in vivo *22*: 463-470 (2008)

Expression of Extracellular Matrix Metalloproteinase (MMP-9), E-Cadherin and Proliferation-associated Antigen Ki-67 and their Reciprocal Correlation in Canine Mammary Adenocarcinomas

MARCIN NOWAK[1], JANUSZ A. MADEJ[1], MARZENA PODHORSKA-OKOLOW[2] and PIOTR DZIEGIEL[2]

[1]*Department of Pathological Anatomy, Pathophysiology, Microbiology and Forensic Veterinary Medicine, Faculty of Veterinary Medicine, Wroclaw University of Environmental and Life Sciences, Wroclaw;*
[2]*Department of Histology and Embryology, Wroclaw Medical University, Wroclaw, Poland*

Abstract. *Background: The purpose of the present study was to determine the expression of the proteins related to tumour metastatic potential, including matrix metalloproteinase (MMP)-9 and E-cadherin, in correlation with the expression of proliferation-associated antigen (Ki-67) in canine mammary adenocarcinomas. Materials and Methods: Material for the studies was obtained during surgery from 35 dogs of various breeds, aged 7 to 16 years. Neoplastic tumours were verified by a pathologist. The studied proteins were detected by immunohistochemical reactions. The microphotographs of the studied tumours were subjected to computer-assisted image analysis using MultiScaneBase V 14.02 software. Results: Expression of MMP-9 was noted in almost 83% of the tumours, expression of E-cadherin in 77% of tumours, while expression of Ki-67 antigen was detected in fewer than 26% of studied tumours. Conclusion: The positive correlation (r=0.375) between expressions of MMP-9 and Ki-67 and negative correlations between E-cadherin and Ki-67 (r=–0.383) as well as between MMP-9 and E-cadherin (r=–0.45) could suggest that expression and biological significance of the studied markers in mammmary adenocarcinomas in dogs resembles the pattern noted in ductal carcinoma, i.e. in the most frequent histological type of malignant tumour in humans. This may point to suitability of the animal model in studies on mechanism of neoplasia and metastases in humans.*

Correspondence to: Marcin Nowak, Ph.D., VMD Department of Pathological Anatomy, Pathophysiology, Microbiology and Forensic Veterinary Medicine, Faculty of Veterinary Medicine, Wroclaw University of Environmental and Life Sciences, Norwida 31, 50-375 Wroclaw, Poland. Tel/Fax: +48 71 328 1567, e-mail: martin75@ozi.ar.wroc.pl

Key Words: MMP-9, E-cadherin, Ki-67 protein, adenocarcinomas, dog.

Throughout the world, mammary carcinoma represents the most frequent malignant tumour both in humans and in dogs. It represents one of the principal causes of tumour-related deaths. Despite the enormous progress in diagnosis and therapy of the tumour, its incidence and mortality remain very high. It should be stressed that at the contemporary level of early diagnosis and with the development of oncological surgery, the primary tumour no longer requires such an extensive therapeutic process as it did just a few years ago. The problem and medical challenge involves the fact that despite extensive efforts and multiple studies, formation of metastases still cannot be controlled and, as a rule, leads to patient deaths.

The prerequisite condition for initiation of the complex process of metastasis involves acquisition by the neoplastic cell of the so-called invasive phenotype. This allows a certain group of tumour cells, selected by cumulation of mutations, to acquire the ability to be transported, infiltrate surrounding tissues and to home in on sites distant from the primary tumour (2).

The first stage in development of a metastasis involves liberation of a neoplastic cell from the parental tumour. The process depends mainly on the strength of junctions between the neighbouring cells, which, in turn, relies on the expression of cellular adhesion molecules (CAM) (2). The four main groups of adhesive molecules include cadherins, selectins, immunoglobulin-like molecules and integrins. These molecules, among which a significant role is played by cadherins, control the extent of adhesion between normal as well as neoplastically altered cells. Moreover, they play an important role as effector and sensor factors in processes of intracellular signal transfer, which allows the extent of adhesion to be regulated depending on the intensity of phosphorylation processes in the cell (3).

Cadherins belong to transmembrane proteins which depend on calcium ions (3, 4). The group includes three types of molecules: E-cadherins (participating in adhesion of epithelial cells), N-cadherins (taking part in adhesion of nervous cells) and P-cadherins (participating in adhesion of placental cells). Cadherins join neighbouring cells by binding to other cadherins or forming homophilic interactions through their *N*-terminal amino acid sequence of His-Ala-Val (HAV). The cytoplasmic domain of E-cadherin binds to the group of reciprocally associated proteins, termed catenins (α, β and γ). The β and γ-catenins compete with each other for a direct binding with E-cadherin. On the other hand, α-catenin binds E-cadherin with F-actin and α-actinin, which form the cell cytoskeleton (5). A disturbed function of the cadherin-catenin complex, resulting from dysfunction and/or deficiency of cadherins or catenins, clearly diminishes the ability of cells to adhere, as well as disturbing their differentiation, which evidently augments the invasive potential of a tumour (5).

The next barrier which has to be overcome by a potentially metastatic neoplastic cell involves the dense network of structural elements of the extracellular matrix (ECM), including the basement membrane (BM). At this stage of development of tumour metastases, a wide group of enzymes classified as matrix metalloproteinases (MMP) play a significant role. In vertebrates, 28 metalloproteinases have been identified, including 22 in man (1, 6). The enzymes disintegrate components of the extracellular matrix, allowing for migration of both normal and neoplastically transformed cells (7).

Metalloproteinases belong to the group of proteases with Zn^{2+} ion- and Ca^{2+} ion-dependent enzymatic activity (8). They are synthesised and released to extracellular spaces as proenzymes mainly by fibroblasts, leukocytes, monocytes, macrophages, neutrophils and endothelial cells, but also by neoplastic cells (8-10). Among others, they participate in the migration of cells in the inflammatory response to damaged tissues, in healing of wounds and in scar development (11, 12). They participate in the movement of cells during growth and they are involved in cyclic changes of the endometrium, in angiogenesis and in bone remodelling (13, 14). Synthesis of MMP is stimulated by many factors, such as vascular endothelium growth factor (VEGF), tumour necrosis factor α (TNF-α), interleukin-1 and prostaglandins (8, 10). Neoplastic cells were also shown to produce a specific factor (the so-called EMMPRIN, an extracellular matrix metalloproteinase inducer) interacting with the neighbouring fibroblasts and stimulating their synthesis and secretion of metalloproteinases (15, 16).

Depending upon the specificity of their target, metalloproteinases are cathegorised into four functional subgroups, including collagenases – MMP-1, -8, -13 and -18 (degrade fibrillary collagen), gelatinases – MMP-2 and -9 (degrade gelatins and elastins), stromyelysins – MMP-3, -10 and -11 (degrade proteoglycans, fibronectins and laminins) and membraneous type metalloproteinases – MMP-14, -15, -16 and -17 (12). High interest, particularly in oncology, is focused on the group of gelatinases, MMP-2 and MMP-9 in particular, the overexpression of which and correlation with prognostically unfavourable factors were demonstrated in female mammary carcinoma (3, 17-20). The proteinases have been shown to be involved with the degradation of collagen type IV, which forms a groundwork of BM in vascular endothelium. Damage to the membrane allows for migration of endothelial cells to the ECM, which is a prerequisite for the development of new blood vessels, also in a neoplastic tumour. In pathological conditions, tumour cells also take advantage of the defects formed in the BM, they pass through the barrier and home in on sites distant from the parental tumour providing the origin for a disseminated neoplastic disease (11). Metalloproteinases (mainly MMP-9), having the capacity of degrading receptors for interleukin-2 on lymphocytes, have been demonstrated to inhibit the immune reactions against neoplastic cells (9).

One of the main elements in appraisal of tumour aggressiveness, including that of mammary carcinoma, is the determination of the level of proliferative activity by estimation of the extent of expression of Ki-67 antigen. This protein belongs to the group of non-histone compounds present in the cell nucleus (21). Its expression can be detected in the early G1-phase of the cell cycle, markedly increases in the S- and G2-phases with a peak in the M-phase and an abrupt disappearance in G0. Thus, the protein is detected only in proliferating cells (22, 23). The ratio of cells displaying the expression of Ki-67 to cells with no Ki-67 expression is termed the proliferative index, reflecting the extent of cell mitotic activity (24).

This study aimed at determining the expression of proteins related to the tumour metastatic potential, *i.e.* MMP-9 and E-cadherin, in correlation with the expression of the proliferation-associated Ki-67 antigen in canine mammary adenocarcinomas. Moreover, we aimed at comparing the obtained results with the results obtained in mammary tumours in humans. This might justify the use of adenocarcinomas in dogs as an experimental model in studies on complex mechanisms which control the development of tumour metastases in humans.

Materials and Methods

Material for the studies was sampled during surgery in 35 dogs of various breeds, aged 7 to 16 years. The tumours were verified as adenocarcinomas by pathologist.

Formalin-fixed, paraffin-embedded tissue was freshly cut (4 μm). The sections were mounted on Superfrost slides (Menzel Gläser, Germany), deparaffinized with xylene and gradually hydrated. Activity of endogenous peroxidise was blocked by 5 min incubation with 3% H_2O_2. Detection of MMP-9, E-cadherin, and Ki-67 antigen expression was preceded by 15 min exposure of the sections

Table I. *Semiquantitative immunoreactive score (IRS) taking into account both the percentage of 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	

in a microwave oven to boiling Antigen Retrieval Solution (DakoCytomation, Denmark) at 250 W. For demonstration of MMP-9, E-cadherin, and Ki-67 antigen expression in the paraffin sections, the following antibodies were used: polyclonal rabbit antibodies (1:100) (DakoCytomation); monoclonal mouse antibodies – clone NCH-38 (1:150) (DakoCytomation); monoclonal mouse antibodies – clone MIB-1 (1:100) (DakoCytomation). The antibodies were diluted in the Antibody Diluent, Background Reducing (DakoCytomation). The sections were incubated with 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). Diaminobenzidine (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 all studied tumours were subjected to computer-assisted image analysis *via* a computer coupled to an 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 MMP-9 and E-cadherin was appraised using the modified semiquantitative IRS scale according to Remmele (Table I) (25). 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 (+), 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 the performed studies on canine mammary adenocarcinomas, the expression of MMP-9 and of E-cadherin as well as Ki-67 antigen was demonstrated (Figures 1-3). The immunohistochemical technique used involves one of the most frequently recommended techniques for detection of MMP-9 and E-cadherin in tumour cells. Using light microscopy, the technique allows the evaluation of both the site of the protein expression and the intensity of the colour reaction.

In our studies, expression of MMP-9 was demostrated in almost 83% of the tumours and expression of E-cadherin in 77% of the tumours, while 23% of the tumours manifested lack of expression of the adhesion molecule. Marked differences were noted in the intensity of expression of the studied proteins. In 40% of the tumours, expression of MMP-9 was appraised at +, in over 31% at ++ and in over 11% at +++. In the case of E-cadherin, in over 28% of the examined tumours expression of the protein was noted at the level of +, in over 11% at ++ and in over 37% at +++. It should be noted that expression of the proliferation-associated antigen, Ki-67, was observed in fewer than 26% of the examined tumours, including over 8% at +, 8% at ++ and 8% at +++. Slightly more than 74% of the examined tumours manifested no expression of the protein. Among the tumours manifesting expression of Ki-67, over 66% of tumours demonstrated in parallel a moderate (3-4 points) to intense (6-12 points) expression of MMP-9. It should be stressed that in over 66% of tumours with expression of Ki-67, no parallel expression of E-cadherin was noted. Moreover, 50% of tumours with intense expression of MMP-9 demonstrated a pronounced parallel expression of Ki-67. On the other hand, in tumours which demonstrated an increased expression of E-cadherin, the expression of Ki-67 antigen was not noted. Among the tumours with intense expression (6-12 points) of MMP-9, only 25% manifested expression of E-cadherin and they did so at a low level (1 point); the remaining 75% of tumours of the group demonstrated no expression of the studied adhesion molecule. In the tumours with a pronounced expression of E-cadherin, strong expression of MMP-9 was never observed.

Statistical analysis conducted for the group of studied tumours using Spearman's correlation demonstrated a positive correlation between the levels of expressions of MMP-9 and Ki-67 ($r=0.375$; $p<0.05$) (Figure 4) and negative correlations between expression of E-cadherin and expression of Ki-67 ($r=-0.383$; $p<0.05$) (Figure 5), as well as between MMP-9 and E-cadherin ($r=-0.45$; $p<0.05$) (Figure 6).

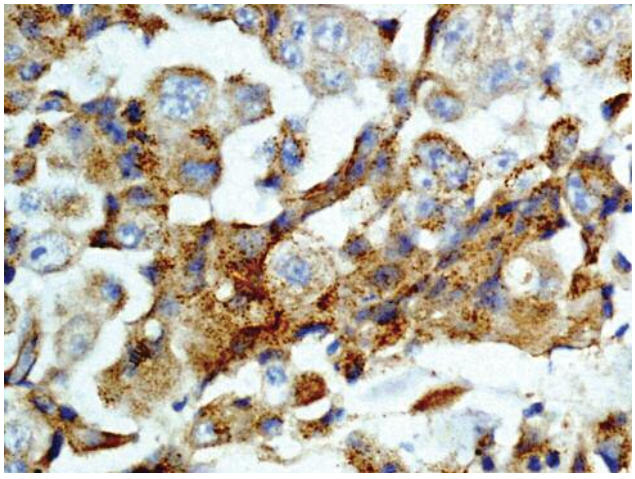

Figure 1. *High expression of MMP-9 in cells of canine mammary gland adenocarcinoma (×400, counterstained with hematoxylin).*

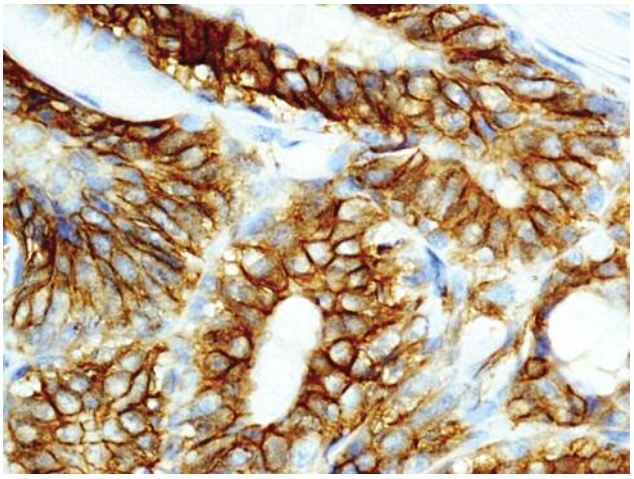

Figure 2. *High expression of E-cadherin in cells of canine mammary gland adenocarcinoma (×400, counterstained with hematoxylin).*

Discussion

In the presented study we have analysed expressions of MMP-9, E-cadherin and of Ki-67 protein in 35 cases of the most frequent canine malignant mammary tumour, namely adenocarcinoma.

In our results, we have noted that the increased expression of the proliferation-associated Ki-67 antigen was accompanied by the reduced expression of the adhesion molecule, E-cadherin. This could suggest that the larger the dividing cell pool in the tumour was, the weaker were their reciprocal junctions and the easier the cells were able to break free from the tumour thus yielding metastases. The extensive role of adhesion molecules in progression of neoplasias and in the development of metastases in human has been confirmed by studies of Asgeirsson *et al.* (4), who analysed E-cadherin expression in histological material from 108 patients with breast cancer. In 64% of studied invasive lobular carcinomas and in 19% of invasive ductal carcinomas, the authors demonstrated a complete loss of expression of the adhesion molecule. Moreover, a lower expression of E-cadherin was accompanied by shortened periods of relapse-free survival. It should be noted that the reduced E-cadherin expression in various types of mammary cancer were also observed by other authors, who demonstrated in addition an association of a reduced expression of E-cadherin with a lower level of α-, β- and γ-catenins (26, 27). Bankfalvi *et al.* (27) examined 142 mammary tumours and demonstrated that a lower expression of E-cadherin clearly correlated with higher levels of tumour histological malignancy as well as with more frequent metastases and shorter total and relapse-free survival. Similarly to studies performed by Matos *et al.* (28) on 77 tumours originating from bitches, the authors showed that tumours in which cells were unable to synthesize these proteins manifested a more aggressive growth, reached a higher size, became ulcerated earlier and yielded metastases more frequently.

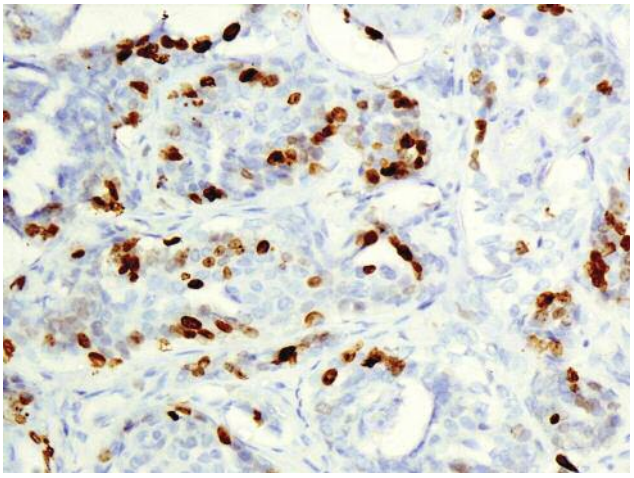

Figure 3. *High expression of Ki-67 in cells of canine mammary gland adenocarcinoma (×200, counterstained with hematoxylin).*

Results obtained by us here and in our earlier studies on mammary cancer and soft tissue sarcomas in dogs, confirmed by numerous studies on histologically similar tumours in humans, support the thesis of E-cadherin as a molecule which suppresses tumour invasiveness (29, 30).

It should also be mentioned that the negative correlation observed in our studies between expressions of E-cadherin and MMP-9 could suggest their coexistence in a tumour under conditions favourable for the development of

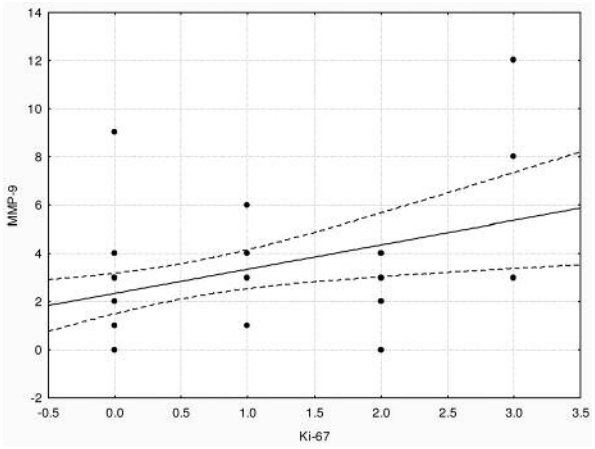

Figure 4. *Correlation between expressions of MMP-9 and Ki-67 antigen in cells of canine mammary gland adenocarcinoma. Correlation coefficient r=0.375; p<0.05.*

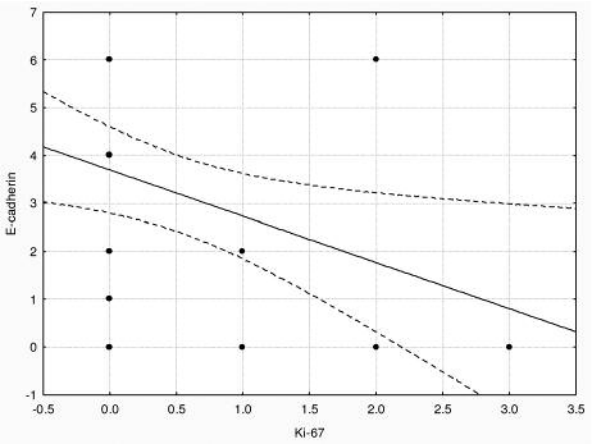

Figure 5. *Correlation between expressions of E-cadherin and Ki-67 antigen in cells of canine mammary gland adenocarcinoma. Correlation coefficient r=–0.383; p<0.05.*

metastases. It is well known that the lower the expression of adhesion molecules (E-cadherin) is, the less pronounced cell adherence is, whereas the more MMP-9 degrading the matrix is present, the easier cell migration from the tumour and infiltration of invasion seems to be. Similar observations were made by Slaton *et al*. (31), who demonstrated that augmented expression of MMP-9 and a reduced expression of E-cadherin correlated with appearance of metastases in human urinary cancer.

The subsequent, very important, observation from our studies involves demonstration of a positive correlation between expression of MMP-9 and expression of the proliferation-associated antigen Ki-67. Concurrent expression of MMP-9 and Ki-67 in cancer cells could indicate tumour aggressiveness. The observations coroborate the results of immunocytochemical studies on mammary cancers in dogs and in humans. Hirayama *et al*. (32) analysed 12 cases of mammary adenocarcinoma in bitches and in every tumour noted an elevated expression of MMP-9, while in non-malignant tumours, adenoma expression of the protein was mostly weak, and in only 25% moderate. Wang *et al*. (33) examined 72 cases of mammary carcinoma in female humans and detected positive correlation between levels of expression of MMP-9 and appearance of metastases in lymph nodes. Apart from mammary carcinomas, similar expression of studied markers and their reciprocal correlations were detected in several other types of malignant tumours. Campos *et al*. (34) examined 125 cases of penile epidermoid carcinoma and found that high expression of MMP-9 was positively correlated with the risk of recurrence of the disease. In a similar way, in oesophageal carcinoma in humans, expression of MMP-9 strongly correlated with the appearance of metastases in the regional lymph nodes (35).

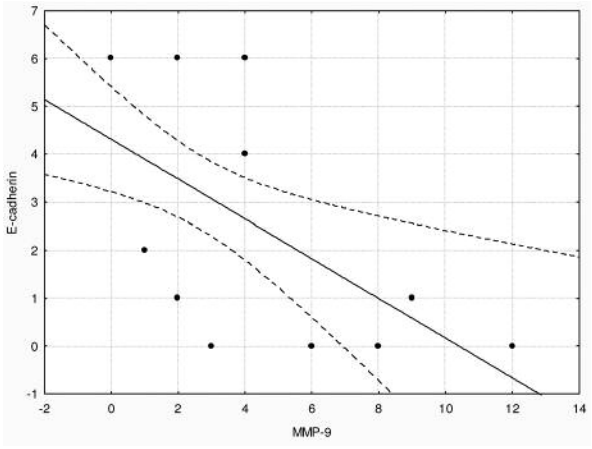

Figure 6. *Correlation between expressions of E-cadherin and MMP-9 in cells of canine mammary gland adenocarcinoma. Correlation coefficient r=–0.450; p<0.05.*

Intensified expression of MMP-9, as well as of the other proteinases was detected in almost all types of cancer in humans, most frequently linked to prognostically unfavourable indices of the disease course, *i.e*. with the degree of clinical advancement, intensity of infiltration and metastasing processes and with a shortened duration of survival (36-42). However, it should be noted that not all the studies point to a negative significance of high MMP-9 expression in neoplastic tissue. Zhang *et al*. (43) analysed 94 cases of invasive breast carcinoma in women and found no correlation between the high MMP-9 expression on one hand and unfavourable prognostic factors on the other, such as tumour size, presence of metastases in lymph nodes or

development of a recurrence. In a similar way, in colon carcinoma, the presence of macrophages containing high amounts of MMP-9 was found to correlate with lower susceptibility to develop metastases (44).

Despite certain variances in the above mentioned results, it should be concluded that the studied proteins exert a significant effect on both the extent/strength of intercellular links (E-cadherins) and on alterations taking place in the extracellular matrix during cell migration (MMP). Although the mechanisms of reciprocal interaction of MMP and ECM in the course of progression in the neoplastic process in either animals or humans remain to be explained in detail, attempts are being made to take advantage of these results in therapy. High expectations are linked to pharmacological control of transformations which take place in the ECM in the course of neoplastic disease, including the application of specific inhibitors of MMP, which might restrict therapy of the disease to excision of the primary tumour and could provide new hope for a complete cure (45, 46).

In summary, it is concluded that the expression and biological significance of the studied markers (MMP-9, E-cadherin and Ki-67) in canine mammary carcinoma resemble the pattern observed in the most frequent histological type of human female mammary malignant tumour, *i.e.* ductal carcinoma. This may point to the suitability of the animal model in studies on mechanisms of neoplasia and of metastasis development in humans.

References

1 Akiyama SK, Olden K and Yamada KM: Fibronectin and integrins in invasion and metastasis. Cancer Metastasis Rev *14*: 173-189, 1996.

2 Epstein RJ: Human Molecular Biology: An Introduction to the Molecular Basic of Health and Disease. Cambridge University Press, Cambridge, pp. 228-233, 2002.

3 Berx G, Staes K, Hengel J, Molemans F, Bussemakers MJ, Bokhoven A and Roy F: Cloning and characterization of the human invasion suppressor gene E-cadherin (CDH1). Genomics *26*: 281-289, 1995.

4 Asgeirsson KS, Jonasson JG, Tryggvadottir L, Olafsdottir K, Sigurgeirsdottir JR, Ingvarsson S and Ogmundsdottir HM: Altered expression of E-cadherin in breast cancer: patterns, mechanisms and clinical significance. Eur J Cancer *36*: 1098-1106, 2000.

5 Sliwowska I and Kopczynski Z: Matrix metalloproteinases – biochemical characteristics and clinical value determination in breast cancer patients. Współcz Okol *9*: 327-335, 2005 (in Polish).

6 Mott JD and Werb Z: Regulation of matrix biology by matrix matalloproteinases. Curr Opin Cell Biol *16*: 558-564, 2004.

7 Sounni NE and Noel A: Membrane type-matrix matalloproteinases and tumor progression. Biochimie *87*: 329-342, 2005.

8 Nagase H and Woessner JF: Matrix metalloproteinases. J Biol Chem *274*: 21491-21494, 1999.

9 Egeblad M and Werb Z: New functions for the matrix metalloproteinases in cancer progression. Nat Rev Cancer *2*: 163-176, 2002.

10 Nagase H: Activation mechanisms of matrix metalloproteinases. Biol Chem *378*: 151-160, 1997.

11 Ray JM and Steller-Stevenson WG: The role of matrix metalloproteinases and their inhibitors in tumor invasion, metastasis and angiogenesis. Eur Respir J *7*: 2062-2072, 1994.

12 Zucker S, Hymowitz M, Conner C, Zarrabi HM, Hurewitz AN, Matrisian L, Boyd D, Nicolson G and Montana S: Measurement of matrix metalloproteinases and tissue inhibitors of metalloproteinases in blood and tissues. Clinical and experimental applications. Ann NY Acad Sci *878*: 212-227, 1999.

13 Yong VW, Power C, Forsyth P and Edwards DR: Metalloproteinases in biology and pathology of the nervous system. Nat Rev Neurosci *2*: 502-511, 2001.

14 Fishman DA, Bafetti LM and Stack MS: Membrane-type matrix metalloproteinase expression and matrix metalloproteinase-2 activation in primary human ovarian epithelial carcinoma cells. Invasion Metastasis *16*: 150-159, 1996.

15 Nabeshima K, Inoue T, Shimao Y and Sameshima T: Matrix metalloproteinases in tumor invasion: role for cell migration. Pathol Int *52*: 255-264, 2002.

16 Tang Y, Nakada MT, Kesavan P, McCabe F, Millar H, Rafferty P, Bugelski P and Yan L: Extracellular matrix metalloproteinase inducer stimulates tumor angiogenesis by elevating vascular endothelial cell growth factor and matrix metalloproteinases. Cancer Res *65*: 3193-3199, 2005.

17 Sheen-Chen SL, Chen HS, Eng HL, Sheen CC and Cheng WJ: Serum levels of matrix metalloproteinase 2 in patients with breast cancer. Cancer Lett *173*: 79-82, 2001.

18 Hanemaaijer R, Verheijen JH, Maguire TM, Visser H, Toet K, McDermott E, O'Higgins N and Duffy MJ: Increased gelatinase-A and gelatinase-B activities in malignant *vs*. benign breast tumors. Int J Cancer *86*: 204-207, 2000.

19 Talvensaari-Mattila A, Paakko P and Turpeenniemi-Hujanen T: Matrix metalloproteinase-2 is associated with survival in breast carcinoma. Br J Cancer *89*: 1270-1275, 2003.

20 Djonov V, Cresto N and Aebersold DM: Tumor cell specific expression of MMP-2 correlates with tumor vascularisation in breast cancer. Int J Oncol *21*: 25-30, 2002.

21 Gerdes J, Shwab U, Lemke H, Baisch H and Wacker HH: Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated witch cell proliferation. Int J Canc 31: 13-20, 1983.

22 Brown DC and Gatter KC: Monoclonal antibody Ki-67: its use in histopathology. Histopathology *17*: 489-503, 1990.

23 Gizinski S, Boryczko Z, Katkiewicz M and Bostedt H: Ki-67 protein as a prognostic factor in mammary gland tumors in female dogs. Medycyna Wet *59*: 888-891, 2003 (in Polish).

24 Barnard NJ, Hall PA, Lemoine NR and Kadarr N: Proliferative index in breast carcinoma determined *in situ* by Ki-67 immunostaining and its relationship to clinical and pathological variables. J Path *152*: 287-295, 1987.

25 Remmele W and Stegner HE: Vorschlag zur einheitlichen Definition eines immunoreaktiven Score (IRS) für den immunohistochemichen Oestrogenrezeptor-Nachweis (ER-ICA) im Mammakarzinomgewebe. Pathologie *8*: 138-140, 1987.

26 Acs G, Lawton TJ, Rebbeck TR, Livolsi VA and Zhang PJ: Differential expression of E-cadherin in lobular and ductal neoplasms of the breast and its biologic and diagnostic implications. Am J Clin Pathol *115*: 85-98, 2001.

27 Bankfalvi A, Terpe HJ, Breukelmann D, Bier B, Rempe D, Pschadka G, Krech R, Lelle RJ and Boecker W: Immunophenotypic and prognostic analysis of E-cadherin and β-catenin expression during breast carcinogenesis and tumor progression: a comparative study with CD 44. Histopathology *34*: 25-34, 1999.
28 Matos AJ, Lopes C, Carvalheira J, Santos M, Rutteman GR and Gartner F: E-cadherin expression in canine malignant mammary tumours: relationship to other clinico-pathological variables. J Comp Pathol *134*: 182-189, 2006.
29 Nowak M, Madej JA and Dziegiel P: Expression of E-cadherin, β-catenin and Ki-67 antigen and their reciprocal relationships in mammary adenocarcinomas in bitches. Folia Histochem Cytobiol *45*: 233-238, 2007.
30 Nowak M, Madej JA and Dziegiel P: Expression of E-cadherin, β-catenin and Ki-67 antigen and their reciprocal correlations in fibrosarcomas of soft tissues in dogs. In Vivo *21*: 751-756, 2007.
31 Slaton JW, Karashima T, Perrotte P, Inoue K, Kim SJ, Izawa J, Kedar D, McConkey DJ, Millikan R, Sweeney P, Yoshikawa C, Shuin T and Dinney CP: Treatment with low-dose interferon-alpha restores the balance between matrix metalloproteinase-9 and E-cadherin expression in human transitional cell carcinoma of the bladder. Clin Cancer Res *9*: 2840-2853, 2001.
32 Hirayama K, Yokota H, Onai R, Kobayashi T, Kumata T, Kihara K, Okamoto M, Sako T, Nakade T, Izumisawa Y and Taniyama H: Detection of matrix metalloproteinases in canine mammary tumors: analysis by immunohistochemistry and zymography. J Comp Path *127*: 249-256, 2002.
33 Wang LH, Liu DY, Chen YJ, Hou L, Wang B, Mao LM and Lu S: Relationship between lymph node metastasis and the expressions of E-cadherin, N-cadherin, and matrix metallo-proteinase in breast carcinoma. Ai Zheng *21*: 965-969, 2002.
34 Campos RS, Lopes A, Guimarães GC, Carvalho AL and Soares FA: E-cadherin, MMP-2, and MMP-9 as prognostic markers in penile cancer: analysis of 125 patients. Urology *67*: 797-802, 2006.
35 Sato F, Shimada Y, Watanabe G, Uchida S, Makino T and Imamura M: Expression of vascular endothelial growth factor, matrix metalloproteinase-9 and E-cadherin in the process of lymph node metastasis in oesophageal cancer. Br J Cancer *80*: 1366-1372, 1999.
36 Curran S and Murray GI: Matrix metalloproteinases: molecular aspects of their roles in tumour invasion and metastasis. Eur J Cancer *36*: 1621-1630, 2000.
37 Vihinen P and Kahari VM: Matrix metalloproteinases in cancer: prognostic markers and therapeutic targets. Int J Cancer *99*: 157-166, 2002.
38 McCawley LJ and Matrisian LM: Matrix metalloproteinases: they're not just for matrix anymore! Curr Opin Cell Biol *13*: 534-540, 2001.
39 Nelson AR, Fingleton B, Rothenberg ML and Matrisian LM: Matrix metalloproteinases: biologic activity and clinical implications. J Clin Oncol *18*: 1135-1149, 2000.
40 Kim JH, Kim TH, Jang JW, Lee KH and Lee ST: Analysis of matrix metalloproteinase mRNAs expressed in hepatocellular carcinoma cell lines. Mol Cells *12*: 32-40, 2001.
41 Tomita T, Fujii M, Tokumaru Y, Imanishi Y, Kanke M, Yamashita T, Ishiguro R, Kanzaki J, Kameyama K and Otani Y: Granulocyte-macrophage colony-stimulating factor upregulates matrix metalloproteinase-2 (MMP-2) and membrane type-1 MMP (MT1-MMP) in human head and neck cancer cells. Cancer Lett *156*: 83-91, 2000.
42 Loukopoulos P, Mungall BA, Straw RC, Thornton JR and Robinson WF: Matrix metalloproteinase 2 and 9 involvement in canine tumors. Vet Pathol *40*: 382-394, 2003.
43 Zhang YG, Du J, Tian XX, Zhong YF and Fang WG: Expression of E-cadherin, beta-catenin, cathepsin D, gelatinases and their inhibitors in invasive ductal breast carcinomas. Chinese Med J *120*: 1597-1605, 2007.
44 Stamenkovic I: Extracellular matrix remodelling: the role of matrix metalloproteinases (review). J Pathol *200*: 448-464, 2003.
45 Maquoi E, Noel A, Frankenne F, Anqliker H, Murphy G and Foidar JM: Inhibition of matrix metalloproteinase 2 maturation and HT1080 invasivenes by a synthetic furin inhibitor, FEBS Lett *424*: 262-266, 1998.
46 Greenwald RA: Thirty-six years in the clinic without an MMP inhibitor. Ann NY Acad Sci *878*: 413-418, 1999.

Received February 12, 2008
Revised April 21, 2008
Accepted April 22, 2008