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

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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 mammary 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.

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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 endometrial matrix, 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), stromelysins – MMP-3, -10 and -11 (degrade proteoglycans, fibronectins and laminins) and membrane 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 peroxidase was blocked by 5 min incubation with 3% H\(_2\)O\(_2\). Detection of MMP-9, E-cadherin, 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 MMP-9, E-cadherin, and Ki-67 antigen expression in the paraffin sections, the following antibodies were used: polyclonal rabbit antibodies – clone NCH-38 (1:100) (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 (++)]. Expression of Ki-67 was evaluated quantitatively by estimation of the percentage of positive cells [0-5% = no reaction (–); 5-25% = weak reaction (+); 26-50% = moderate 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 immuno-histochemical 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 demonstrated 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).
**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 numerous 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 mammmary 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.

Results obtained by us here and in our earlier studies on mammmary 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
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 corroborate 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).

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 metastasising 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


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