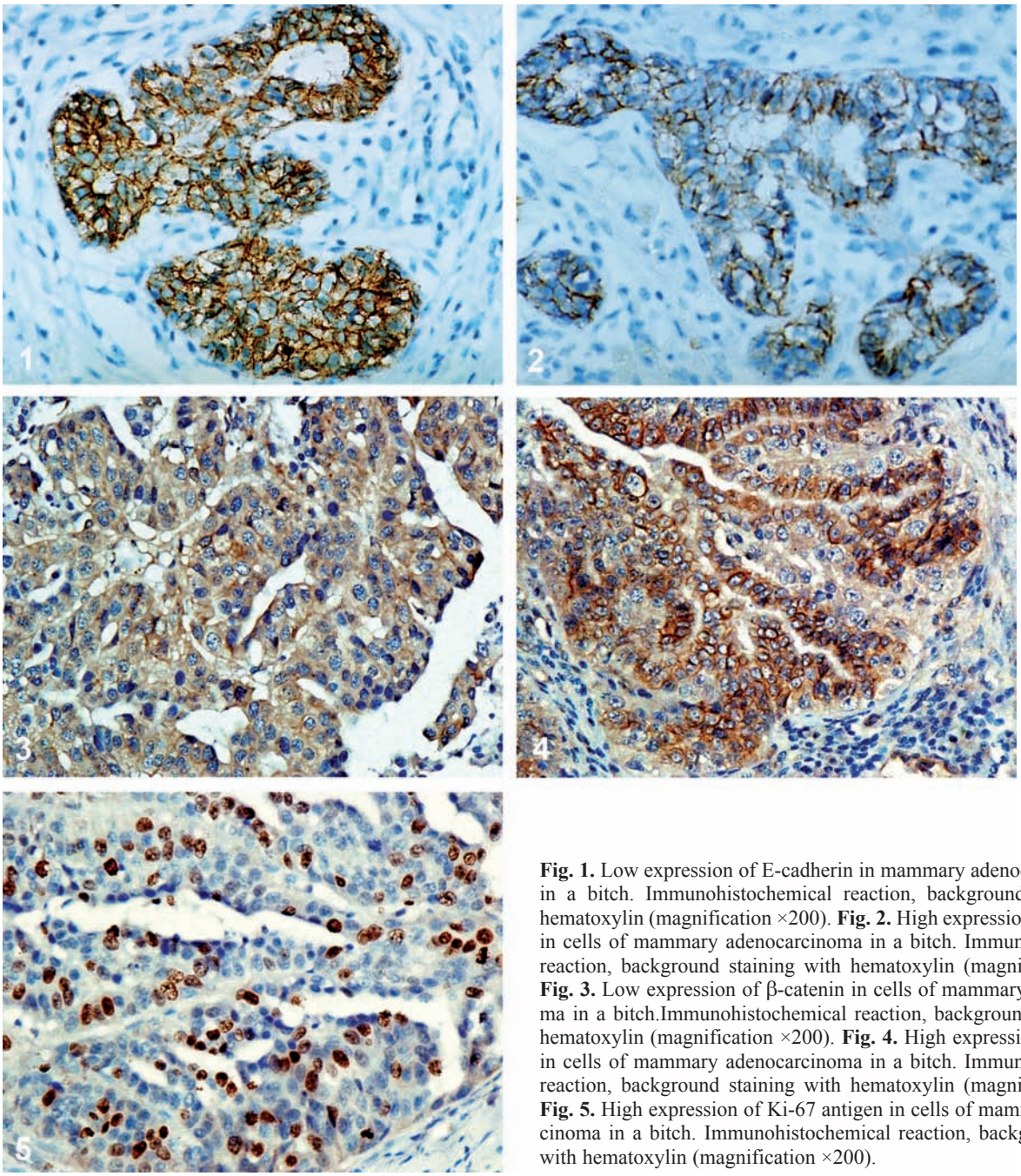

Fig. 1. Low expression of E-cadherin in mammary adenocarcinoma cells in a bitch. Immunohistochemical reaction, background staining with hematoxylin (magnification ×200). **Fig. 2.** High expression of E-cadherin in cells of mammary adenocarcinoma in a bitch. Immunohistochemical reaction, background staining with hematoxylin (magnification ×200). **Fig. 3.** Low expression of β-catenin in cells of mammary adenocarcinoma in a bitch.Immunohistochemical reaction, background staining with hematoxylin (magnification ×200). **Fig. 4.** High expression of β-catenin in cells of mammary adenocarcinoma in a bitch. Immunohistochemical reaction, background staining with hematoxylin (magnification ×200). **Fig. 5.** High expression of Ki-67 antigen in cells of mammary adenocarcinoma in a bitch. Immunohistochemical reaction, background staining with hematoxylin (magnification ×200).

expression of β-catenin (6-12 pts.) manifested in parallel high expression of E-cadherin. It should be noted that in over 37% tumours with high expression of β-catenin a low or absent expression of E-cadherin was detected.

Statistical calculations involving the entire group of examined tumours using Spearman's correlation test demonstrated strong positive correlation between expression of β-catenin and that of Ki-67 antigen (r=+0.712) (Fig. 7), but no correlation between expression of E-cadherin and that of Ki-67 antigen (r= -0.028). Also, no correlation could be disclosed between expressions of E-cadherin and β-catenin (r= -0.032).

Discussion

Extensive role of adhesion molecules in neoplastic progression in humans was proved in studies of, *i.e.*, Asgeirsson *et al.* [5], who analysed E-cadherin expression in histological material obtained from 108 patients with breast cancer. In 64% examined invasive lobular cancers and in 19% invasive ductal cancers the authors detected complete loss of expression of the

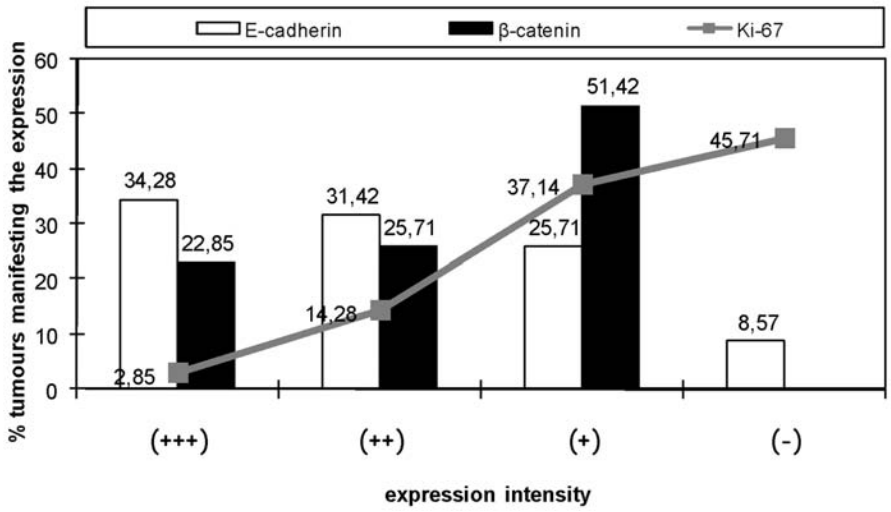

Fig. 6. Distribution of expression intensities of E-cadherin, β-catenin and Ki-67 antigen in mammary adenocarcinomas in bitches.

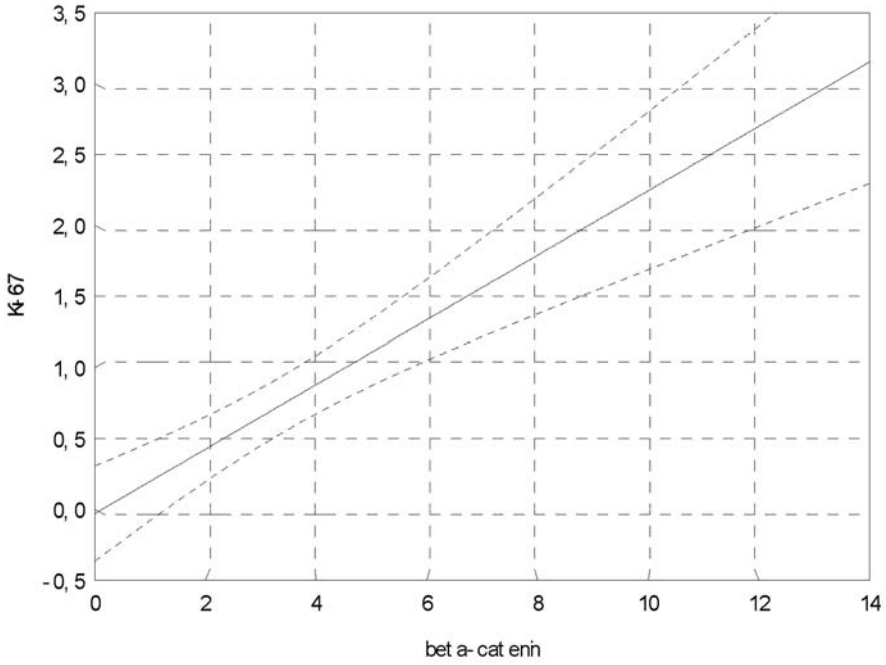

Fig. 7. Correlation between expressions of β-catenin and Ki-67 antigen in mammary adenocarcinomas in bitches. Correlation coefficient, $r=0.712$; $p<0.05$.

adhesion molecule. Moreover, the lowered expression of E-cadherin was accompanied by shorter relapse-free survival periods. It should be noted that a decreased expression of E-cadherin in various types of mammary cancer was detected also by other authors, who - in addition - pointed to clear association of a decreased expression of E-cadherin with reduced expression of α, β and γ catenins [8,9]. Similarly to mammary cancer, also in pulmonary adenocarcinoma reduction in the membrane complex of E-cadherin/β-catenin clearly correlated with low degree of histological differentiation and high Ki-67 index. Low expression of β-catenin was also noted to significantly abbreviate survival of the patients [10]. Also in cases of intrahepatic cholangiocarcinoma Settakorn *et al.* [11] demonstrated reduced membraneous expression of E-cadherin and β-catenin in, respectively, 61% and 58% examined tumours. Bankfalvi *et al.* [9] and Lim and Lec [12] examined neoplastic mammary tumours and found that a decreased expression of E-cadherin and β-catenin evidently correlated with higher degrees of histological malignancy of a tumour and with more frequent metastases, shorter total and relapse-free survival periods in the patients. In turn, Bukholm *et al.* [13] in studies on 176 mammary tumours documented higher expression of E-cadherin and β-catenin in tumours of lower diameter. Other authors who studied mammary tumours in dogs proved that tumours with cells manifesting reduced expression of the proteins demostrated a more aggressive growth, reached higher dimensions, became ulcerated earlier and developed metas-

Table 1. Semi-quantitative IRS scale (according to Remmele), taking into account both percentage of positive cells (**A**) and intensity of the reaction colour (**B**), the final result of which represents a product of the two variables (A × B).

A	B
0 pts. – no cells with positive reaction	0 pts. – no reaction colour
1 pt. – up to 10% positive cells	1 pt. – reaction colour of low intensity
2 pts. – 11-50% positive cells	2 pts. – moderately intense reaction colour
3 pts. – 51-80% positive cells	3 pts. – intense reaction colour
4 pts. – over 80% positive cells	

tases more frequently [14]. It should, however, be mentioned that studies were also published in which no significant relationships was noted between expression of E-cadherin and catenin on one hand and tumour size, condition of lymph nodes and total survival on the other [15]. Studies of Kovacs *et al.* [16] should also be noted, in which expression of E-cadherin was detected in most (81%) cases of invasive mammary cancers. Moreover, the authors detected no correlation between expression of E-cadherin and tumour size or degree of histological malignancy. As evident from the above, results of studies on expression of E-cadherin and β-catenin were frequently divergent and contradictory.

The studied by us tumours which have manifested moderate or intense expression of E-cadherin have demonstrated also a low proliferative potential, demonstrated by absent or low expression of Ki-67 antigen. The result may suggest that E-cadherin suppresses tumour invasiveness. This suggestion seems confirmed by results of Brunetti *et al.* [17], who studied mammary tumours in bitches and found that reduced expression of E-cadherin and β-catenin was significantly associated with the progression from noninfiltrating to highly infiltrating tumours, but not with proliferation or survival. We have found also that augmented proliferative potential of adenocarcinomas has been associated also with increased expression of β-catenin which, in line with the current views, may become transplaced to cell nucleus and may accelerate proliferation of tumour cells [3]. The hypothesis may be confirmed by our results, in which a pronounced positive correlation has been documented between cytoplasmic/nuclear expressions of β-catenin and Ki-67 antigen.

Summing up it should be noted that expression and biological behaviour of studied markers of mammary adenocarcinomas in bitches have resembled the patterns observed in the most frequent histological type of mammary neoplastic lesions in women, *i.e.*, in ductal cancers [18-20]. This may indicate usefulness of applying the animal model (mammary tumours in dogs) in studies on mechanisms of invasiveness and metastasis development in human tumours.

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