Extracellular matrix in canine mammary tumors

with special focus on versican, a versatile extracellular proteoglycan

Ildikó Erdélyi

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De extracellulaire matrix van mammatumoren van de hond met bijzondere aandacht voor versican, een veelzijdig proteoglycan
(met een samenvatting in het Nederlands)

Extracelluláris mátrix a kutya emlõdaganataiban
különös tekintettel egy sokoldalú extracelluláris proteoglikánra, a verzikánra
(Magyarnyelvû összefoglalóval)

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“Our destination is never a place but rather a new way of looking at things.”

Henri Miller

to my mother

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Preface

As a veterinary undergraduate in the last decade of the past millennium I was captivated by the colorful and complex microscopical world. Soon I devoted myself to veterinary pathology. Already as a veterinary student I had special interest in tumor diagnostics and wrote my student thesis on the "Immunohistochemical evaluation of canine tumors". After graduating in 1997 I spent one year as a trainee and 3 years as a staff member of the Department of Pathology under the guidance of Professor Vetési Ferenc who played a large role to further deepen my special devotion to pathology. In those years I examined hundreds and hundreds of canine mammary tumor biopsies and struggled to establish their precise histological classification and grading. It almost seemed impossible at the beginning since these tumors have the most versatile histomorphology of all the tumor types I have ever encountered. This was the time when I turned to Dr Vass Laszló, a human pathologist who is one of the greatest experts in human breast cancer pathology in Hungary. This was an exciting period when I learned a lot about breast cancer pathology. However, it was also the crucial point when I faced that there were several differences between human and canine mammary tumors, thus we can not understand the complex and unique histogenesis and behavior of canine mammary tumors based on comparative analysis with human breast cancer. Knowing that there is a huge need for a comprehensive research in several aspect of mammary tumor biology of dogs I decided to look around for a PhD program in this field. I was very fortunate to be accepted for a one year Marie Curie European Union Fellowship to perform research at the Department of Veterinary Pathology in Utrecht under the supervision of Bert Nederbragt in the challenging new field of focus in cancer research (that I have never even heard till then): The Extracellular Matrix! Soon I became obsessed with the topic and thanks for Professor Jaap van Dijk decision I could continue my first year research in the Department with an additional 2.5 years PhD fellowship that finally resulted in this thesis.

CHAPTER **1**

General Introduction: The role of extracellular matrix in tumor biology. Scope and outline of the thesis

Extracellular matrix and cancer

In the last few decades of the 20th century the main focus in cancer research was on the genetically deranged cancer cells. This has lead to the discovery of mutations that produce oncogenes with dominant gain of function and tumor suppressor genes with recessive loss of function in human and animal tumor cells. We now understand that one of the main hallmarks of cancer is the acquired growth signal autonomy of the tumor cells, in large part because of the prevalence of dominant oncogenes that act by mimicking normal growth signaling.[1] The dynamic nature between cell growth and cellular interactions with the extracellular matrix (ECM) has been relatively long recognized. However, the extensive and in-depth analysis of the role of tumor environment, including stromal fibroblasts, endothelial cell and ECM molecules, became popular only in the last decade and evolved to a rapidly developing field of cancer research, particularly in humans. Understanding how the ECM participates in the normal and abnormal growth is fundamental to understand cancer. It is clear that the rapidly increasing new data in this field add further layers of complexity of what we know of tumor biology and initiated a more complex approach to understand tumor development and behavior. After 10 years of rapid advances in ECM research several initial concepts on the role of tumor ECM have been modified and new ones evolved and clearly this is just the beginning!

The complex "world" of the ECM

Composition and function with special regard to proteoglycans

The ECM is like a biological reservoir containing many components that provide a variety of functions to the enveloped cells. It is composed of a collection of insoluble protein fibrils and soluble polymers. The *insoluble fibrils* are made of two types of structural proteins: collagen and elastin. Collagens make up about 30% of the body's proteins and they are synthesized by a wide variety of cells. There are many types of collagens classified (roman numerals) in the order they were discovered. Types I, II, III, V, and XI are known as the fibrillar collagens because they form fibrils. The remaining types of collagens are described as non-fibrillar because they form sheets or meshworks. Each type of collagen has its characteristic distribution in tissues. For example type IV collagen forms a

meshwork (not fibers) of procollagen molecules matted together to form the basal lamina of basement membranes and type II collagen is mostly located in hyaline and elastic cartilage where its primary function is to resist pressure. (In Chapter 3 we investigate the expression pattern of cartilage biomarkers in canine mammary tumors, including type II collagen.) The *soluble polymers* consist of three families of macromolecules: glycosaminoglycans (GAGs), proteoglycans (PGs), and cell adhesive glycoproteins. These macromolecules are made up of long carbohydrate chains linked to proteins and bind water with high affinity forming a hydrated gel-like ground substance of the ECM. They form interactions with each other and the insoluble fibers of the extracellular matrix.

The ECM provides a mechanical framework that distributes the stresses of movement and gravity while maintaining the structural integrity of the body's tissues. The ECM also provides a physicochemical environment, water and ions, for the body's cells. This environment supports cell mobility within the connective tissue scaffold (cells of the tissues adhere to components of the ECM). Thus, the ECM is the medium through which nutrients and chemical messengers can freely diffuse. Furthermore, increasing body of evidence suggests that ECM can actively regulate cellular processes such as growth, differentiation, apoptosis, adhesion, migration, invasion and gene expression.[2-4] These cellular events, in turn, regulate both physiological processes such as embryonic development, tissue morphogenesis and angiogenesis and also pathological processes such as transformation and metastasis.[2-6] In normal circumstances the ECM controlled signaling and the intercommunication of the neighboring cells ensures that cells divide and differentiate in their home territory of each specific tissue. Therefore, tumor cells must remodel the matrix and alter the normal cell-cell conversation to escape the control and to generate a new and optimal microenvironment that facilitates their proliferation, invasion and survival. In fact, tumor cells cross-talk and cooperate with stromal cells by interchanging growth factors (e.g. transforming growth factor-ß and epidermal growth factor), cytokines and chemoattractans (e.g. interleukin-6 and scatter factor/hepatocyte growth factor), angiogenesis factors (e.g. vascular endothelial growth factor and basic fibroblast growth factor), and proteases (e.g. matrix metalloproteinases and urokinase plasminogen activator). This allows them to modulate and activate the surrounding ECM, which in turn induces and promotes selection and expansion of neoplastic cells.[7-8] Recent studies suggest that the oncogenic activity of the tumor stroma reflects many different processes, including

alteration of the ECM composition; altered growth factor activity, mainly mediated by TGF-ß, whose effect includes phenotypic changes in adhesion, migration, differentiation and cell death, and altered expression of receptors that mediate cell-cell interaction [9]; and direct cellular communications with the ECM, especially ß1-integrins.[10] Together, these findings point to the significant contribution of stromal alterations to tumorigenesis.

Proteoglycans: classification, structure and role in tumorigenesis

Among the components of ECM, proteoglycans (PGs) are a family of complex macromolecules characterized by the presence of one or more polysaccharides chains covalently linked to a polypeptide backbone. The predominant polysaccharides are known as glycosaminoglycans (GAG) and consist of repeated disaccharide units. Several PGs are among the largest and most complex molecular structures in mammalian cells. Although they are originally named and categorized on the basis of the GAG substituent, increasingly they are being identified as members of gene families that encode their different core proteins. To date, more than 30 distinct genes have been identified in the mammalian genome which code for the protein cores. PGs are found predominantly in ECM or associated with the cell surface of most eukaryotic cells where they bind to other matrix- and cell-associated molecules. The matrix PGs, broadly defined as proteoglycans secreted into the pericellular matrix, can be separated into three groups: the basement membrane PGs, the hyalectans (PGs interacting with hyaluronan and lectins), and the small leucine-rich PGs.[11,34-36] The highly interactive nature of PGs derives for a large part from their structural diversity, which arises from variations in polysaccharide type, size and composition as well as core protein primary sequence, domain arrangement, and degree of sulfation and distribution of polysaccharide chains. Furthermore, N- and O-linked glycoprotein-type oligosaccharides or more than one type of GAG chain may be attached to the same core protein, enhancing the diversity of structure. Although there are common features among the GAGs, six distinct classes are recognized based on fine structure differences due to specificity in composition and sulfation, epimerization or N-acetylation modifications: chondroitin sulfate (CS), dermatan sulfate, heparin and heparin sulfate, keratan sulfate, hyaluronan (hyaluronic acid, hyaluronate, HA). Hyaluronan is very different from the other five types of GAG in that it is not sulfated or covalently linked to a protein core. It is structurally similar to the other GAGs and consists solely of repeating

disaccharides of N-acetylglucosamine and glucuronic acid. Although hyaluronate can be considered to have the least complex structure of all the GAGs, the polysaccharide chains can reach molecular weights of 10^5-10^7 Da, a feature important to the biological function of hyaluronan. (In Chapter 2 and 3 we describe the presence and tissue distribution of chondroitin sulfate, HA and also two of the hyalectans, namely versican and aggrecan in canine mammary tumors.)

Considering the complexity of proteoglycan molecules, often having modular core protein domains and posttranslational modifications that vary with developmental setting, the various steps of synthesis and processing are most likely highly regulated. Furthermore, regulation of proteoglycan expression is even more complex as they frequently are expressed transiently by multiple cell types and in different developmental time frames. Elucidation of cell- and developmental-specific control elements that regulate the expression of these complex macromolecular families is only beginning.

The exact function of many PGs is not or only poorly understood. However, it is known that these complex molecules fulfill a variety of biological functions i.e. matrix organization, ionic filtration, modulation of growth-factor activities, cell growth and differentiation, regulation of collagen fibrillogenesis and skin tensile strength, neurite outgrowth etc. Recently, one of the main focuses in PG research is on the signaling activity of PGs. It has been discovered that some of the large molecular weight PGs can signal biological function besides being structural molecules.[11,34-36] Clearly, these molecules contain signaling sequences within their structure. Growth factor repeats, arginine, glycine, aspartic acid (RGD) sequences, and other signaling sequences may be released by proteolysis; yet how the cascade of different proteases and their inhibitors in the ECM might regulate the release of this information is not clear. Furthermore, it is becoming clear that PGs play a central role also in several events of tumor development and progression, such as tumor cell proliferation and invasion. In human tumors the presence of altered PG composition was reported as early as 1939 by Meyer and Chaffee.[12] Since than altered PG and GAG expression, particularly increased levels of sulfated-GAG and chondroitin-PGs has been reported in a variety of human tumor types, including human breast cancer. However, many of the tumor PGs have not been identified or have not been studied extensively. Since PGs are rather tissue specific, it is likely that each tumor expresses an abnormal PG profile in comparison to its normal counterparts.

Hyalectans, with special focus on Versican

Versican is the largest member of hyalectans, a family of large aggregating chondroitin-sulfate PGs that interact with hyaluronan and lectins. The other family members are aggrecan, neurocan and brevican. Each of these PGs has a characteristic tissue distribution, with aggrecan prominent in cartilage, neurocan and brevican in the central nervous tissues.[11] However, versican is expressed in various tissues e.g. the central and peripheral nervous system, blood vessels in normal and tumor tissues, dermis, and the proliferative zone of the epidermis, and embryonic tissue.[13]

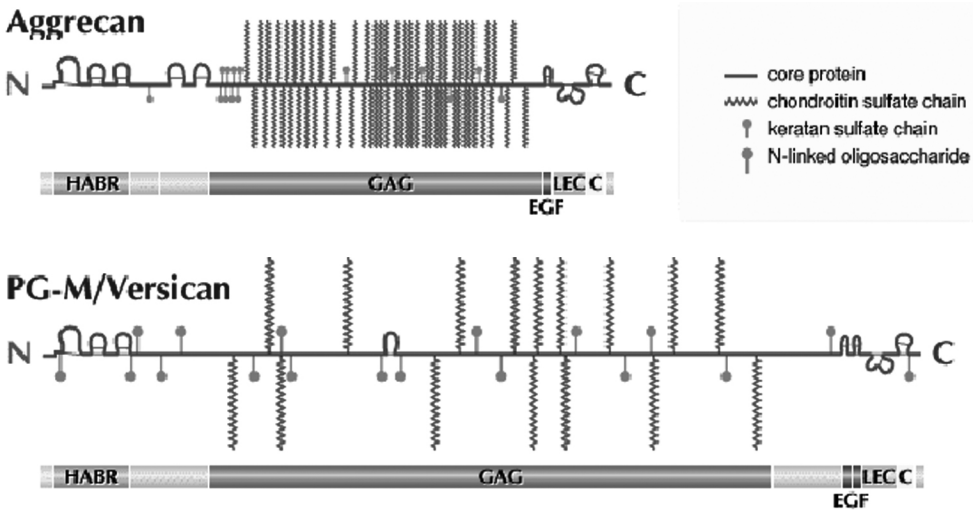

Figure 1: A structural model of aggrecan and versican. The simplified schematic representations show the organization of peptide domains in aggrecan and versican. HABR: hyaluronic acid-binding region; GAG: glycosaminoglycan-attachment domain; EGF: epidermal growth factor-like repeat; LEC: C-type lectin-like module; C: complement regulatory protein-like module. The G1-domain of versican contains one Ig repeat followed by two consecutive link protein modules (tandem repeats), which are involved in mediating the binding to HA. The central GAG-binding domain consists of two large subdomains, designated GAG-α and GAG-β, that are encoded by two alternatively spliced exons.[14,15] The C-terminal globular (G3) domain consists of several structural motifs such as two EGF-like repeats, a lectin-like motif (also called as carbohydrate recognition domain), and a complement binding protein-like module. These motifs are characteristically found in the selectins that are adhesion receptors regulating leukocyte-homing and extravasation of inflammatory sites.

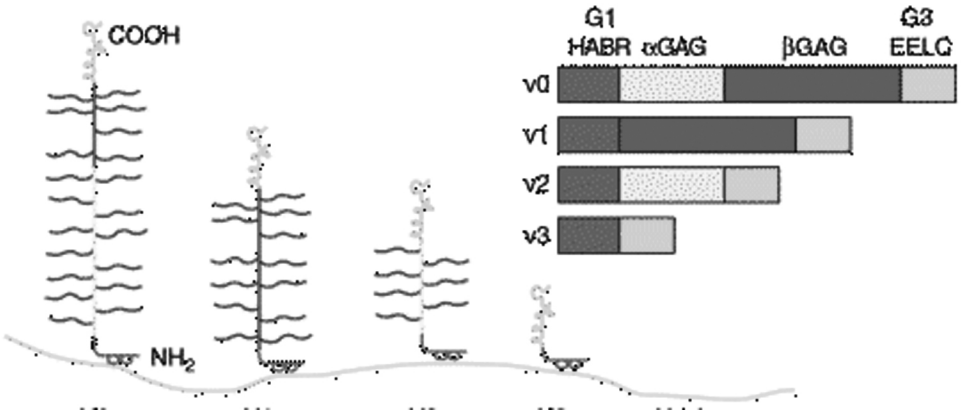

Figure 2. A model of the different isoforms generated by alternative splicing of the mRNA transcript for versican. All isoforms interact with hyaluronan and thus are capable of forming different sized versican–hyaluronan aggregates, which in turn determines, in part, tissue volume. Different colors denote specific domains in the gene and in the protein product. Purple = hyaluronan binding region (HABR); YELLOW = the α GAG exon and protein product; RED = β GAG exon and the protein product; GREEN = two epidermal growth factor repeats (EE), a lectin binding domain (L) and a complement regulatory region. The glycosaminoglycan chains are shown in blue. In human versican the number of potential GAG attachment sites is the following: 17-23 for V0, 12-15 for V1, 5-8 for V2, and 0 for V3.

These PGs share structural and functional similarities at both the genomic and protein levels. They have a tridomain structure: an N-terminal globular domain (G1) that binds hyaluronan with great affinity, a central domain of variable length with multiple sites for GAG (chondroitin-sulfate or dermatan sulfate) and O-linked oligosaccharide attachment, and a C-terminal globular domain (G3) that binds lectins and several other molecules. The detailed schematic representations of the structure of aggrecan and versican domains are shown in Figure 1. The versican domains and their binding partners (Figure 1, Appendix) and splice variants (Figure 2) are described also in the following chapters.

Alternate splicing and various degrees of glycanation and glycosylation make hyalectans appropriate molecular bridges between cell surfaces and the ECM.[11,16,17] It is likely that versican forms a molecular link between lectin-containing glycoproteins at the cell surface and extracellular HA. Similar to aggrecan, versican forms PG aggregates with link protein (LP) and HA.[18] Because HA is bound to the cell surface via its CD44 receptor, versican may also stabilize a large supramolecular complex at the plasma membrane zone. The large

proteoglycan aggregates provide the tissues with a unique gel-like property and increased resistance to mechanical forces through water absorption. Moreover, the aggrecan-based complexes regulate the differentiation of chondrocytes by storing extracellular signaling molecules involved in differentiation and distributing them to target cells.[19] However, the tissue distribution and *in vivo* function of versican aggregates remain to be clarified.

As a result of alternate exon usage, versican exists in at least four isoforms, namely V0, V1, V2 and V3. The protein products of each mRNA transcript differ dramatically in length and in number of CS chains attached, and therefore may have different functions. In fact, the different splice variants of versican, such as that of the other members of the hyalectan family, are often expressed in developmentally-specific and tissue-specific manner supporting that each splice variants has specific biological roles.[20,21] In general, connective tissues express predominantly the V1 variant. Although V1 versican is also the principal expressed form during prenatal brain development, it is replaced by the V2 isoform, which is the characteristic versican isoform in the adult brain. The V0 and especially the V3 variant are known to be expressed only in a limited number of tissues.

After more than a decade of the discovery of versican increasing body of evidence reveals that the function of this PG is far beyond a simple structural and biomechanical role in a variety of tissues, instead, versican affects several crucial biological events in development and disease. The detailed description of the versatile functions of versican in different developmental and other cellular events is beyond the scope of this introduction. Therefore, in the following, I briefly summarize the main functions of versican (in cell adhesion, proliferation, migration and survival) with focus on its role in tumor biology:

Early studies show that versican interferes with the attachment of embryonic fibroblasts to various substrates, including fibronectin, laminin, and collagen. The inhibition of cell adhesion by versican depends upon the CS chains attached to the central domain of V0, V1 and V2 splice variants.[22,23] In addition, the anti-adhesive activity of versican can be partly attributed to HA-binding via the G1 domain. Furthermore, versican interacts with CD44 through chondroitin sulfate chains and this interaction is believed to stabilize the cell surface associated versican-HA complexes.[24] In response to growth factors, e.g. TGF-ß and platelet derived growth factor (PDGF) there is an increase in versican complexes resulting in the expansion of a highly viscoelastic pericellular matrix that excludes

interaction of the cell surface with other matrix molecules. This ultimately affects cellular adhesion, proliferation and migration.[25-27] It explains, at least in part, the association of versican with proliferative cell phenotypes and its increased expression in a variety of tumor types, such as breast-, prostate-, and colon cancer, melanoma and brain tumors.[28-32] In fact, increased concentration of stromal versican is an independent predictor of outcome for patients with early-stage prostate cancer and increased versican expression in the peritumoral stroma is a strong predictor of a shorter relapse-free survival in breast cancer and also associated with an increased risk of developing metastatic prostate and breast cancer.[29,30]

Furthermore, besides the HA-binding G1 domain, the C-terminal G3 domain of versican has been also reported to promote cell proliferation directly through the EGF sequences.[33] Moreover, Zheng and his colleagues (2004) have recently reported that the expression of a versican G3 construct in astrocytoma cells enhances tumor growth and blood vessel formation in nude mice.[34] They also showed that G3 stimulated the expression of fibronectin and vascular endothelial growth factor (VEGF) and formed complex with these two molecules. The complex significantly enhanced endothelial cell adhesion, proliferation and migration. These findings implicate that promotion of angiogenesis may be another mechanism by which versican enhances tumor growth via the G3 domain and targeting versican G3 fragments could be a potential anti-angiogenic treatment, thus anticancer therapy.

Versican seems to play an important role also in cell survival. It has been reported to be involved in reducing oxidant injury through an enhancement of cell-matrix interaction.[35] Another recent and interesting study of Sheng and colleagues (2005) showed that while V1 versican (containing the GAGβ subdomain) enhances proliferation of NIH3T3 fibroblasts and renders cells resistant to apoptosis, the V2 variant (containing the GAGα subdomain) exerted an inhibitory effect on cell proliferation and had no effect on apoptosis.[36] They showed that the GAGβ domain is responsible for the activation whereas the GAGα domain plays a role in the suppression of epidermal growth factor receptor (EGFR) expression and its downstream signal pathway. Moreover, V1 and V2 alter proliferation differently, not only through the EGFR pathway, but also through cell cycle-related proteins. These findings imply that the GAGα and GAGβ domains play different roles in cell proliferation and survival, ascribing distinct biological activities to the V1 and V2 isoforms. The opposing effects of V1 and V2

on cell proliferation suggest that a dynamically balanced expression pattern of these two isoforms may provide a suitable extracellular environment for normal proliferation and survival of cells.
The V3 variant that lacks CS chains has been reported to have distinctly different effects on cell migration than CS-bearing versican variants. For example, overexpression of V3 in rat smooth muscle cells significantly down-regulates cell migration and proliferation and this is hypothesized to occur via the displacement of versican V0/V1 from the cell surface.[37]
In summary, versican synthesis is a highly regulated process that causes changes in cell adhesiveness, proliferation and survival and may either facilitate or inhibit cellular migration. Furthermore, the recent advances in versican research have elucidated that versican not only presents or recruits molecules to the cell surface, but also modulates the expression levels of genes and coordinates complex signal pathways.

Proteolysis of ECM

The ECM not only provides the cells with a structural scaffold, it also acts as a reservoir for embedded cytokines and growth factors and harbors cryptic information within molecules that make up the ECM network. As already mentioned above tumor cells must remodel the matrix to escape its control and to be able to proliferate and migrate out of the home territory of normal cells in tissue. Proteolysis is a major factor leading to changes in the ECM. The proteolytic enzymes principally disrupt ECM structures such as basement membranes and degrade ECM components promoting the spread of tumor cells by removing the physical barrier to cell migration. However, these enzymes have also other substrates such as cytokines, growth factor receptors, adhesion molecules which may also promote tumor cell proliferation and invasion.[38] In fact, the extracellular proteases have been shown to be essential to cell transformation, migration and differentiation. Yet, the underlying mechanisms remain uncertain. It may be the release of important sequences from ECM molecules, the shedding of receptors during change in cellular phenotype, or the release of mechanical tension that provides the essential signal to cells.[39,40]

Enzymes

The principal enzymes that degrade the ECM include matrix metalloproteinases (MMPs), cystein proteases (cathepsin B), aspartic proteases (cathepsin D) and serine proteases (elastase, urokinase plasmin activator). In Chapter 5 we primarily focus on the metabolic mapping of MMPs in canine mammary tumors, therefore, in the following I briefly introduce these enzymes, respectively.

MMP*s* comprise a large family of extracellular enzymes that share common structural features, particularly those regions involved in the regulation of proteolytic activity. They are a subgroup of a much larger metalloproteinase superfamily, which also includes the adamalysin proteases or ADAMs (proteins with disintegrin and metalloprotease domain) and ADAMTS (an ADAM with a thrombospondin-like motif) proteases, among others.[41,42] To be classified as a MMP, a protein must have at least two conserved motifs, namely the pro-domain and the catalytic domain. The catalytic domain has a HEXGH motif responsible for ligating zinc, which is essential for catalytic function.[43] In addition to the conserved regions, MMPs have a variety of specialized domains that contribute to substrate specificity and recognition or interaction with other molecules. Based on their structure and substrate specificity they have traditionally been divided into four main groups of closely related members: the interstitial collagenases (MMP-1, MMP-8, MMP-13) stromelysins (MMP-3, MMP-10, MMP-11, MMP-7) and membrane-type MMPs (MT-MMPs). MMP-7 (matrilysin-1) together with MMP-26 (matrilysin-2) are often classified as a separate subgroup, namely matrilysins, or placed into a separate group with other known MMPs than the members of the traditional main four groups of MMPs. Table 1 in Appendix describes the classification and substrate preference of MMPs.

The catalytic activity of MMPs is regulated at four points -gene expression, compartmentalization (pericellular accumulation of the enzymes), pro-enzyme (latent form) activation and enzyme inactivation- and is further controlled by substrate availability and affinity. MMPs are secreted in latent form (as pro-enzymes) and require specific proteolytic activation.[43] Most MMPs are activated extracellularly, with exception of MMP-11 and MT1-MMP, which are activated prior to secretion intracellularly by furin-like proteases.[44] This activation step serves as a regulatory element. MMP-activity is further regulated by MMP inhibitors (TIMPs) and expression of molecules, which present the active enzyme on the cell surface.[44,45] However, most MMPs are converted into active forms in a specific multistep activation process that is known as the “cystein switch”. A

conserved unpaired cystein residue in the pro-domain forms a coordinate bond with zinc ion at the active site. Cleavage of the pro-domain results in opening the active site by disruption of the zinc-cystein bond and is followed by loss of the N-terminal pro-domain.[46] For most MMPs, proteolytic activation is initiated in the extracellular space by serine proteases such as plasmin and urokinase plasmin activator, neutrophil elastase and by other members of the MMP family members, especially MMP-2 and MMP-13, by MT1-MMP cleavage of the pro-enzyme.[44,48,49] However, little is known about the activation induced by other factors including other MMPs, cytokines, trypsin, serine proteases, reactive oxygen species, hypoxia, c-erbB ligands, and leukocyte elastase.[50,51] Some activated MMPs can further activate other pro-MMPs.

Because MMPs degrade all the components of the ECM, they have been extensively studied in the context of modulating matrix function. Because of the similarity in their metalloproteinase domains, there is potential for functional overlap within the MMP family as well as overlap between other metalloproteinase families. MMPs are generally produced by cells at very low levels. In contrast, in case of ECM remodeling, under either physiological or pathological circumstances, increased expression and activity of the enzymes can be induced.[48] Physiologically, MMPs play a role in normal tissue remodeling e.g. in embryonic development, wound healing, angiogenesis, ovulation and mammary gland involution. Increased MMP activity seems to contribute to various pathological processes including osteoarthritis, rheumatoid arthritis, pulmonary emphysema, and tumor growth, invasion and metastasis.[48,52,53] MMP expression has been shown in a variety of tumor types including carcinomas of breast, colon, lung and prostate.[54-57] In addition, the plasma and urine levels of MMPs are elevated in patients with cancer compared with healthy subjects.[58] MMPs in tumor tissues are produced not only by malignant tumor cells but also by stromal fibroblasts and inflammatory cells.[59] These cells may produce cytokines and proteins that induce the production of MMPs by surrounding cells, creating extracellular network of MMP secretion and activation.[60-62] Furthermore, analyses of cellular components derived from primary tumor tissues or their corresponding lymph node metastases demonstrated that expression of MMPs is increased in the metastatic tissues, indicating that MMP expression is a component of the metastatic process.[63] However, little is know about the *in vivo* activity of MMPs in tumor tissues and their metastases. There is clinical evidence, though, that over-production of these enzymes confers a poor prognosis in patients with a variety

of malignancies (reviewed in reference 64). Besides ECM remodeling in tumor invasion MMPs also influence tumor initiation and risk for neoplastic development. For example, there is a formation of mesenchymal-like tumors in mice transgenically expressing MMP-3 in normal mammary epithelial cells.[65] The role of MMPs in tumor development is further complicated by the balance of these proteins in relation to TIMPs. TIMPs have various antioncogenic functions and the expression of TIMPs has been associated with a less aggressive tumor behavior and favorable prognosis in cancer patients. (For further details on TIMPs see reference 66).

It is becoming increasingly clear that matrix degradation is not the only function of MMPs. In fact, several reports in the last decade have suggested or demonstrated that various MMPs can modulate the activity of a variety of non-matrix proteins. For example, several MMPs, such as MMP-1, MMP-2, MMP-3, MMP-7 and MMP-11 directly modulate the activity of several growth factors and chemokines, such as transforming growth factor (TGF)-$ß_1$, tumor necrosis factor (TNF)-α, insulin-like growth factor (IGF)-1, epidermal growth factors, fibroblasts growth factors (FGFs), and monocyte chemoattractant protein (MCP)-3.[67-73] In addition, fragments of matrix proteins released by MMP-mediated proteolysis can act as chemoattractants for distant cells and their proteolytic activity on ECM substrates uncovers or releases cryptic sites of ECM macromolecules that function to modulate a cellular response.[74,75] Last but not least, a functional link between E-cadherin and MMP expression has been demonstrated recently by several studies.[76-79] These studies demonstrate a direct role of E-cadherin/catenin complex in the regulation of MMPs and implicate this regulation in the expression of an invasive phenotype by tumor cells. In conclusion, MMPs should therefore not be viewed solely as proteinases of matrix catalysis, but as extracellular processing enzymes critically involved in cell-cell and cell-matrix signaling (for review see also reference 80).

ECM of canine mammary tumors/ Scope and outline of the thesis

Mammary tumors are the second most common tumors after skin tumors and the malignant mammary tumors are the most common malignant neoplasia in the female dog. The incidence in dogs is three time as high as in women.[81] They show great histomorphologic heterogeneity that makes the accurate tumor classification and grading often difficult. (The latest WHO Classification of canine mammary tumors is shown in the Appendix.[82]) In addition to epithelial cells,

they frequently have proliferating spindle cells of uncertain origin (complex tumors) and cartilagenous or osseous ECM (mixed tumors). There are conflicting ideas on the origin of both spindle cells and cartilage/bone of complex and mixed tumors. The spindle cells have been traditionally assigned a myoepithelial origin [83], but their immunophenotype described in the literature by different authors are conflicting.[84,85] There are four main lines of thinking on the origin of cartilage and bone in mixed tumors: (1) stem cell origin [86,87], (2) metaplasia from myoepithelial cells [84,88,89], (3) metaplasia from epithelial cells [90], and (4) metaplasia from connective tissue [85]. However, up to now there are no data published that would provide direct evidence for any of these suggestions.

The active role of the ECM and its remodeling enzymes in regulation of mammary gland morphogenesis has been shown and recently reviewed by Fata and colleagues.[91] However, understanding how ECM imparts morphoregulatory signals to mammary epithelium is of importance also in carcinogenesis. In fact, several studies in the last decade showed that changes in the composition and integrity of the ECM are associated with several human tumor types, such as human breast cancer.[92,93] Among several other ECM proteins the expression and composition of PGs also change in breast cancer.[92] Although the ECM of canine mammary tumors, particularly that of complex and mixed tumors is abundant and shows great heterogeneity very little attention has been paid to identify its components and organization. In fact, only few studies provide some useful data on the GAG composition [94], and fibronectin, tenascin and collagen content of these tumors [95-98] and there are no data regarding the nature and distribution pattern of PGs. As early as 1979 Palmer and Monlux demonstrated that the ECM of complex mammary tumors of dogs is particularly rich in GAGs.[99] However, in the next 20 years no further studies have been conducted to reveal the biological significance of the accumulating GAGs in these tumors or to identify the exact type of the GAGs. In 1999, in accordance with findings in human breast cancer, Hinrichs et al identified chondroitin sulfate as the predominant sulfated GAG present in canine complex and mixed tumors.[94] They showed that chondroitin sulfate (CS) accumulates predominantly in the tumor stroma, particularly around clusters of epithelial tumor cells, suggesting that tumor cells may induce CS production by stromal fibroblasts. However, the nature of PG that carries the CS side-chains remained to be elucidated. Therefore, as a first step, the aim of this thesis was to investigate whether versican, a member of the CS-PGs (hyalectans) is co-expressed with CS in canine mammary tumors (Chapter 2). We choose to

investigate versican because it was the most probable candidate among the CS-PGs, since enhanced versican expression has been already observed in a number of tumor types. Moreover, several studies within the last few years have confirmed a significant role for versican in regulating cell adhesion, survival, proliferation, migration and extracellular matrix assembly, indicating an overall importance of this PG in key events during tumor development and progression (see above; for review see also reference 100). Furthermore, the other CS-PGs are not likely candidates because they are rather tissue specific, aggrecan being expressed mainly in cartilage, neurocan and brevican in the nervous tissues.[11] Based on the findings in Chapter 2, we addressed the question whether the high expression of versican in the myoepithelial-like spindle cell proliferations and myxoid tissues relates to cartilaginous differentiation of these specific tissues, and whether these tissues may serve as precursor tissue of the ectopic cartilage in mixed tumors. Therefore, in Chapter 3 we investigated by immunohistochemistry the co-expression of versican with the cartilage biomarkers collagen type II, aggrecan, link protein (LP) and 3B3 neoepitope (neopitope of chondoitin-6- sulfate) in myoepithelial-like spindle cell proliferations and myxoid tissues. The relation of mRNA expression and protein accumulation of versican, collagen type II and aggrecan has also been studied. Furthermore, the phenotype of spindle cells in complex and mixed tumors, which are most likely involved in the ectopic cartilage formation, has been characterized by immunohistochemistry. In addition, to determine whether versican and/or aggrecan co-localize *in vivo* with HA and LP we have stained our tumor samples simultaneously for these molecules. In Chapter 4 one of our main objectives was to determine if versican is expressed in multiple isoforms (splice variants) in canine mammary tumors and cell lines. Furthermore, the results of the immunohistochemical study on versican expression (Chapter 2) strongly indicated that the protein core of versican is proteolyticly cleaved in canine mammary tumors, thus the presence of catabolic products of versican has also been studied.

An important hallmark of cancer includes altered expression of ECM-degrading proteases. However, in canine mammary tumors little is known on the expression, localization and *in vivo* activity of these proteases. In Chapter 4 our main objective was the metabolic mapping of MMPs and cathepsin B in these tumors. We focused on proteases that potentially may cleave proteoglycans and

therefore may be involved in the proteolytic processing of the core protein of versican.

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CHAPTER 2

Immunohistochemical evaluation of versican, in relation to chondroitin sulfate, in canine mammary tumors

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Abstract

The expression of increased amounts of versican, a chondroitin sulfate proteoglycan, in neoplastic tissues may play a role in promoting tumor cell proliferation and migration. This study investigated the immunolocalization of versican in normal and neoplastic canine mammary tissues, using antibodies 12C5 and 2B1, against different epitopes of the protein core of versican. Antibody CS56, recognizing chondroitin sulfate (CS), was used to investigate the relation between versican and CS, which accumulates in canine mammary tumors. We found enhanced versican expression in both benign and malignant tumors, appearing in three main patterns: in periductal tissues, probably in association with basement membranes of ducts; in peripheral invasive areas of malignant tumors; and in spindle cell proliferations and myxoid areas of complex and mixed tumors. The 12C5 and 2B1 immunoreactivities co-localized in all types of tumors, and could be improved by chondroitinase digestion. The only exception was the abundant extracellular matrix (ECM) of spindle cell proliferations, particularly in myxoid areas of complex and mixed tumors, which displayed intense and diffuse 12C5 immunoreactivity and patchy or absent 2B1 and CS56 immunoreactivities; versican immunoreactivity could not be enhanced by chondroitinase digestion. The results indicate that versican is one of the extracellular matrix components characteristic of canine mammary tumors. It appears likely that in complex and mixed tumors versican exists in at least two forms, one of them lacking the CS chains and the 2B1 epitope. Furthermore, the enhanced versican expression in the invasive areas of malignant tumors indicates the involvement of this proteoglycan in tumor cell invasion.

Introduction

Among the multiple components of the extracellular matrix (ECM) in neoplastic tissues, the proteoglycans (PGs), such as versican, are in a central position because of their role in cell adhesion and proliferation.[1] Versican, also called PG-M, belongs to the family of large aggregating chondroitin sulfate (CS)-containing PGs [1,2], which also includes the cartilage-derived aggrecan, and two smaller PGs expressed in nervous tissues, namely neurocan and brevican.[3] These PGs have common features such as the tridomain structure of the protein core: a highly similar hyaluronan-binding domain (G1) at the N-terminal region; a central domain bearing the glycosaminoglycan (GAG) attachment sites; and a selectin-like domain (G3) composed of epidermal growth factor (EGF)-like, lectin-like, and complement regulatory protein (CRP)-like elements at the C-terminal globular region.[4] Versican has a strong hyaluronan (HA) binding affinity via the G1 domain [5] and its G3 domain has been shown to bind tenascin-R [6], fibulin-1 [7], fibrillin-1 [8], β1-integrin [9] and several carbohydrates [10]. The central domain of versican is subdivided into two large subdomains, designated GAG-α and GAG-β that are encoded by two alternatively spliced exons.[11] As a result of alternative splicing the mammalian versican exists at least in four isoforms, namely V0, V1, V2 and V3, which may express different functions. The largest V0 isoform is known to contain both αGAG and βGAG exons, V1 containing the βGAG exon, V2 having the αGAG, and V3 lacking any GAG subdomain. Thus far, protein products have been demonstrated for the V0, V1, and V2 forms which differ in length and in number of attached CS chains.[12,13]

Versican localises in a variety of human and animal tissues.[14,15] It has been identified in the loose connective tissue of various organs, often associated with the elastic fiber network, in smooth muscle, cartilage, veins and elastic arteries, and in the central and peripheral nervous system.[16,17] It has also been detected in the proliferative zone of the epidermis and has been proven to be involved in hair follicle development.[18-19] The exact function of versican remains elusive, although the molecule appears to have several functions. It may be involved in intercellular signaling through its lectin- and EGF-like sequences and may take part in cell recognition via interaction with other ECM components and cell surface molecules.

Abnormal versican deposition has been observed in a number of tumor types, including human colon [20], prostate [21] and breast cancer [22,23], and the pleomorphic

adenoma of the salivary glands [24]. Furthermore, it has been described in brain tumors [25] and in malignant melanoma [26], as well as in some other non-epithelial neoplasms [27]. It has been suggested that the versican-rich extracellular matrices exert an anti-adhesive effect on cells, thus facilitating tumor-cell migration and invasion.[28]

Mammary tumors are the most frequent tumors in bitches and are notorious for their complexity in histological pattern and biological behaviour. On the basis of the cell types and ECM components present in the tumor, they are classified histologically as simple, complex and mixed tumors. Simple tumors are composed of only one type of proliferating epithelial cells. In complex tumors, the luminar epithelium and the spindle-shaped cells of possible myoepithelial origin largely contribute to tumor formation. The stroma of mixed tumors is characterized by the presence of cartilage and bone, and tumorous epithelial and mesenchymal cells can proliferate simultaneously. There is only scarce literature concerning the PG content of canine tumors. In mammary gland tumors, Palmer and Monlux [29] have reported the presence of a tumor stroma rich in GAGs in complex carcinomas and Hinrichs et al demonstrated an enhanced expression of CS, in particular in complex and mixed tumors[30]. However, which CS-containing PGs are present in canine mammary tumors and the biological significance of these ECM components still remains to be elucidated. Therefore, in the present work we have performed immunohistochemical labelling to identify the large interstitial PG, versican, as a possible candidate for containing CS in mammary tumors [22], with two anti-versican monoclonal antibodies in a large number of mammary tumors. The relation between the distribution of versican and CS was also studied.

Materials and Methods

Antibodies

The antibodies used in the present study were: (1) monoclonal antibody 12C5 raised against versican isolated from human brain (Developmental Studies Hybridoma Bank, Iowa City, IA, USA) and known to recognise the HA-binding domain of versican [31]; (2) monoclonal antibody 2B1 against versican, purified from human yolk sac tumor (Seikagaku Co., Tokyo, Japan) [32]; and (3) monoclonal antibody CS56 (Sigma Chemical Co., St Louis, MO, USA), which reacts with

chondroitin sulfate but not with dermatan sulfate [33], was used to confirm the presence and distribution of CS.

Tissues

The mammary tissues examined in the present study were derived from 75 dogs and were resected surgically or obtained from the archives of necropsies of the Department of Veterinary Pathology, Utrecht University. The samples included inactive and lactating (secretory) normal mammary tissues, benign and malignant tumors. One out of the 7 lactating mammary tissues was derived from a pregnant dog and 4 were derived from pseudopregnant dogs with clinical lactation. There was no clinical information available concerning the ovarian cycle of the remaining two dogs with lactating (secretory) mammary tissues. The histological type and number of the examined tissues are summarized in Table 1.

Table 1. Histological diagnosis of the 81 canine mammary tissues examined

HISTOLOGY	NUMBER OF TUMORS
Normal mammary tissue (n=10)	
Inactive	3
Lactating*	7
Benign tumor (n=37)	
Simple adenoma	
Tubulopapillary	12
Complex adenoma	15
Mixed tumor	10
Malignant tumor (n=34)	
Simple carcinoma	
Tubulopapillary	12
Solid	6
Anaplastic	4
Complex carcinoma	7
Mixed tumor with malignant epithelial component	5

*6 out of the 7 lactating mammary tissues also showed some regression.
Classification according to WHO criteria.[34]

Tissues were fixed immediately after surgery in 10% neutral-buffered formalin and embedded in paraffin, after which 5 µm sections were cut and stained with hematoxylin and eosin (H&E). Tumors were classified from the H&E-stained sections according to the recent WHO classification.[34]
Follow-up of most animals was not available in this retrospective study. Malignancy was based on the evaluation of histologic features, predominantly on the growth pattern and cellular morphology. The term of infiltrating carcinoma was used in case of clear signs of stromal invasion of tumor cells regardless of the presence or absence of lymph/vascular invasion or lymph node metastasis.

Immunohistochemistry
Immunohistochemistry was performed using an indirect immunoperoxidase staining procedure, applying the avidin-biotin based technique (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA, USA). After deparaffinisation and rehydration, the sections were washed in Tris-HCl buffer (0.25 M, pH 8.0). All incubation steps, except where stated, were performed at room temperature. Proteolytic demasking of antigens by trypsin increased the intensity of immunostaining for versican with antibody 2B1, but not with antibody 12C5 and did not improve CS immunolabelling with antibody CS56. Thus sections were incubated with 0.1 % trypsin (Dako A/S, Glostrup, Denmark) in 0.01 M PBS with 0.1% CaCl2 (pH 7.8) at 37°C for 20 minutes only before the use of 2B1 antibody. We also used chondroitinase ABC (from Proteus vulgaris; Sigma Chemicals) digestion before the application of the primary antibodies. Digestion with chondroitinase ABC was performed at 37°C for 2 hours with 0.5 U/ml of the enzyme in 0.25 M Tris buffer (pH 8.0) containing 0.18 M sodium chloride, 0.05% bovin serum albumin (BSA), 0.1 M 6-amino-n-caproic-acid and 5 mM benzamidine hydrochloride to inhibit protease activity. To control the specificity of chondroitinase pretreatment control slides were treated at the same time with the same protease inhibiting buffer without enzymes. All sections were rinsed with S-PBS (PBS with 0.03% saponine), soaked in 0.3% H_2O_2 in S-PBS for 30 minutes to inhibit the activity of endogenous peroxidase, rinsed again, and then allowed to react with 10% normal horse-serum in 1% BSA-containing S-PBS, followed by overnight incubation with diluted primary monoclonal antibodies, 2B1 (1:1000), 12C5 (1:50) and CS56 (1:200), at 4°C. After incubation and washing, the sections were treated with biotinylated horse anti-mouse immunoglobulin diluted 1:125 in S-PBS, followed by incubation with avidin-biotin

complex according to the manufacturer's instructions (Vector Laboratories). Reactions were visualised using 0.5% 3,3-diaminobenzidine tetrahydrochloride (Sigma Chemicals) and 0.3% H_2O_2 diluted in Tris/HCl buffer (0.05 M, pH 7.4), during a 10-minute incubation step. Sections were counter-stained with Mayer's hematoxylin and mounted. As negative controls, normal mouse serum was employed for the reaction instead of primary antibodies.
Slides were assessed for staining intensity and location of immunoreactivity within the tissues (Table 2 and 3) by two experienced observers (EI and HN) simultaneously. The intensity and pattern of the staining were graded using an empirical semi-quantitative system: (-), no reaction; (+), weak reaction; (++), moderate reaction; (+++), intense reaction; (+++d), intense diffuse reaction.

Results

The results of immunohistochemical analyses of versican and CS expression in all types of mammary tissues examined are summarized in Table 2.
In skin covering mammary tissues, versican immunoreactivity with antibodies 12C5 and 2B1 was frequently noted at the dermoepidermal junction as a moderate to strong discontinuous line along the epidermal basement membrane and as a weak fibrillar staining in the immediate superficial dermis. Furthermore, reactivity could be seen in connective tissue sheets, and in necks and dermal papillae of hair follicles in accordance with the observation in human skin.[19] The same structures were stained for CS, but CS immunoreactivity was often more intense and widespread compared to versican. In general, large blood vessels showed immunoreactivity with all three antibodies in all three layers of their muscular walls, but in a number of cases staining for versican was observed only in perivascular fibrous elements. The adventitia of small blood and lymph vessels was also immunostained. These positively-stained structures in skin and also blood vessels served as internal positive controls in our samples.

Normal mammary tissues
The histological characteristics of the normal mammary gland during the ovarian cycle are comparable in women and female dogs.[35,36] In this study none of the normal mammary tissues showed appreciable versican immunoreactivity with antibodies 12C5 and 2B1 (Figure 1A). CS was widely present in most of the

lactating (lobuloalveolar secretory tissue) and regressing (alveolar involution), but not in the inactive mammary tissues (branched ductal system with quiescent epithelium).

Table 2. Immunohistochemical labelling with antibodies

SITE	12C5	2B1	CS56
Normal mammary tissues			
Inactive	-	-	-
Lactating			
Intralobular connective tissue	-	-	++
Periductal tissues	-	-	+++
Cytoplasm of secretory epithelial cells	-	-	+++
Benign Tumors			
Fibrous connective tissue	++/-	++/-	+++
Periductal tissues	++/+++	++/+++	+++
Cytoplasm of neoplastic epithelium	-	-	++/-
Malignant Tumors			
Fibrous connective tissue	+/-	+/-	+++
Periductal tissues	++/-	++/-	+++
Peripheral invasive area	+++/-	+++/-	+++
Cytoplasm of neoplastic epithelium	-	-	++/-
Benign and Malignant Complex and Mixed Tumors			
Myoepithelial-like spindle cells proliferations			
Poor ECM			
Abundant, myxoid ECM	+++	+++	+++
Cytoplasm of spindle cells	+++d	++/-	++/-
Cartilage matrix	+++	+++	+++
Perichondral tissues	-	-	+/++
Bone	++	++	++
	-	-	-

-: no reaction; +: weak reaction; ++: moderate reaction; +++: intense reaction; +++d: intense, diffuse reaction

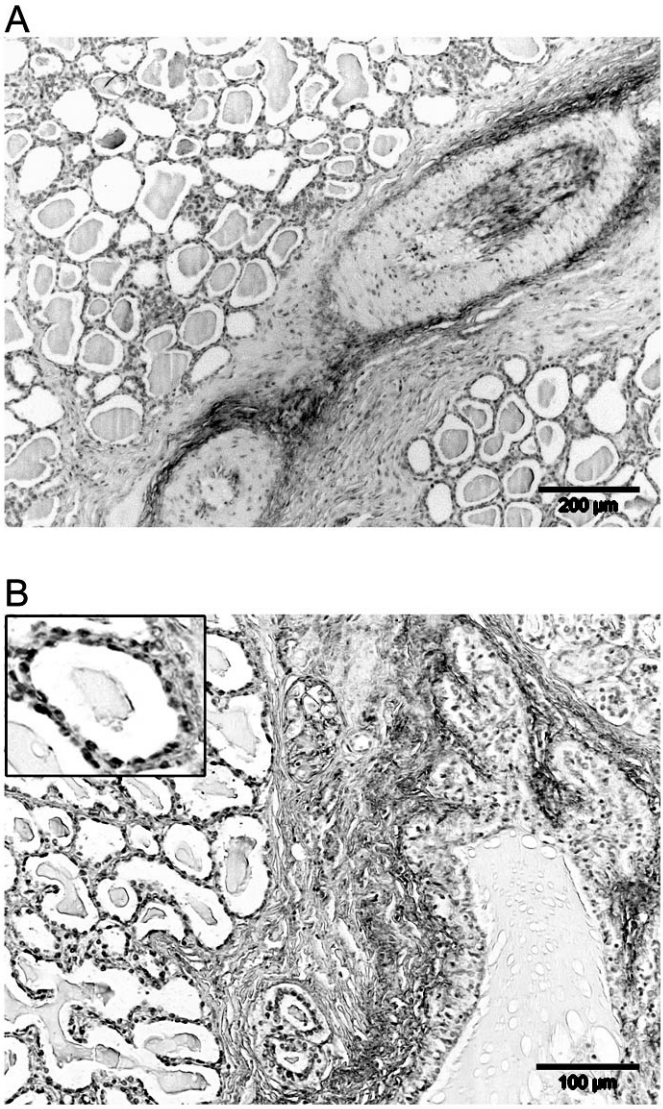

Figure 1. *Immunolabelling of normal mammary tissues*
(A) Lack of versican expression in a normal mammary tissue. Reactivity in blood vessels served as internal positive control (antibody 12C5; bar represents 200 μm). **(B)** CS expression surrounding an extralobular duct that blends with the staining in the adjacent connective tissue and intense cytoplasmic reactivity of the luminal epithelial cells (inset) in secretory alveoli (antibody CS56; bar represents 100 μm).

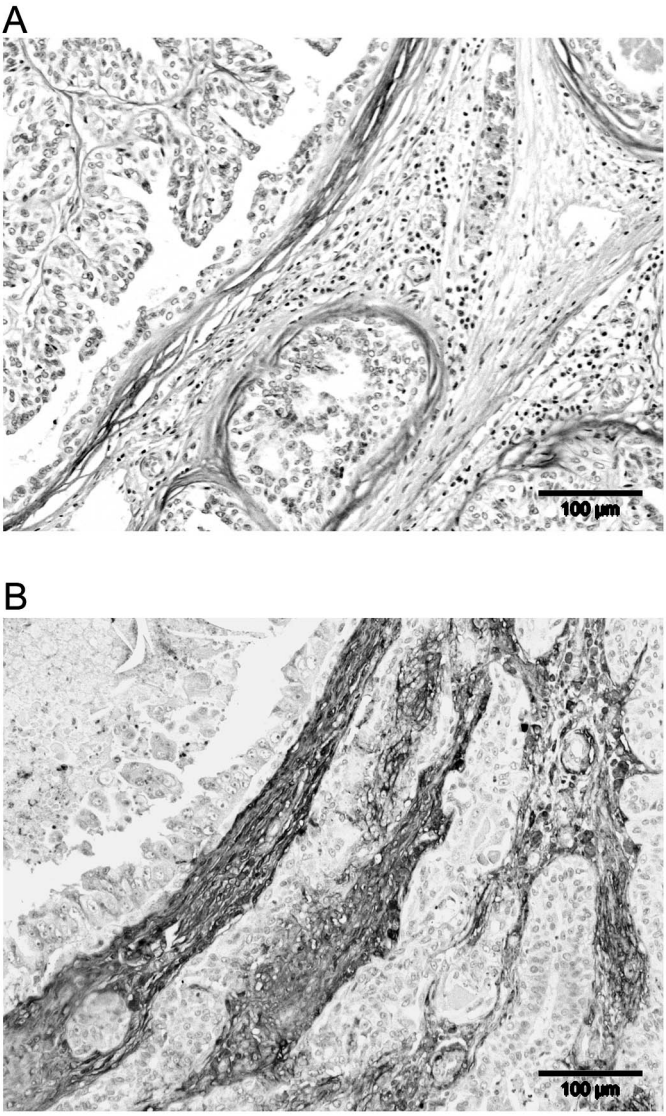

Figure 2. *Immunolabelling of periductal tissues in benign tumors*
(A) Intense versican expression in concentric layers surrounding ducts in a tubulopapillary adenoma. Note also the staining in the loose connective tissue of papillary projections (antibody 2B1). **(B)** CS reactivity is not restricted to the periductal tissues, but is intense and widespread throughout the intratumoral fibrous tissue (antibody CS56). Bars represent 100 μm.

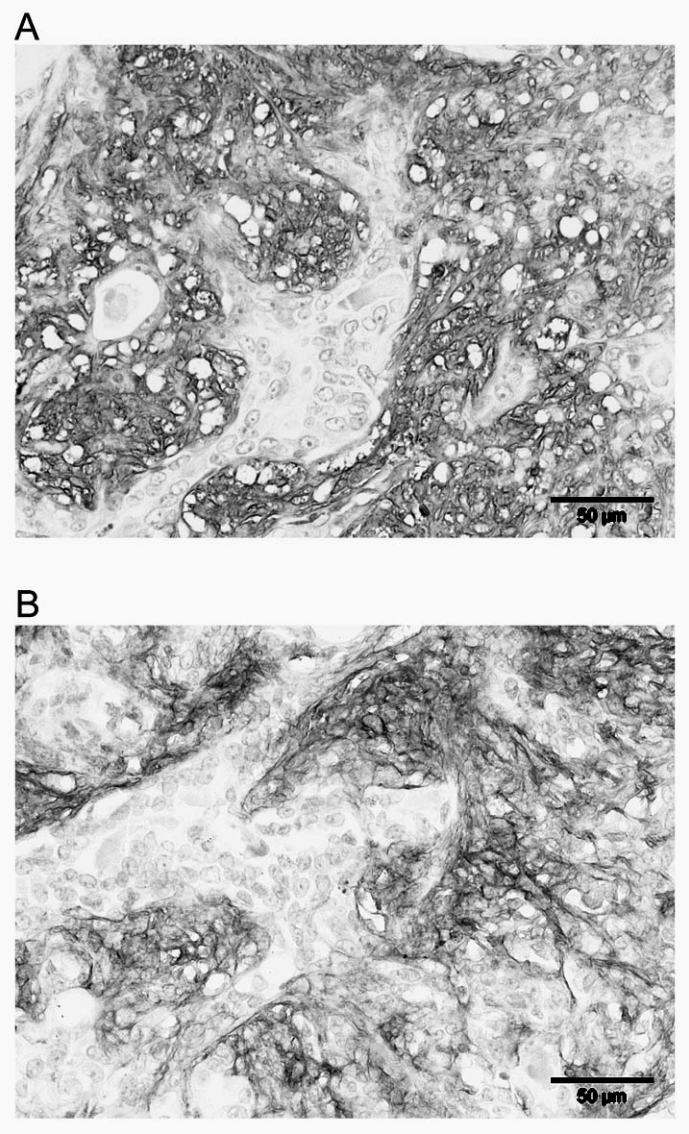

Figure 3. *Immunolabelling of complex and mixed tumors*
Intense versican immunoreactivity in cell-rich myoepithelial-like spindle cell proliferations of a complex adenoma. There is no significant difference in the labelling with **(A)** antibody 12C5 and **(B)** antibody 2B1. Bars represent 50 µm.

Consistently noted patterns of CS immunoreactivity were the following: moderate to intense cytoplasmic staining of luminal epithelial cells almost exclusively in secretory differentiated lobules; reactivity of the intralobular connective tissue with more intense staining in the immediate periacinar and periductal structural elements; and prominent periductal reactivity surrounding extralobular ducts that blended with staining in the adjacent connective tissue (Figure 1B). In the immunoreactive connective tissue the cytoplasm of interstitial fibroblasts was also positively stained.

Benign tumors

In general, fibrous connective tissues within benign tumors displayed moderate to intense immunoreactivity with antibodies 12C5 and 2B1 in the immediate periductal tissues. Intense reactivity appeared as a concentric layer surrounding large extralobular ducts (Figure 2A). In most of the tubulopapillary adenomas the periductal reactivity extended into the loose connective tissue of papillary projections. CS showed a more widespread and homogenous distribution throughout the fibrous connective tissue (Figure 2B). In the immunoreactive areas of fibrous connective tissue in all types of benign tumors the cytoplasm of interstitial fibroblasts was often immunostained with all three antobodies. Furthermore, apical cytoplasmic staining for CS, but not for versican, was noted in the lining epithelium of several papillary projections. Removal of CS side chains from the protein core of versican by chondroitinase ABC treatment significantly improved the intensity of staining with antibodies 12C5 and 2B1 in the above mentioned areas, but completely demolished CS labelling with CS56 antibody, as was expected.

In complex adenomas immunoreactivity to versican was most prominent in the ECM and in the cytoplasm of a large portion of spindle cells within the areas of myoepithelial-like spindle cell proliferation. In those areas where myoepithelial-like spindle cells formed cell-rich bundles accompanied by a relatively small amount of ECM, there was no considerable difference observed in the intense immunoreactivity to versican with antibodies 12C5 and 2B1 (Figure 3A,B); the intensity of staining could be enhanced after chondroitinase ABC digestion. In these areas immunoreactivity to CS was rather similar to that to versican. In ECM-rich myxoid areas with few characteristic polygonal-cells (Figure 4A), the abundant ECM showed not only intense but completely diffuse and homogenous staining with antibody 12C5, whereas it was only partially stained with antibody

2B1, often displaying a distinct halo around cells (Figure 4B,C). Moreover, 2B1 immunoreactivity was completely absent in some of the myxoid matrices. Immunoreactivity to CS was patchy or absent in these areas (Figure 4D). Interestingly, in abundant myxoid ECM the 12C5 immunostaining invariably appeared intense and diffuse even without chondroitinase ABC treatment. Morover, the intensity of 12C5 and 2B1 immunostainings in myxoid ECM could not be improved further by chondroitinase ABC digestion. Cytoplasmic staining of spindle and polygonal shaped cells, both in ECM-poor and ECM-rich areas, was detected with all three antibodies. Summarizing, in spindle cell proliferations the intensity and extension of staining with all three antibodies showed some variation, the only exception being 12C5 immunostaining in myxoid ECM.

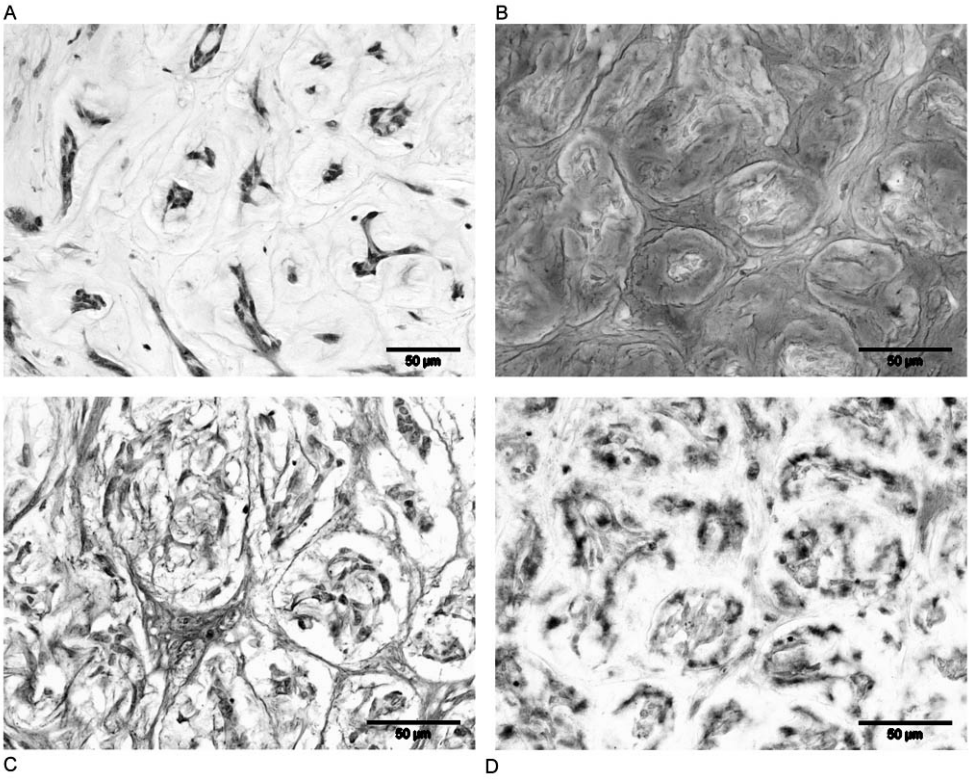

Figure 4. *Immunolabeling of myxoid areas in complex and mixed tumors*
ECM-rich myxoid tissue characterized by spindle- and polygonal-shaped cells in serial slides of a benign mixed tumor. **(A)** H&E staining. The abundant ECM is stained **(B)** intensely and diffusely with antibody 12C5 and **(C)** only partially with antibodies 2B1 and **(D)** CS56. Bars represent 50 µm.

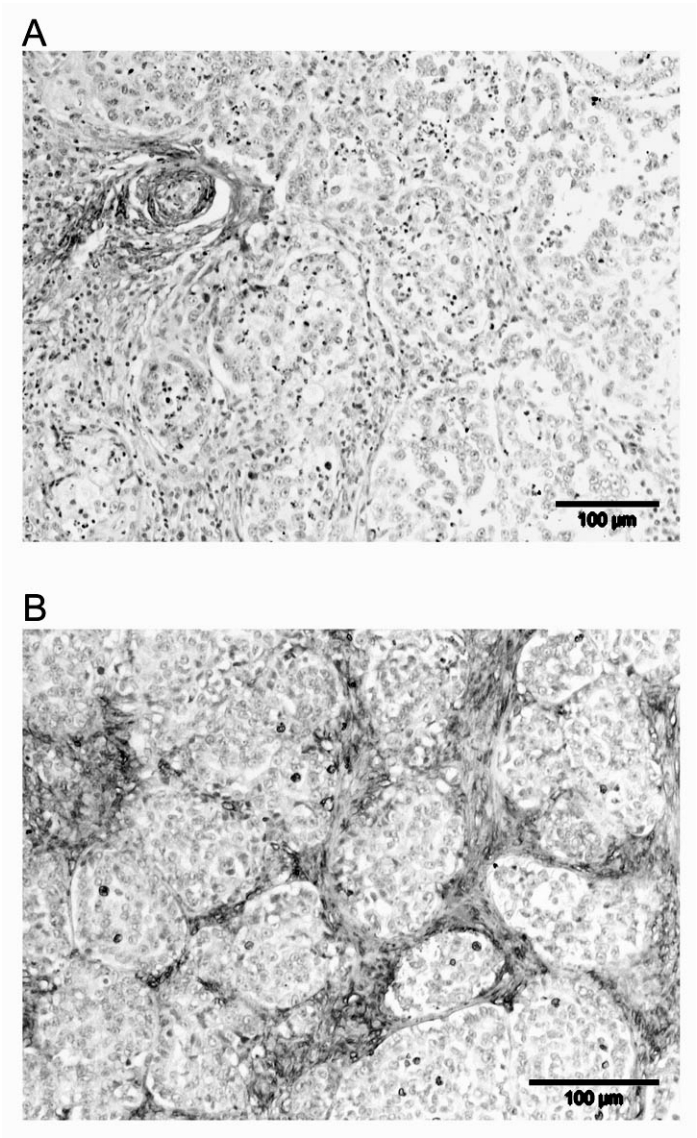

Figure 5. *Immunoreactivity in the fibrous connective tissue within malignant tumors*
In a simple carcinoma **(A)** versican reactvity is mostly restricted to the vessel wall and perivascular tissues (antibody 2B1), whereas **(B)** CS reactivity is present throughout the intratumoral fibrous tissue (antibody CS56). Bars represent 100 μm.

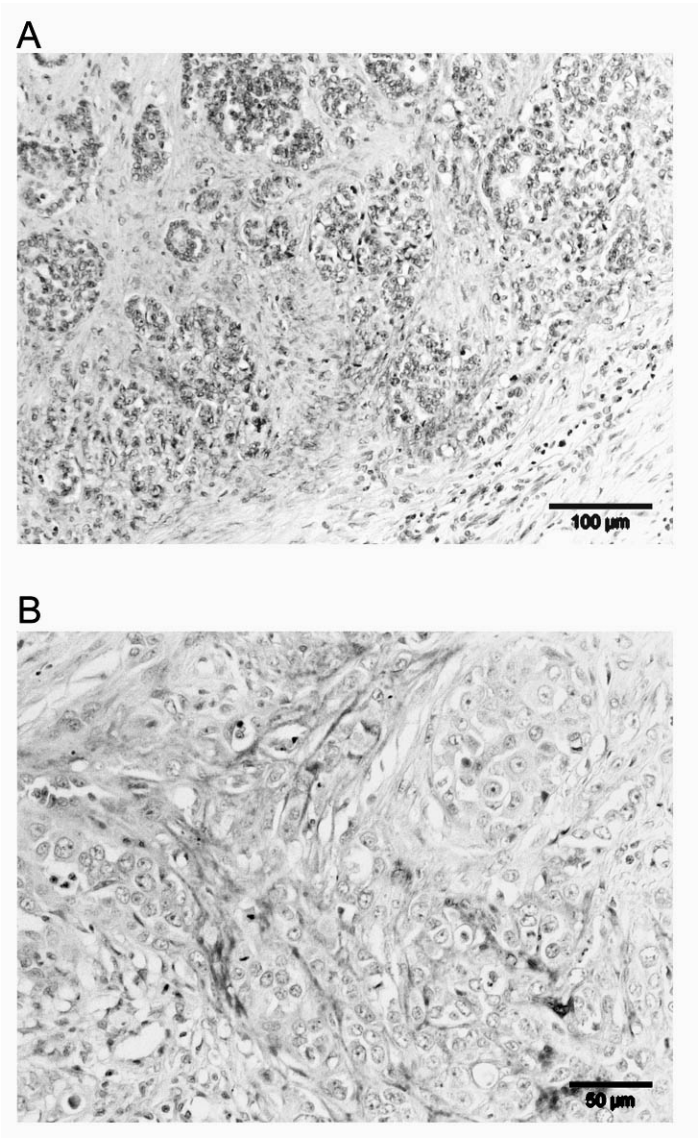

Figure 6. *Versican immunoreactivity in peripheral invasive areas*
Enhanced versican expression in the mesenchymal tissue around invading nests and clusters of tumor cells. Not all interfaces between neoplastic epithelium and mesenchymal tissue show appreciable immunoreactivity to versican. **(A)** antibody 2B1; bar represents 100 μm. **(B)** antibody 12C5; bar represents 50 μm.

In benign mixed tumors the myoepithelial-like spindle cell proliferations and myxoid areas showed immunoreactivity with all three antibodies, with the same distribution and intensity as was decribed in complex adenomas. Dense matrices of cartilage tissues were slightly to moderately reactive to CS with more intense staining in perilacunar regions, whereas appreciable versican immunoreactivity with antibodies 12C5 and 2B1 was mostly restricted to perichondral tissues. In perichondral tissues versican reactivity was improved after chondroitinase ABC digestion. No immunoreactivity to versican or to CS was seen in bone tissues.
In summary, fibrous connective tissues within benign tumors displayed a more widespread CS distribution compared to versican. However, in periductal and perichondral tissues and in cell-rich spindle cell proliferations, versican immunostaining was also intense with both 12C5 and 2B1 antibodies. Interestingly, the myxoid ECM showed intense and diffuse immunoreactivity solely with antibody 12C5.

Malignant tumors
Immunohistochemical labelling of malignant tumors demonstrated heterogenous immunoreactivity to versican both in terms of distribution patterns and staining intensity between different types of tumors. Benign spindle cell proliferations, myxoid and chondroid tissues within malignant complex and mixed tumors displayed the same intense immunoreactivity to versican as in their benign counterparts. The chondroitinase ABC pretreatment convincingly improved the staining for versican with both anti-versican antibodies in all the stained tissue elements, with the exception of myxoid ECM in complex and mixed tumors, such as in benign tumors.
In general, fibrous connective tissues within malignant tumors showed low or no expression of versican (Figure 5A; Table 3). However, in the peripheral invasive areas 2B1- and 12C5-reactive elements were seen in the mesenchymal tissue closely around nests and clusters of tumor cells, although not all interfaces between invading neoplastic epithelium and mesenchymal tissues showed appreciable immunoreactivity to versican (Figure 6A,B; Table 3). In highly invasive carcinomatous tissues and anaplastic carcinomas, both 2B1 and 12C5 labellings were most prominently positive in this area and around lymph vessels filled with metastatic carcinoma cells, but no appreciable staining of the peri- or intratumoral fibrous connective tissue was found. Furthermore, immunoreactivity with both anti-versican antibodies closely approximated the contours of large

ducts, comprising irregular concentric rings with several discontinuities. This periductal staining contrasted with the immunoreactivity located in the loose connective tissue of intraductal papillary projections and in the fibrous septa within tumors, where no or only moderate and scattered immunoreactivity was found. No cytoplasmic staining of the neoplastic epithelial cells with antibody 12C5 and 2B1 could be detected in any types of carcinomas examined. In contrast to the distribution of versican, homogenous and intense CS immunoreactivity was consistently detected throughout the fibrous stroma of all types of malignant tumors (Figure 5B). The most intense CS staining was observed in mesenchymal tissues closely interwoven with clusters of carcinoma cells, in periductal fibrous elements, and in sclerotic stromal tissues. The cytoplasm of neoplastic cells was often immunostained for CS. Similar to benign tumors, cytoplasmic staining of interstitial fibroblasts was seen with all three antibodies.

Complex carcinomas, a tumor type that arises from the transformation of both luminal epithelial and myoepithelial components, displayed the most widespread versican expression of all malignant tumors. As was seen in complex adenoma, myoepithelial-like spindle cell proliferations reacted intensely with both anti-versican antibodies and also with CS56 antibody; however, reactivity with antibody 12C5 was more diffuse and homogeneous in the abundant ECM, particularly in myxoid areas, than with antibodies 2B1 and CS56. The chondroid and myxoid areas of mixed tumors with malignant epithelial component, expressed both versican and CS in the same pattern as in benign mixed tumors. The carcinomatous component of mixed tumors displayed similar reactivity to versican and to CS as in simple and complex carcinomas.

Staining intensity and percentages of all types of infiltrative tumors with reactivity to versican and CS within the peripheral invasive zone and/or the intratumoral connective tissue are summarized in Table 3.

In summary, malignant mammary tumors expressed versican in the periepithelial mesenchymal tissues, in particular surrounding large ducts and in the peripheral invasive areas. Furthermore, benign spindle cell proliferations, and benign myxoid and chondroid tissues within malignant complex and mixed tumors expressed versican similar to their benign counterparts.

Table 3. Immunohistochemical labeling of infiltrative tumors

HISTOLOGY	INTRATUMORAL CONNECTIVE TISSUE				PERIPHERAL INVASIVE AREAS			
	Versican		CS		Versican		CS	
	Staining Intensity	R (%)	Staining Intensity	R (%)	Staining Intensity	R (%)	Staining Intensity	R (%)
Tubulopapillary carcinoma (n =8)	+	3/8 (37.5%)	++/+++	8/8 (100%)	+/++	7/8 (87.5%)	++/+++	8/8 (100%)
Solid carcinoma (n = 6)	-	0/6 (0%)	++/+++	6/6 (100%)	++/+++	6/6 (100%)	++/+++	6/6 (100%)
Anaplastic carcinoma (n = 4)	-	0/4 (0%)	++/+++	4/4 (100%)	++/+++	4/4 (100%)	++/+++	4/4 (100%)
Complex carcinoma (n = 5)	+	2/5 (40%)	++/+++	5/5 (100%)	+/++	4/5 (80%)	++/+++	5/5 (100%)
Mixed tumor with carcinoma (n = 5)	+	1/5 (20%)	++/+++	5/5 (100%)	+/+++	5/5 (100%)	+/+++	5/5 (100%)

R (%) = Ratio and percentages of infiltrative tumors with reactivity to versican and CS within the intratumoral connective tissues and peripheral invasive zone

Discussion

An increased stromal accumulation of chondroitin sulfate in canine mammary tumors compared to the normal tissues has been previously reported.[30] The focus of this study was to determine whether versican, a large CS-containing PG, is present in mammary tumors of dogs, thus partly or entirely being responsible for the elevated levels of CS. We also tackled the question as to whether in canine mammary tumors, as in a number of human cancers [21,26], the presence of versican relates to malignant behaviour.

Our results showed enhanced versican deposition in the ECM of both benign and malignant mammary tumors, especially in complex and mixed tumors, compared to the non-neoplastic mammary tissues. In fact, no immunoreactivity to versican could be detected in inactive or lactating (pregnancy or pseudopregnancy) normal mammary tissues. CS was not present in inactive mammary tissues, but appeared in lactating and regressing tissues; apical cytoplasmic CS staining of luminar epithelial cells could be detected almost exclusively in secretory differentiated lobules. These results may reflect changes in CS expression in the normal mammary tissues of dogs induced by ovarian hormones. Involvement of sGAGs and PGs in mammary tissue morphogenesis and differentiation of different species is well known from the literature.[37-38] Changes in the ECM of the normal human breast, such as altered concentration and distribution of CS during the menstrual cycle have also been reported [39], suggesting that the ECM profile of the normal breast tissue is under hormonal regulation. The lack of versican from non-neoplastic mammary tissue indicates the involvement of other CS- PGs than versican in the morphological changes of the normal mammary gland of dogs, but identification of these ECM compounds awaits further studies.

This study has revealed an increased versican expression in neoplastic mammary tissue; however, we found variations in patterns of immunoreactive versican between certain histological types of tumors. The fibrous connective tissue within all types of tumors displayed a more widespread CS distribution compared to versican, indicating that the CS accumulation cannot be entirely attributed to the presence of versican.

The three main patterns of versican accumulation, in periductal tissues, in peripheral invasive areas of malignant tumors and in spindle cell and myxoid areas, will be further discussed.

Periductal staining pattern of versican

All types of mammary tumors showed versican immunoreactivity in a periductal pattern, in particular surrounding large pre-existing extralobular ducts, probably in association with their basement membranes. In these areas versican co-localised with CS. Similar findings have been described in human benign breast tumors where the basement membrane component of the duct epithelium was reactive with antibody 2B1.[22] In human yolk sac tumors basement membrane components were also stained positively to 2B1.[40] As versican is associated with basement membranes, the co-localisation of versican and CS in the immediate periductal tissues in our study strengthens the basement membrane origin of positively-stained tissue elements for CS surrounding epithelial structure in canine mammary tumors as described by Hinrichs and colleagues.[30] Versican has been shown to be associated with components of the elastic tissues.[18,17,41] In the study of Nara et al, focal elastosis in breast carcinomas was noted in periductal spaces and was found to be reactive to versican, as well as to collagen IV and laminin, indicating that the elastosis masses were composed of basement membrane components.[22] Our finding that 12C5 and 2B1 immunoreactivities often appeared as a concentric layer, with some variability in thickness, encompassing large ducts, may also be explained by the expression of versican in elastic tissues surrounding these ducts. It appears likely that versican is a component of tumor basement membrane in mammary tumors of dogs, although it is uncertain how it contributes to the basement membrane formation.

Versican immunoreactivity in peripheral invasive areas of malignant tumors

In the peripheral invasive areas the remarkable staining of the periepithelial mesenchymal tissue by the two anti-versican antibodies contrasted with the lack of immunoreactivity of fibrous connective tissues within these tumors. The localization of versican in the invasion front may be related to its anti-adhesive effects on cells, a property that has been shown to reside both in the CS side-chains [42] and in the core protein [43]. It has been reported that versican can stimulate cell proliferation via two mechanisms: through two EGF-like motifs in the G3 domain which play a role in stimulating cell growth [44], and through the G1 domain [43], which destabilises cell adhesion and facilitates cell growth. It has been proposed that the effects of the G1 domain on cell proliferation can be explained by its strong HA-binding affinity, resulting in a local increase in HA concentration, which will destabilize cell adhesion and increase cell

proliferation.[43] Preliminary data suggest an overlapping distribution of HA with versican and CS in the peripheral invasive areas of canine mammary tumors (our data, not shown), consistent with the findings in human breast cancer [22]. The co-existence of HA and versican in the peripheral invasive areas indicates that versican may perform its function by binding to HA in these areas. Consequently, the formation of an ECM rich in versican and HA provides a highly hydrated environment, which may facilitate local cancer cell proliferation and migration by decreasing cell-matrix adhesion. The promotion of tumor cell migration through the ECM may also facilitate formation of distant metastases. This is supported by the findings of Ricciardelli and colleagues [21,23], where patients with prostate and breast tumors containing versican-rich peritumoral stroma experienced shorter relapse-free intervals than patients with versican-poor tumors. They suggested that the peritumoral location of versican and its implied involvement in tumor spread is indicative of the production of soluble mediators by more aggressive cancer cells which regulate deposition of versican into the ECM by fibroblastic cells. In accordance with the findings in human breast and prostate cancer [21-23] the cytoplasm of neoplastic luminal epithelium did not contain immunoreactive versican in canine mammary tumors, confirming that versican is a product of stromal cells. These cells might possibly be interstitial fibroblasts, but we showed that myoepithelial-like spindle cells were also able to produce versican since they were immunostained for versican in complex and mixed tumors (see below).

Versican immunoreactivity in myoepithelial-like spindle cell proliferations and myxoid areas

In both benign and malignant complex and mixed tumors, immunoreactive versican was most abundantly present in the areas of myoepithelial-like spindle cell proliferation. 12C5 and 2B1 immunoreactivities substantially co-localised in all types of mammary tumors with the exception of ECM-rich spindle cell proliferations and myxoid areas. Interestingly, the abundant ECM of these areas was only partially stained or not stained at all with antibodies 2B1 and CS56, whereas 12C5 immunoreactivity was intense, completely diffuse and homogeneous. Furthermore, the intensity of versican staining could not be enhanced after chondroitinase ABC digestion in these areas. Taken as a whole these observations suggest that in canine complex and mixed mammary tumors versican carries little or no CS side chains and lacks the 2B1 epitope in the

abundant ECM of spindle cell proliferations and myxoid areas. The lack of CS could also explain that chondroitinase ABC digestion has no effect on the intensity of versican immunoreactivity in these tissues. The form of versican carrying little or no CS-side chains and lacking the 2B1 epitope may be generated by enzymatic cleavage, separating the HA-binding region from the 2B1 epitope- and CS-bearing domain of the molecule. It has been shown that members of the large CS-containing PG family are susceptible to partial proteolytic cleavage by matrix metalloproteinases (MMPs) and by a sub-group of the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) proteases occurring endogenously under pathological conditions.[45-47] Enzymatic digestion of these proteins often results in separating the HA-binding region from the chondroitin sulfate-rich region of the molecules.[48] It is likely that the HA-binding region remains attached to the HA in the matrix while the rest of the molecule, including the large CS-bearing domain, is degraded further and removed from the tissues.

Several extracellular matrix proteins have a diversity of molecular forms as a result of alternative splicing.[13] Isoforms of these proteins have been shown to differ in function and to be regulated developmentally and/or in tissue-dependent manners.[42,19] Alternative splicing of CS attachment domains (GAGα and GAGβ) yields multiforms of the versican core protein in several species.[18,13] Therefore, the unique pattern of 12C5 immunoreactivity in ECM-rich spindle cell masses and myxoid areas could also be explained by alternative splicing of versican, yielding a short form of the core protein without CS attachment domain and lacking also the 2B1 epitope. However, all the currently known splice variants of versican contain the 2B1 epitope. A transcript encoding a short form of the core protein, V3 was detected in various mouse and human tissues including brain, stomach and liver, using polymerase chain reaction (PCR).[49] DNA sequences of these PCR products suggested that V3 had no CS attachment domain; therefore it may be classified as a glycoprotein and not a proteoglycan. V3 has no CS attachment region, and thus lacks the large size and high charge density, but retains the HA-binding domain of the large isoforms; this form may therefore have a unique function. In fact, *in vitro* studies have recently shown that V3 overexpression in arterial smooth muscle cells, retrovirally transduced with rat V3, alters their smooth muscle cell phenotype and results in increased adhesion and inhibition of migration and proliferation of these cells. It has been suggested that V3 may exert these effects through changes in pericellular coat

formation, either by competing with larger isoforms for HA-binding, or by altering other components of the pericellular matrix.[50]
In summary, the present findings showed that versican is one of the extracellular matrix PG characteristics of canine mammary tumors. The enhanced versican expression both in benign and malignant canine mammary tumors indicates that it cannot be considered as a general marker for malignancy. However, the high versican expression in the invasive zones of infiltrating malignant tumors suggests a possible involvement in tumor invasion. Furthermore, it appears likely that in complex and mixed tumors versican exists in different forms, one of them lacking the CS attachment domain and the 2B1 epitope. The latter form of versican may be a result of proteolytic cleavage by MMPs and/or ADAMTS proteases or might be a splice variant.

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CHAPTER ***3***

Expression of versican in relation to chondrogenesis-related extracellular matrix components in canine mammary tumors

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Abstract

Versican plays a role in tumor cell proliferation and adhesion and may also regulate cell phenotype. Furthermore, it is one of the pivotal proteoglycans in mesenchymal condensation during prechondrogenesis. We have previously demonstrated accumulation of versican protein in myoepithelial-like spindle cell proliferations and myxoid tissues of complex and mixed mammary tumors of dogs. The objective of this study was to investigate whether the high expression of versican relates to prechondrogenesis in these tissues. Therefore, we aimed to identify cartilage markers, such as collagen type II and aggrecan both at mRNA and protein level in relation to versican. The neopitope of chondoitin-6-sulfate (3B3) known to be generated in developing cartilage has been investigated by immunohistochemisty and a panel of antibodies was used to characterize the phenotype of cells that are involved in cartilage formation. In addition, co-localization of versican with hyaluronan and link protein was studied.

RT-PCR revealed upregulation of genes of versican, collagen type II and aggrecan in neoplastic tissues, especially in complex and mixed tumors. Immunohistochemistry showed the expression of cartilage biomarkers not only in the cartilagenous tissues of mixed tumors, but also in myoepitheliomas and in the myoepithelial-like cell proliferations and myxoid areas of complex and mixed tumors.

The results show the cartilagenous differentiation of complex tumors and myoepitheliomas and indicate that the myxoid tissues and myoepithelial-like cell proliferations are the precursor tissues of the ectopic cartilage in mixed tumors. Furthermore, we suggest that cartilage formation in canine mammary tumors is a result of (myo)epithelial to mesenchymal transition.

Introduction

The extracellular environment has an active and complex role in modulating tissue development and differentiation, including histomorphogenesis of neoplastic processes.[1] Among proteoglycans (PGs), a chondroitin sulfate PG, versican, seems to have a prevailing importance to modulate cell adhesion and to promote tumor cell proliferation and invasiveness.[2-3] Several studies, particularly within the past few years, have confirmed a significant role for versican in regulating cell phenotype.[2,4,5] As an example, it is one of the pivotal PG in mesenchymal condensation during prechondrogenesis, and the epidermal growth factor-like motifs of the G3 domain have been shown to stimulate chondrocyte proliferation and to modulate chondrocyte morphology.[4,6,7] The effect of versican on chondrocyte differentiation has been attributed to disassembly of actin filaments in chondrocytes.[4]

Similar to the other members of the chondroitin sulfate (CS)-PG family, like aggrecan, versican has a core protein with a globular domain both at the N- and C-terminal region and a central CS-attachment domain.[8-10] The N-terminal domain of both versican and aggrecan specifically recognizes and binds hyaluronan (HA) and this interaction is stabilized by link-protein (LP).[11-13] The stable versican-HA complexes increase the viscoelastic nature of the pericellular matrix, creating a loose highly hydrated environment that supports a cell-shape change necessary for proliferation and migration.[14,15]

The matrix synthesis and organization in canine mammary tumors have not been thoroughly investigated. On the basis of the cell types and ECM components present in the tumor, they are classified histologically as simple, complex and mixed tumors. The latter two types are the most common types of benign canine mammary tumors and histologically they show high similarity with the pleomorphic adenoma of the human salivary gland. In contrast to simple tumors that are composed of only one type of proliferating epithelial cell, complex and mixed tumors are characterized by proliferating spindle shaped cells of possible myoepithelial origin (termed as myoepithelial-like spindle cells) and by the appearance of a myxomatous substance in addition to epithelial cells (Figure 4.1). Furthermore, the stroma of mixed tumors is characterized by the presence of cartilagenous tissue and bone.[16] Traditionally, the cartilage of mixed tumors has been considered to originate from myoepithelial cells [17-19], but there are

suggestions arguing against this hypothesis in favor of stem cell[20,21], fibroblastic [22] or epithelial origin [23].

Our previous immunohistochemical study demonstrated a particularly high versican expression in the myoepithelial-like spindle cell proliferations and myxoid areas of complex and mixed tumors.[24] In the present study, we addressed the question whether the high expression of versican indicates early cartilage differentiation of these specific tissues. Therefore, in addition to versican, we aimed to identify cartilage biomarkers, such as collagen type II, aggrecan and a neoepitope of chondroitin-6-sulfate (3B3 neoepitope) in myoepithelial-like spindle cell proliferations and myxoid tissues. The relation of mRNA expression and protein accumulation of versican, collagen type II and aggrecan in mammary tumors has also been studied. In addition, because of the conflicting ideas of the cell type of origin of spindle cell in myoepithelial-like spindle cell proliferations and myxoid tissues we characterized the phenotype of these cells using a panel of antibodies to epithelial and mesenchymal cytoplasmic markers. Lastly, to determine whether versican co-localizes in vivo with HA and LP we have stained these tumors simultaneously for these molecules.

Table 1. Histological diagnosis of the canine mammary tissues examined

HISTOLOGY OF MAMMARY TISSUES (n=58)	NUMBER OF TISSUES
Normal mammary tissue	2
Benign tumor (n=45)	
Simple adenoma	
Tubulopapillary	4
Myoepithelioma[a]	3
Complex adenoma	16
Mixed tumor	22
Malignant tumor (n=11)	
Simple carcinoma	
Tubulopapillary	3
Solid	3
Complex carcinoma	2
Mixed tumor with malignant epithelial component	2
Carcinosarcoma	1

Classification according to WHO criteria.[16]
[a]One of the tumors showed focal malignancy at cellular level.

Table 2. Oligonucleotide primers used for RT-PCR

Target gene (primer name)	Forward (F) and reverse (R) primer sequence (5' - 3')	Product size (bp)	GenBank accession number/Location of primers	PCR conditions
Versican (VG3)	GTAGAAAATGCCAAGACCTT	186	D32039 (*h*) F: 1786-1805 R: 1971-1952	94°C, 30 s 50°C 30 s 72°C, 30 s
	AGAATAAGTCCTTTGGTATG			
Collagen type II (Col2)	CTGCCAACGTCCAGATGACC	347	AF023169 (*c*) F: 4129-4148 R: 4457-4475	94°C, 30 s 62°C 30 s 72°C, 30 s
	GCAGACAGGCCCGATGTCC			
Aggrecan (AG3)	CCTCCCCTACAGATGATGCT	166	U65989 (*c*) F: 6170-6189 R: 6335-6316	94°C, 30 s 57°C 30 s 72°C, 30 s
	CGGTAGCTGGGAAGGCATAA			
GAPDH	AACCATGAGAAGTATGACAAC	429	BC020308 (*h*) F: 444-464 R: 872-853	94°C, 30 s 53°C 45 s 72°C, 30 s
	CTCAGTGTAGCCCAGGATGC			

Locations of primers are given based on the corresponding canine or human cDNA sequence as indicated (*h*, human; *c*, canine).

Materials and methods

Tissues

The 58 mammary tissues examined in the present study were derived from 52 dogs and were resected surgically or obtained from the archives of necropsies of the Department of Veterinary Pathology, Utrecht University. The histological type and number of the examined tissues are summarized in Table 1. A part of those tissues that were collected at the time of the surgery was fixed in 10% neutral-buffered formalin and a part of them was frozen in liquid nitrogen under RNase free conditions and stored at – 80°C before use. After 24 h formalin fixation tissues were embedded in paraffin, after which 5 μm sections were cut and stained with hematoxylin and eosin (H&E). Tumors were classified from the H&E-stained sections according to the recent WHO classification.[16]

Semi-quantitative RT-PCR

Hematoxylin and eosin stained cryosections from the snap frozen mammary tumors were examined by light microscope prior to RNA isolation to ensure tissue specificity and to avoid RNA extraction from the surrounding normal tissues. Total RNA was extracted from the tissues (100mg) using Trizol Reagent (Invitrogen, Breda, The Netherlands) according to the manufacturer's instructions and treated with Dnase I (DNA-free kit; Invitrogen) to prevent amplification of genomic DNA. Quality of total RNA was evaluated with electrophoresis in agarose gel and by A_{260}/A_{280} ratio. The reverse transcription of 900 ng of total RNA was carried out using M-MLV reverse transcriptase (Invitrogen) with oligo$(dT)_{12-18}$ primers (Amersham Biosciences, Piscataway NJ, USA) in a total volume of 20 μl. One microliter of cDNA was subsequently amplified in a volume of 50 μl using Taq DNA polymerase (Promega Co., Madison, WI, USA) and primers for versican, collagen type II and aggrecan. The PCR was initiated with denaturation at 94°C for 3 min followed by 30-40 cycles of reaction as detailed in Table 2 depending on the sequence of the primer pairs used. Primers for PCR amplification were generated from GenBank sequences. BLASTN searches were conducted on all primer nucleotide sequences to ensure gene specificity. Negative controls (without reverse transcription and water blank) were negative. For a semi-quantitative analysis of the PCR products, cDNA concentrations in each sample were evaluated during the exponential phase of amplification (30-40 cycles). 10 microliter of PCR products were analyzed on 2%

agarose gel, stained with ethidium bromide. The specificity of each PCR product was confirmed by sequence analysis (BigDye Terminator Cycle Sequencing Ready Reaction Kit; ABI PRISM 310).

Densitometric analysis

After electrophoreses agarose gels were photograped with ImageMaster VDS (Pharmacia Biotech) connected to LISCAP Capture Application Software (Version 1.0). Image analysis of the detected bands was performed using Molecular Analyst PC Software (Bio-Rad Laboratories). For each target gene, mRNA levels were normalized to GAPDH and used for statistical analysis.

Statistical analysis

Analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test was used to compare tissue-dependent expression levels of versican, collagen type II and aggrecan between different histological types of tissues, including one normal mammary tissue, two simple and three complex adenomas, five mixed tumors, three simple carcinomas and 1 myoepithelioma. All data of two measurements are expressed in Box-Wisker plots, showing the tissue-dependent expression profile of each gene analyzed. A value of $P<0.05$ was considered to be statistically significant.

Monoclonal antibodies

The following monoclonal antibodies were used in the present study: (1) **12C5** raised against versican isolated from human brain (Developmental Studies Hybridoma Bank /DSHB/, Iowa City, IA, USA) and known to recognize the HA-binding domain of versican.[25] (2) **II-II6B3** raised against chicken collagen type II (DSHB). (3) **IC6** raised against hyaluronic acid binding region at the N-terminal end of rat chondrosarcoma proteoglycan (DSHB) and known to recognize an epitope shared by both the G1 and the G2 domains of aggrecan.[26] It slightly cross reacts with non-cartilagenous hyaluronic acid binding proteoglycans; however tissues other than cartilage do not give immunoreactivity by immunohistochemistry [27,28], therefore we applied this antibody for immunolocalization of aggrecan. (4) **3B3** recognizes an epitope in native CS chains that possess a saturated hexuronic acid residue adjacent to 6-sulfated *N*-acetyl galactosamine at the non-reducing terminus of the CS GAG [29] (ICN

Biomedicals, Inc, Ohio, USA). (5) **8A4** recognizes link protein isolated from hyaline cartilages of several species (DSHB).
Furthermore, monoclonal antibodies against vimentin (V9; Biogenex, San Ramon, CA, USA), cytokeratin-14 (LL02; Biogenex), broad spectrum cytokeratins (CK 5,6,8,17 and probably 19; MNF116; DAKO, Denmark), smooth-muscle actin (A4; Biogenex) and S-100 protein (rabbit polyclonal antibody; DAKO) were used to characterize the cell phenotype in myoepithelial-like spindle cell proliferations, in myxoid and cartilage tissues.

Histochemical staining and immunohistochemistry
Safranin-O staining was used to detect the sulfated-PG content and distribution in our samples. Immunohistochemistry was performed using an indirect immunoperoxidase staining procedure, applying the avidin-biotin based technique (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA, USA). After deparaffinisation and rehydration, the sections were washed in Tris-HCl buffer (0.25 M, pH 8.0). All incubation steps, except where stated, were performed at room temperature (RT). Chondroitinase ABC (from *Proteus vulgaris*; Sigma Chemicals) digestion was performed, before the application of primary antibodies 12C5, IC6, II-II6B3 and 8A4, at 37°C for 2 hours with 0.5 U/ml of the enzyme in 0.25 M Tris buffer (pH 8.0) containing 0.18 M sodium chloride, 0.05% bovin serum albumin (BSA), 0.1 M 6-amino-*n*-caproic-acid and 5 mM benzamidine hydrochloride to inhibit protease activity. To control the specificity of enzyme pretreatment control slides were treated at the same time with the same protease inhibiting buffer without chondroitinase. In case of HA-staining control slides were treated with hyaluronidase (*Proteus vulgaris;* Seikagaku Co., Tokyo, Japan), at 60°C for 2h with 100 TRU/ml of enzyme in 0.1M sodium-acetate buffer pH 5.5., before the application of HABP to control the specificity of the staining. All sections were rinsed with S-PBS (PBS with 0.03 % saponine), soaked in 0.3% H_2O_2 in S-PBS for 30 min to inhibit the activity of endogenous peroxidase, rinsed again, and then allowed to react with 10% normal horse-serum in 1% BSA-containing S-PBS, followed by overnight incubation with diluted primary monoclonal antibodies 12C5 (1:100), II-II6B3 (1:100), 1C6 (1:100), 3B3 (1:150), 8A4 (1:100); LL02 (1:50), and anti-S-100 protein (rabbit polyclonal antibody; 1:300) at 4°C. One hour incubation was applied for primary antibodies V9 (1:250), MNF116 (1:200), A4 (1:1200) at RT. After incubation and washing, the sections were treated with biotinylated horse anti-mouse immunoglobulin diluted 1:125 in

S-PBS, followed by incubation with avidin-biotin complex according to the manufacturer's instructions (Vector Laboratories). Reactions were visualized using 0.5% 3,3-diaminobenzidine tetrahydrochloride (Sigma Chemicals) and 0.3% H_2O_2 diluted in Tris/HCl buffer (0.05 M, pH 7.4), during a 10-minute incubation step. Sections were counter-stained with Mayer's hematoxylin. As negative controls, normal mouse serum was employed for the reaction instead of primary antibodies.
Slides were assessed for staining intensity and location of immunoreactivity within the tissues (Table 3). The intensity and pattern of staining in the ECM were graded using an empirical semi-quantitative system: (–), no reaction; (+/–) weak focal reaction; (++/–) moderate focal reaction; (+++/–), intense focal reaction; (+) weak diffuse reaction, (++) moderate diffuse reaction, (+++) intense diffuse reaction.

Results

Semi-quantitative RT-PCR

The gene expression profiles of versican, collagen type II and aggrecan in normal mammary tissue and in different types of mammary tumors were detected by the semi-quantitative RT-PCR method. In general, all the three genes were upregulated in neoplastic tissues compared to the normal mammary tissue, and showed a substantial peak in complex adenomas and mixed tumors. However, similar to the normal mammary tissue, aggrecan was not expressed in simple adenomas and simple carcinomas (Figures 1, 2).
Out of the three target amplicons versican and collagen type II first appeared as faint bands in the normal mammary tissue, indicating very low expression levels, whereas, aggrecan expression could not be detected in the normal mammary gland. The expression of versican and collagen type II increased in simple adenomas compared to the normal mammary tissue, and they peaked in complex adenomas and mixed tumors. Versican was highly expressed also in simple carcinomas. Interestingly, similar to the normal mammary tissues low levels of collagen type II expression were detected in simple adenomas and carcinomas. Significantly higher versican expression compared to the normal mammary tissue appeared in complex adenomas (P= 0.008), mixed tumors (P= 0.001) and simple carcinomas (P= 0.035), whereas collagen type II expression was significantly

increased in complex adenomas (P< 0.001) and mixed tumors (P= 0.001). Compared to simple adenomas significantly higher versican expression was detected in mixed tumors (P= 0.01), while the expression of collagen type II was significantly higher in both complex adenomas (P< 0.001) and mixed tumors (P< 0.001).

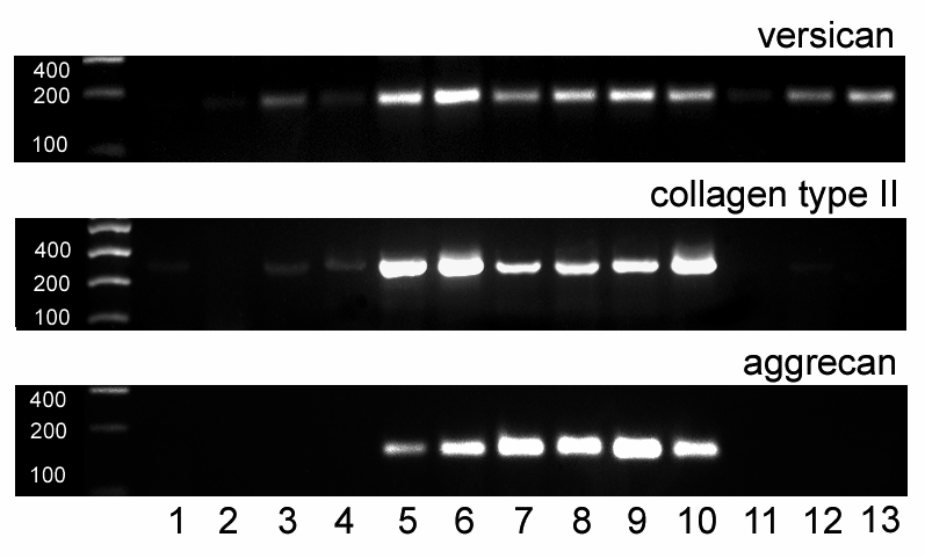
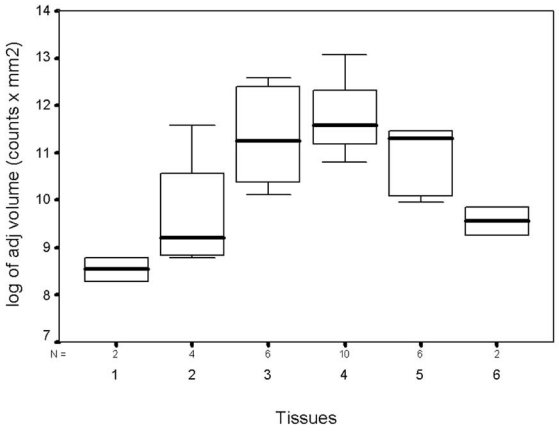


Figure 1. Gene expression profile showed by RT-PCR analysis of versican, collagen type II and aggrecan in normal mammary tissue (1-2), in simple adenomas (3-4), in complex adenomas (5-6), in a myoepithelioma (7), in mixed tumors (8-10) and simple carcinomas (11-13). One μg of total RNA from each sample was DNase 1-digested, reverse transcribed with oligo (dT) as primer and 1 μl of the first strand cDNA reaction was then amplified using respective specific sets of primers by PCR (30 cycles). 10 μl of respective amplicons were detected by electrophoresis in 2.0% agarose gel stained with ethidium bromide. Expression profiles for the respective genes were similar for 30, 35, 40 cycles (note shown) of PCR. Note that the faint bands of versican in lane 1-2, and the faint bands of collagen type II in lane 1-4 and 11-13 are not obvious in this Figure, which represents 10 μl of PCR products. The values measured with densitometry were then normalized to the housekeeping enzyme, GAPDH levels.

Figure 2. Tissue-dependent semi-quantitative gene expression profile of versican (2.1), collagen type II (2.2) and aggrecan (2.3). Normal mammary tissue (1), simple adenoma (2), complex adenoma (3), mixed tumor (4), simple carcinoma (5), myoepithelioma (6). N= number of measurements. The results are shown as Box-Wisker plots, which represent the relative mRNA expression of target gene normalized to GAPDH level of the samples.

2.1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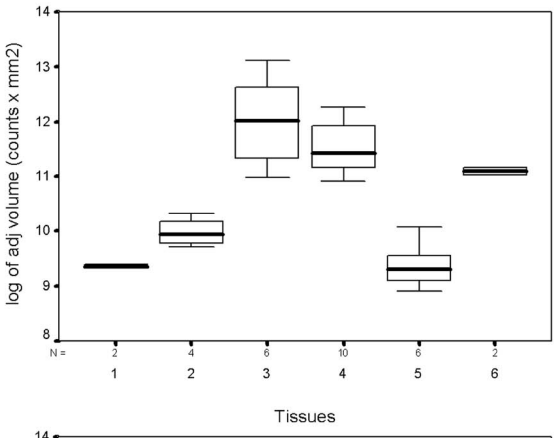
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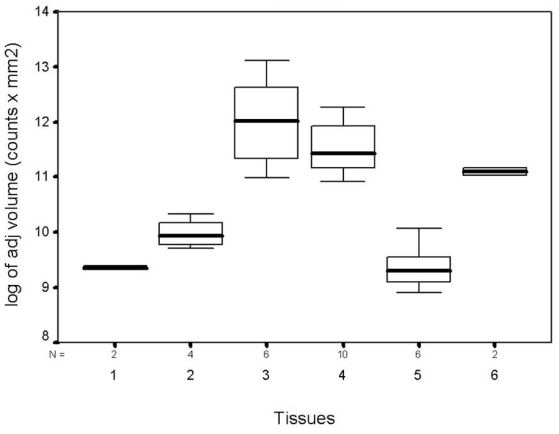
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The semi-quantitative RT-PCR revealed aggrecan expression in complex and mixed tumors. The expression both in complex adenomas ($p < 0.001$) and mixed tumors ($p < 0.001$) was significantly higher compared to the normal mammary tissues and to the simple adenomas and carcinomas. An interesting result was the relatively high expression of all the three genes also in the myoepitelioma.
In summary, the results show a significant upregulation of all the three genes in complex and mixed tumors, compared to the normal mammary tissue. Versican expression was significantly higher also in simple carcinomas. The high expression level of collagen type II and aggrecan in complex adenomas and in the myoepithelioma suggest a cartilagenous potential of these tumors.

Immunohistochemistry and histochemical stainings
As a first step, to detect the distribution of sulfated-PGs characteristic of cartilage matrix, we performed Safranin-O staining in our samples. Safranin-O stained exclusively the ECM of myxoid (Figure 3.1) and precartilagenous tissues (Figure 3.2) similar to the cartilage ECM (Figure 3.3), indicating a common feature of their ECM concerning the sulfated-PG content. The results of immunohistochemical analysis are summarized in Table 3.

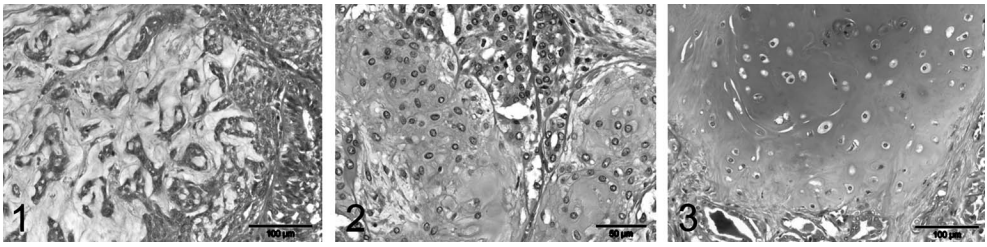

Figure 3. Safranin-O stained exclusively the ECM of myxoid- (1), precartilagenous- (2), and cartilage tissues (3) in complex and mixed tumors indicating a common feature concerning their sulfated-PG content. Bars represents 100μm (1, 3) and 50μm (2).

Skin and normal mammary tissues
In skin adjacent to mammary tissues, moderate to strong staining for versican, hyaluronan (HA) and link protein (LP) was frequently noted at the dermoepidermal junction and in the hair follicles in accordance with the observation in human skin.[30,31] In general, the walls of large blood vessels and/or the perivascular tissues were often stained for versican and HA.

Table 3. Tissue-dependent immunohistochemical expression of ECM components

SITE	VERSICAN	COLLAGEN II	AGGRECAN	3B3 NEOEPITOPE*	HA	LP
Normal mammary tissues	-	-	-	-	-, +	-
Simple adenoma						
Tubulopapillary	++/-	-	-	-	++	-
Myoepithelioma	+++	+/-	+/-	++/-	++	+++
Benign and malignant complex and mixed tumors						
Connective tissue stroma	++/-, +++/-	-	-	-	++	++/-
Myoepithelial-like spindle cell proliferations	++,+++	++/-	+/-	++/-	++	++
Abundant, myxoid ECM	+++	++, +++/-	++, +++/-	++, +++/-	+++	+++
Precartilagenous tissues	+/-	++, +++	++, +++	+++	++	++, +++
Cartilage	-	+++	+++	+++	+	++, +++
Perichondral tissues	++	-	-	-	++	+
Simple carcinoma	+++/-	-	-	-	++, +++/-	++/-
Carcinosarcoma						
Connective tissue stroma	+++/-	-	-	-	++, +++/-	++/-
Malignant cartilage component	-	+++	+++	+++	+	++, +++

- no reaction; +/- weak focal reaction; ++/- moderate focal reaction; +++/- intense focal reaction; + weak diffuse reaction; ++ moderate diffuse

These positively stained structures in skin and also blood vessels served as internal positive controls for these markers. The normal mammary tissue was used as positive control for broad-spectrum cytokeratin (bsp CK) cytokeratin-14 (CK14), smooth-muscle actin (SMA), vimentin and S-100 protein staining. The resting myoepithelial cells were immunoreactive to each of these markers, whereas, the luminal epithelium was strongly reactive to bsp CK and the fibroblasts to vimentin. Collagen type II, aggrecan and 3B3 neoepitope were not detected in the skin or in the normal mammary tissues. In normal mammary tissues versican and LP were not expressed, while HA appeared weakly in the connective tissues (data not shown).

Benign and malignant mammary tumors

Simple adenoma and carcinoma

The main purpose of this study was to investigate the expression of cartilage biomarkers, HA and LP in complex and mixed canine mammary tumors in relation to versican. However, as control tissues we studied also few simple adenomas and carcinomas. As expected, simple tumors did not stain for any of the cartilage biomarkers. Similar to versican [24], HA was frequently detected as moderate staining in the connective tissue stroma. Focal weak staining for LP could also be detected in the connective tissue of some of the simple carcinomas. Although according to the latest WHO classification of canine mammary tumors myoepitheliomas belong to simple adenomas [16], we describe the expression pattern in these tumors separately (see below).

Benign and malignant complex and mixed tumors

In the connective tissue of complex and mixed tumors the staining pattern of all the markers was similar to that described for simple tumors.

As we reported earlier, versican immunoreactivity was diffuse, moderate to intense in the ECM of myoepithelial-like spindle cell proliferations and myxoid tissues of complex and mixed tumors (Figure 4.2) .[24] Compared to versican, HA and LP displayed similar staining pattern in these areas (Figure 4.3-4). Moderate focal immunoreactivity to 3B3 neoepitope and collagen type II and weak focal reactivity to aggrecan could also be detected in the ECM of myoepithelial-like spindle cell proliferations of almost all cases (not shown). Moreover, moderate focal immunoreactivity to 3B3 neoepitope appeared in the cytoplasm of myoepithelial-like spindle cells. The abundant ECM of myxoid tissues stained

moderately to intensely for collagen type II (Figure 4.5), aggrecan (Figure 4.6) and also for 3B3 neoepitope (Figure 4.7).

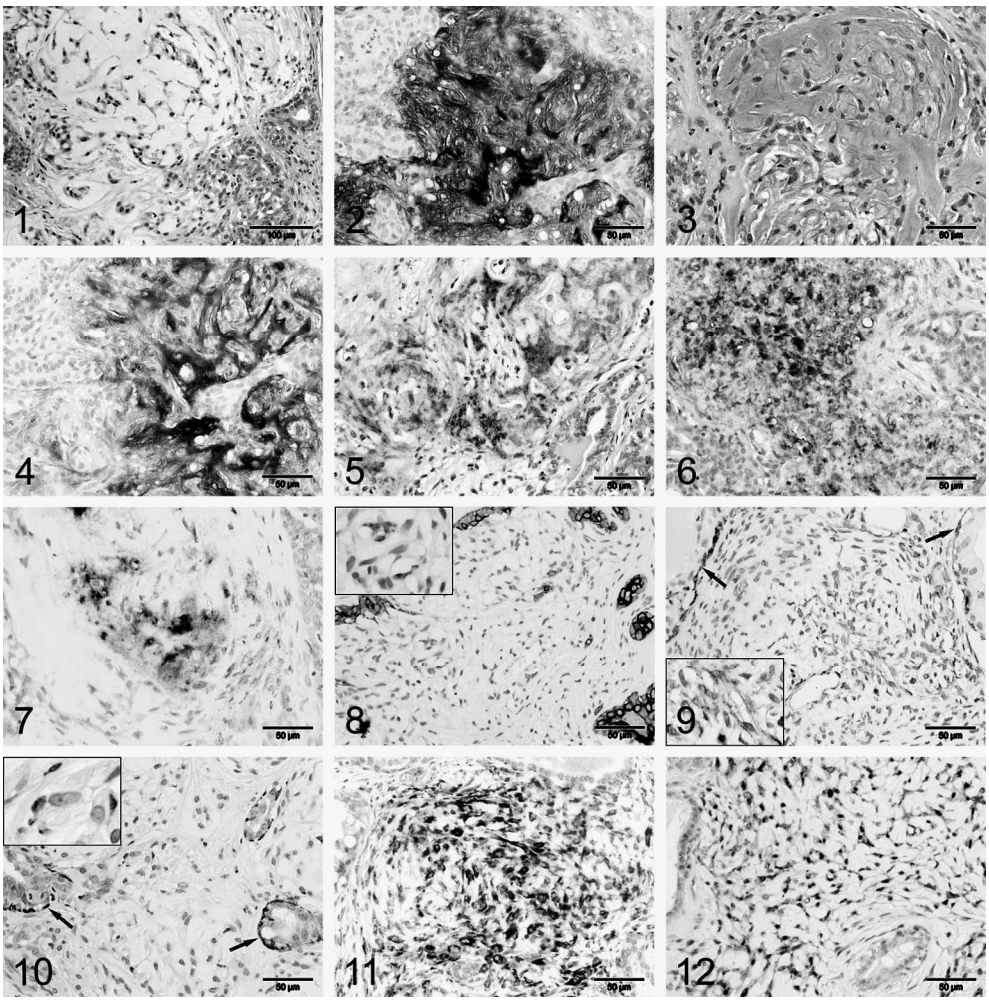

Figure 4. Immunostainings of myxoid tissues in complex and mixed tumors. The myxoid tissue is characterized by spindle- and polygonal-shaped cells embedded in abundant ECM (1) H&E staining; bar represents 100μm. The abundant ECM is stained intensely and diffusely for versican (2), HA (3) and LP (4); moderately to intensely for collagen type II (5), aggrecan (6) and 3B3 neoepitope (7). A low percentage of spindle-shaped tumor cells in myxoid tissues stained for bsp CK (8), CK14 (9) and SMA (10). Expression of vimentin (11) and S-100 protein (12) by these cells is higher. In Figure 4.8, 4.9 and 4.10 the insets shows positively stained cells; note the arrows that point out the positively stained myoepithelial layer surrounding epithelial structures. Bars represent 50 μm.

With some variation (~10-50 %) the neoplastic cells of the myoepithelial-like spindle cell proliferations and myxoid tissues expressed bsp CK (Figure 4.8), CK14 (Figure 4.9) and SMA (Figure 4.10), whereas expression of vimentin (Figure 4.11) and S-100 protein (Figure 4.12) was higher (~ 50-100%).

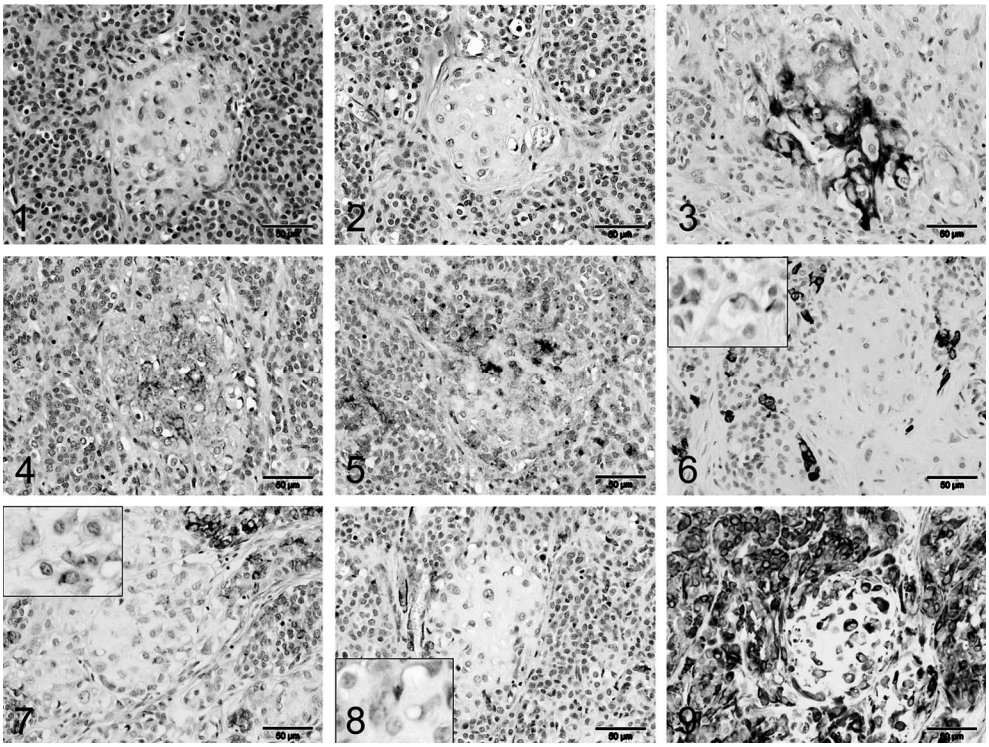

Figure 5. Immunolabelling of early cartilage tissues in serial slides of a mixed tumor. H&E (1). Immunoreactivity to versican was restricted predominantly to the peripheral areas (2), whereas reactivities to collagen type II (3), aggrecan (4) and 3B3 neoepitope (5) were moderate to intense in the ECM of precartilagenous tissues. Note that reactivity to 3B3 neoepitope appears also in the cytoplasm of tumor cells in and around the precartilagenous tissues. A small percentage of cells shows immunoreactivity to bsp CK (6), CK14 (7) and SMA (8); the insets in Figure 5.6, 5.7 and 5.8 show immunoreactivity of few cells. Immunoreactivity to vimentin (9) was present in a high number of tumor cells both in and surrounding the precartilagenous tissues. Bars represent 50 μm.

In those areas where early cartilage differentiation (precartilage) could be recognized on the basis of histomorphological features (Figure 5.1) immunoreactivity to versican was lower (Figure 5.2), whereas reactivity to collagen type II (Figure 5.3), aggrecan (Figure 5.4) and 3B3 neoepitope (Figure

5.5) became moderate to intense. Staining for HA was moderate, and LP showed moderate to intense diffuse reaction. In the early cartilage tissues neoplastic cells gradually lost their CK14, bsp CK and SMA expression towards the more differentiated cartilage. In fact, only a small percentage of cells reacted with these markers (Figure 5.6-8) in early cartilage tissues, particularly at the peripheral, less differentiated areas. In cartilage tissues, displaying histomorphological characteristics of a more differentiated cartilage, versican expression was restricted to the perichondral tissues while collagen type II, aggrecan, 3B3 neoepitope and also LP expression were intense throughout the cartilage matrix. Cytoplasmic immunoreactivity to 3B3 neoepitope was consistently present in tumor cells surrounding precartilagenous (Figure 5.5) and cartilage tissues. In some cases scattered CK14 positive cells were seen at the periphery of the cartilage, but similar to bsp CK and SMA, CK14 was not expressed in cartilage tissues. Expression of vimentin (Figure 5.9) and S-100 by neoplastic cells remained very high both in the precartilagenous and cartilage tissues.
Interestingly, besides the intense immunoreactivity to versican, we observed a low to moderate expression of all cartilage markers also in myoepitheliomas (Figure 6.2-3). Neoplastic myoepithelial cells highly expressed all the cytoplasmic markers, but CK14 appeared the most intense and in the highest percentage of tumor cells (Figure 6.1).

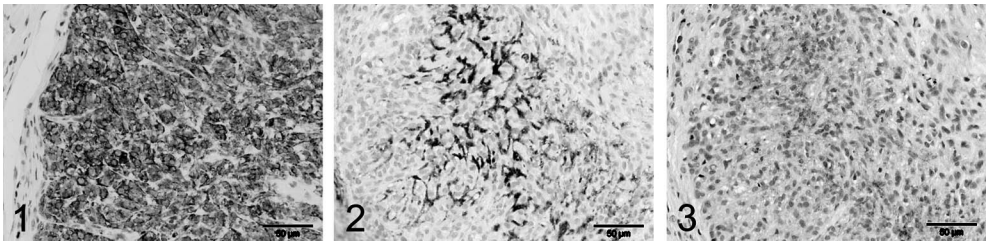

Figure 6. Immunolabelling of myoepitheliomas. CK14 was strongly expressed almost in 100% of the neoplastic myoepithelial cells (1). Intense immunoreactivity to versican (2) and patchy, weak to moderate immunoreactivities to aggrecan (3), collagen type II and 3B3 (not shown) were also present. Bars represent 50 μm.

In summary, versican and all the cartilage biomarkers studied showed no expression in the normal mammary tissues, but were all expressed in tumor

tissues with a characteristic distribution pattern. As we already described in our previous study [24], the highest versican expression appeared in the myoepithelial-like spindle cell proliferations and myxoid tissues of complex and mixed tumors, but its expression was restricted to the periphery of the cartilage of mixed tumors. Besides versican all cartilage markers could also be detected in the myoepithelial-like spindle cell proliferations and myxoid tissues although to a lower level and their expression was the highest in the matrices of cartilagenous tissues of mixed tumors. These immunohistochemical findings were in line with the expression pattern of versican, collagen type II and aggrecan showed by RT-PCR analysis. However, immunoreactivity to versican and collagen type II could not be seen in normal mammary tissues and immunoreactivity to collagen type II did not appear in simple adenomas and carcinomas despite the low level expression of their mRNA in these tissues.

Discussion

Identification of the ECM components in complex and mixed mammary tumors may contribute to the understanding of ectopic cartilage formation in mixed tumors. Up to now only few papers provided some valuable data on the GAG composition and on the cartilage-specific collagen content of these tumors.[32-35] In our previous study we showed that versican accumulates in myoepithelial-like spindle cell proliferations and myxoid tissues of complex and mixed canine mammary tumors.[24] Since versican is one of the main PG in mesenchymal condensation during prechondrogenesis [6,7], we hypothesized that versican accumulation in myoepithelial-like spindle cell proliferations and myxoid tissues relates to early cartilage differentiation.

Here we present evidence that versican and cartilage markers, such as collagen type II, aggrecan and 3B3 neoepitope expressed not only in the cartilage tissues of mixed tumors, but also in the myoepithelial-like spindle cell proliferations and myxoid tissues of both complex and mixed tumors and also in myoepitheliomas. These findings with special regard to the high expression of versican and cartilage markers in the ECM of complex and mixed tumors suggest a progressional sequence of chondrogenic differentiation from myoepithelial-like spindle cell proliferations and myxoid areas to cartilage of mixed tumors. Moreover, the results show that the expression pattern of ECM components

during ectopic chondrogenesis in canine mammary tumors shows remarkable similarity to that of fetal cartilage development. First, versican is known to be expressed in the undifferentiated mesenchymal cells of the early limb bud during the onset of prechondrogenic condensation and its expression becomes restricted to the periphery of the newly formed cartilage.[6,36] This process is inversely correlated with a dramatic upregulation of the cartilage-specific CS-PG, aggrecan, during the establishment and maturation of the chondrocytic phenotype.[7] In accordance with these findings, in complex and mixed canine mammary tumors versican is highly expressed in spindle cell proliferations and myxoid areas of complex and mixed canine mammary, which are the prechondrogenic tissues according to our hypothesis. In the cartilagenous tissues of mixed tumors it is mostly restricted to the perichondral tissues and predominantly occupied by aggrecan and collagen type II. Second, the expression of collagen type II and aggrecan in the spindle cell proliferations and myxoid tissues of complex and mixed tumors show that these tissues in fact undergo a chondroid differentiation. These results are consistent also with a recent study where aggrecan deposition has been demonstrated not only in the chondroid matrix, but also in the myxoid stroma of the pleomorphic adenoma of the salivary gland.[37] Third, the high expression of 3B3 neoepitope not only in the precartilagenous and cartilage tissues of mixed tumors, but also in the ECM and in the cytoplasma of neoplastic (myo)epithelial cells in complex and mixed mammary tumors indicates the cartilagenous potential of these specific tissues. This is in accordance with previous studies in which the expression of 3B3 neoepitope in cartilage tissue development and disease was observed, in particular when tissues are growing and remodelling. [29,38]

According to our phenotyping results 10-50 % of myoepithelial-like spindle cells and spindle cells of myxoid tissues consistently co-expressed bsp CK, CK14, and SMA, indicating a myoepithelial origin. Furthermore, our finding that the SMA and CK14 positive neoplastic spindle cells often express the cartilage specific 3B3 neoepitope and are embedded in a cartilagenous matrix displaying collagen type II, aggrecan and 3B3 immunoreactivity suggest a chondroprogenitor potential of these cells. In the cartilagenous tissues of mixed tumors the strong expression of vimentin and S-100 protein in the neoplastic cells was maintained or even further upregulated while the expression of bsp CK, CK14, and SMA expression was mostly restricted to cells located in the peripheral areas of cartilagenous tissues. Similar observations have been previously reported that in mixed mammary

tumors the spindle-and star-shaped myoepithelial cells proliferating out of the basement membrane in the tumor stroma show arrested expression of keratins and SMA, whereas their expression of vimentin and S-100 protein is maintained or even upregulated.[19,39] In conclusion, the pattern of molecular alterations presented here shows a chondroid differentiation of myoepithelial-like spindle cell proliferations and myxoid tissues and suggests a myoepithelial to mesenchymal transition of the neoplastic cells during ectopic chondrogenesis in canine mammary mixed tumors.

In two of the cartilagenous tumor, we found a direct association between nonspindle-shaped neoplastic epithelial cells and cartilagenous tissues, without the presence of either myxoid tissue or spindle cell proliferation (see Figure 5). Interestingly, these neoplastic epithelial cells expressed not only bsp CK, but also vimentin and S-100 protein; moreover, a large percentage of the cells expressed CK14 and SMA. This expression profile indicates that these neoplastic epithelial cells also exhibit at least a partial myoepithelial differentiation program. It has been shown that the myoepithelial lineage is derived directly from the normal luminal epithelial lineage.[40] Our finding confirms what has been recently described in human breast cancer, that even though the neoplastic epithelial cells first and foremost express luminal epithelial keratins, some of the cells still retain their intrinsic ability to switch, at least partially, to a myoepithelial differentiation program.[41] Taking this finding into consideration, it is likely that in canine mammary tumors the cartilage formation is a result of (epithelial→) myoepithelial→ mesenchymal (chondroid) transition process.

An intriguing result of this study was the expression of collagen type II and aggrecan both at protein and mRNA level in myoepitheliomas, which further strengthens the chondrogenic potential of myoepithelial cells. Furthermore, an unexpected finding was the expression of collagen type II mRNA, although at a low level, also in the normal mammary tissue and in simple tumors. This might be explained by the presence of chondroprogenitor cells, e.g. resting myoepithelial or stem cells, expressing collagen type II A, a splice variant of collagen type that is characteristic of chondroprogenitor cells in fetal chondrogenesis.[42] For an adequate explanation further studies are necessary, since low level of collagen type II is also synthesized in certain noncartilage tissues, e.g. during development in fetal tissues.[43]

The semi-quantitative RT-PCR revealed a significantly higher level of versican mRNA in simple carcinomas compared to the normal mammary tissues. This is

inline with our previous study showing local increase in versican deposition in the invasive zone of malignant tumors. In that study we discussed the possibility of the presence of versican-HA aggregates that could facilitate local cancer cell proliferation and migration by decreasing cell-matrix adhesion.[24] Recently, it has been proven that similar to the aggrecan-HA aggregates the interaction of versican with HA is often stabilized by LP. Therefore, the present study addressed also the issue whether versican co-localizes in vivo with HA and LP. Immunohistochemistry showed co-localization of versican with HA and link protein, although the latter two molecules displayed a wider distribution. Therefore, it is likely that in canine mammary tumors versican exerts its function by binding to HA, and this interaction is stabilized by link protein. Extracellular matrices rich in versican-HA complexes provide a highly hydrated environment that promotes the proliferation and migration of cancer cells.[44,45] Furthermore, the versican-HA surrounds cells serves as an important mechanism for controlling cell shape and cell division.[14,15,46] This could be also of major importance during ETM conversion; therefore, in mammary tumors where epithelial cells seem to convert to chondrocytes. Regulatory mechanisms responsible for chondroid differentiation in canine mammary tumors need to be further investigated.

In summary, it has been shown here that myoepithelial-like spindle cell proliferations and myxoid tissues of complex and mixed mammary tumors display cartilagenous differentiation and the neoplastic spindle cells of these specific tissues have myoepithelial origin. These findings indicate that the common way of cartilage formation in canine mammary tumors is sequential to the formation of myxoid tissues and/or spindle cell proliferations with myoepithelial origin. Furthermore, we suggest that the high expression of versican in these tissues may relate to early events of cartilage differentiation and cellular changes leading to mesenchymal phenotypes of myoepithelial cells. Whether versican plays a decisive role in this process remains to be established in further experiments.

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CHAPTER 4

Multiple forms of versican in canine mammary tumors and cell lines

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Submitted

Abstract

We previously reported that versican is one of the characteristic extracellular matrix proteoglycans of canine mammary tumors. We now identify the precise expression pattern of versican mRNA splice variants and their protein products to be V1, V0 and V3 variants, with a prevalence of V1/V0 versican in mammary tumors and mammary cell lines. Real-time PCR and Western blot analysis revealed increased mRNA and protein expression of V0/V1 versican in tumor cells lines compared to the normal mammary fibroblasts. The myoepithelial-like tumor cells lines expressed the highest levels of versican mRNA splice variants of all the cell lines tested. Furthermore, several catabolic products of versican were detected including a 70 kDa fragment known to be generated by ADAMTS proteases. This versican fragment was particulary abundant in myxoid tissues of complex and mixed tumors.

We suggest that the large V0/V1 variants, derived in large part from the myoepithelial-like cells, contribute to the formation of a highly hydrated extracellular environment that is optimal for tumor cell proliferation, migration and differentiation in canine mammary tumors. We further propose that versican degradation occurs as part of the tumor process and that specific fragments of versican may possess similarly important and distinct biological roles in tumor cell-matrix interactions.

Introduction

There is an increasing body of evidence that proteoglycans (PG) are involved in tumor development. A member of the large chondroitin sulfate PG (CSPG) family, namely versican, is present in a number of tumor types [1-5] and has a been shown to influence tumor cell behaviour and phenotype.[6,7] Similar to the other members of the CSPG family, the structure of versican follows a domain template. The amino-terminal globular domain (G1) contains Ig-like and hyaluronan (HA)-binding elements and binds HA with high affinity.[8,11] The carboxy-terminal globular domain (G3) consists of a C-type lectin domain, two epidermal growth factor (EGF) domains and a complement regulatory region. The central glysosaminoglycan (GAG) attachment domain that carries several chondroitin sulfate (CS) chains is encoded by two large exons. The subdomain encoded by exon 7 is called GAGα, the other subdomain encoded by exon 8 is GAGß.[8] As a result of alternative splicing in exon 7 and 8, versican exists in at least four isoforms, namely V0, V1, V2 and V3. The largest V0 isoform is known to contain both GAGα and GAGß, V1 contains the GAGß, V2 has the GAGα, and V3 lacks any GAG subdomain. The protein products of each mRNA transcript differ in length and in number of CS chains attached [9,10], and therefore may possess different properties. Functions shared by versican isoforms are likely to be associated with HA-binding via the amino-terminus [11], and binding of tenascin-R [12], fibrillin-1 [13] and other extracellular molecules by the C-terminal domain.[14,15] Versican isoforms may therefore act as bridges of different length, cross-linking matrix molecules and play also a cental role in assembly of the pericellular matrix. Furthermore, it has been recently suggested that versican not only presents and recruits molecules to the cell surface, it also modulates the expression of genes and coordinate complex signal pathways. [16-18]

Versican influences cell adhesion and proliferation. The anti-adhesive properties of versican have been at least partly attributed to the interaction of the G1 domain of the molecule with HA. The versican-HA complexes, which are stabilized by link protein in tissues, are believed to increase viscoelasticity and to exclude interaction of the cell surface with other matrix molecules that affect cellular adhesion and proliferation.[19-21] On the other hand the EGF sequences in the G3 domain of versican have been proven to directly enhance cell proliferation.[22] Thus, versican expression is associated with a proliferative cell phenotype that may explain its overexpression in several developing tissues and in a variety of tumors.

Canine mammary tumors are histologically classified as simple, complex and mixed tumors. Histologically the complex and mixed tumors show high similarity with the pleomorphic adenoma of the human salivary gland. In contrast to simple tumors that are composed of only one type of proliferating epithelial cell, complex and mixed tumors are characterized by proliferating spindle shaped cells of possible myoepithelial origin (termed as myoepithelial-like spindle cells) and by the appearance of a myxomatous substance in addition to epithelial cells. Furthermore, the stroma of mixed tumors is characterized by the presence of cartilagenous tissue and bone.[23]

We have previously reported an elevated expression of versican in canine mammary tumors compared to the normal mammary tissues, particularly in the invasive areas of malignant tumors and in the myoepithelial-like spindle cell proliferations and myxoid areas.[5,24] In the present study, one of our main objectives was to determine if multiple isoforms of versican exist in canine mammary tumors and cell lines. Furthermore, our previous immunohistochemical study indicated a proteolytic processing of the protein core of versican.[5] This finding prompted us to further investigate the presence of catabolic products of versican in canine mammary tumors. Although very little is known about versican degradation in tissues, it is known that a sub-group of the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) proteases are capable of cleaving versican.[25-27] These proteases have been identified in a number of tumor types, but their functional relevance in malignancies remains elusive.[28-30] Here we determined if canine mammary tumors contained increased levels of versican degradation products generated by ADAMTS proteases. To our knowledge this is the first study showing an extensive ADAMTS-mediated versican degradation in tumor tissues.

Materials and Methods

Tissues

Twelve mammary tumors were resected surgically and a portion of the tissues was fixed in 10 % neutral-buffered formalin. Another part of the tissues was snap frozen in liquid nitrogen under RNase free conditions and stored at – 80°C before use. After 24 hours formalin fixation, the tissues were embedded in paraffin, after which 5μm sections were cut and stained with haematoxylin and eosin

(H&E). Tumors were classified from the H&E-stained sections according to the recent WHO classification [23], although we applied some modification when noted. The 8 benign tumors were classified as simple adenoma (n=3), epitheloid myoepithelioma (n=2), benign complex-mixed tumor (n=3). The group of benign complex-mixed tumors consisted of two complex adenomas with abundant myxoid ECM and few focal areas displaying histomorphological characteristics of early cartilage differentiation and one benign mixed tumor characterized with cartilage and bone tissue in the ECM. The 4 malignant tumors were classified as simple carcinomas; however they showed heterogeneity in their histomorphology, degree of malignancy and in metastatic characteristics. One out of the four carcinomas was a myoepitheloid carcinoma (see Figure 2) without metastasis to the lymph nodes or any organs. One of them was an anaplastic carcinoma with metastasis to lymph nodes and several organs. The remaining two simple carcinomas displayed solid-tubulary growth pattern with invasive growth into the tumor stroma, but without metastasis to the lymph nodes or any organs.
For immunohistochemical analysis of the the ADAMTS cleaved versican products, an additional 14 paraffin embedded mammary tissues were obtained from the archives of necropsies of the Department of Veterinary Pathology, Utrecht University. These samples included 2 normal mammary tissues, 2 simple adenomas, 4 benign complex and mixed tumors, 2 simple carcinomas, 4 malignant complex and mixed tumors. (The term malignant mixed tumor we use corresponds to the mixed tumor with malignant epithelial component in the recent WHO classification.[23])

Cell lines and cell culture

The tissue/tumor of origin and the known phenotypical characteristics of the cell lines used in this study are summarized in Table 1. The canine mammary cell lines coded P217, SH15, P95/168 were previously isolated in our laboratory.[31-33] While the morphology and phenotype of the P217 and SH15 cell lines have been well described [31-32], little is known of the characteristics of P95/168 cell line. Therefore, in this study we characterized these cells by immunocytochemistry and electron microscopy (Table 1). The canine mammary tumor cell lines CMT-8 (also called CMT-U335) and CMT-3 (also called 229)[34], CMT-U309, CMT-U304[35] have been previously well described by Hellmen and colleagues.

Table 1. Description of the canine mammary cell lines

CELL LINES	TISSUE/TUMOR TYPE OF ORIGIN	CELL TYPE*	PHENOTYPICAL CHARACTERISTICS
P217	Normal mammary tissue	Stromal fibroblasts	vimentin + (100%), SMA + (5-20%); versican -/+
SH15	Simple carcinoma with spleen metastases	Malignant epithelial cells	E-cadherin, α-, β-, and γ-catenins +, CK +, vimentin +, vesican +; loose colonies, invasive behaviour into collagen type 1 gel and in chick heart invasion assay
CMT-3 (229)	Atypical benign mixed tumor	Atypical epithelial cells	CK +, vimentin +, desmin +/- Ultrastructure: desmosomes, secretory vesicles, microvilli, duct-like and alveolar-like structures in collagen gel; tendency to polarity and lumen formation
P95/168	Malignant mixed tumor	Malignant myoepithelial/ fibroblast-like spindle cells	CK + (~20-30%), SMA + (~10-20%), vimentin +, vesican + , HA + Ultrastructure: no desmosomes, secretory vesicles, cytoplasmic microfilaments
CMT-U304	Spindle cell tumor	Myoepithelial-like spindle cells	CK5, 6 and 18, CK8, CK14 moderately + at the first cell passage; vimentin strongly +, SMA moderately +; elongated cells, no cell contacts; Ultrastructure: rich in RER, thin cytoplasmic filaments in abundance; very low growth rate
CMT-U309	Spindle cell tumor	Atypical myoepithelial-like spindle cells	CK5, 6, and 18: single cells strongly +; SMA, vimentin, CK 8: slightly to moderately +; elongated cells, some very large with several nuclei; 22 h cell doubling time
CMT-8 (CMT-U335)	Osteosarcoma	Malignant spindle cells	vimentin +, CK5, 6, and 18 -, desmin and neurofilaments -

*The cell type described is based on the tissue of origin, on the cell morphology in monolayer cultures and on their previously [30-34] or presently described immunohistochemical characterization and behaviour. Abbreviations: CK-cytokeratin, SMA-smooth muscle actin, HA-hyaluronan.

The cells were cultured in 75 cm^2 tissue culture flask in Dulbecco's modified essential medium (DMEM), 10% fetal calf serum and 1% penicillin/streptomycin sulfate at 37 °C, 5% CO_2 up to the second passage when cells were plated on 100-mm dishes (5×10^5 cells/dish) for RNA isolation.

Immunocyto/histochemistry and Electron microscopy

Cell-lines- $2x10^4$ normal fibroblasts (P217) and $1x10^4$-$1x10^5$ tumor cells were cultured on multi-chambered glass slides (LAB-TEK II Chamber Slide, Nalge Nunc International, Naperville, IL, USA) in DMEM with 10% FCS. After 72 hours slides were rinsed in PBS pH 7.4 and fixed in methanol. Each cell line was stained for versican (monoclonal antibody 2B1, 1:1000, Seikagaku Co., Tokyo, Japan). The P95/168 cell line was also stained for cytokeratin (MNF116, broad-spectrum cytokeratin, 1:50; DAKO, Denmark), smooth-muscle actin (1A4, 1: 500; Biogenex, San Ramon, CA, USA) and vimentin (V9, 1:200; Biogenex).

Tissues- In addition to versican expression, we previously described the phenotype of the tumor cell populations in our tumor samples by immunohistochemical staining for broad-spectrum cytokeratin, cytokeratin 14 (CK14), smooth muscle actin (SMA) and vimentin.[24] In this study immunostaining was performed for the above markers in one simple carcinoma that was not characterized previously. Furthermore, twenty-six mammary tissues (see *Tissues*) were examined for the presence of DPEAAE neoepitope generated by ADAMTS mediated cleavage in the ß-GAG region of versican core protein. The rabbit polyclonal antibody (Versican, V1/V0 Neo, 1:2500; Affinity Bioreagents) is known to recognize this neoepitope at Glu^{441} on V1 isoform and at Glu^{1428} on V0 isoform.[26] All immunocyto/histochemical procedures were performed using the streptavidin-biotin based technique (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA, USA), as described previously.[5,24] The corresponding negative control slides were incubated with Mouse IgG1 serum (X0931, DAKO) or normal rabbit serum (in the same protein dilution as the primary antibody) instead of the primary antibody.

Electron microscopy- The P95/168 cell line was also studied ultrastructurally. Monolayer cultures were prefixed in Karnovsky's and postfixed in 2% OsO_4, buffered in 0.1 M cacodylaat PH 7.4, for 2 hours. Subsequently, the samples were dehydrated in acetone (50, 70, 80, 96, and 100%), immersed overnight in 100% acetone/ durcupan (epoxy) 1:1, immersed in pure durcupan for 2 hours and

embedded in durcupan. Ultrathin sections were cut and examined in an electron microscope (Philips CM 10).

RNA Isolation

Cell cultures- Upon reaching confluence, cells were harvested and total RNA was isolated using TriZol solution (Life Technologies Inc. GIBCO BRL, Rockville, MD, USA) following manufacturer's instructions.

Tissues- The frozen tissues were first pulverized with a Bio-pulverizer™ (Research Products International Corp., Mt. Prospect, Illinois, USA), then added to TriZol solution and immediately homogenized using a Polytron. The subsequent RNA isolation steps were performed as per the manufacturer's instructions. RNA integrity was confirmed with gel electrophoresis and by A_{260}/A_{280} ratio. The RNA concentration of each sample was determined by UV spectrophotometry.

Conventional RT-PCR

Expression of the main mRNA versican splice variants, V0, V1, V2 and V3 were analysed by reverse transcriptase–polymerase chain reaction (RT-PCR). As far as the authors are aware, this is the first study analyses of the expression of versican mRNA splice variants in canine tissues.

Primer design- Rat versican V3 sequence (Genbank AF072892) was entered into the dog BLAT genome search at the UCSC Bioinformatics web site (http://genome.ucsc.edu/cgi-bin/hgBlat). The result "details" with the highest homology included the sequence for the entire coding sequence of dog Versican on Chromosome 3. Blue highlighted sequences of the dog genome corresponding to transcribed exons of dog V3 were pieced together and blasted back to the rat V3 sequence (www.ncbi.nlm.nih.gov/BLAST/). Missing nucleotides in the dog V3 were found by looking 5' or 3' to the blue highlighted exons and finding matching sequence to the rat. Dog V0, V1, and V2 versican sequences were predicted in the same manner using human versican V0 sequence (Genbank U16306). Completed sequences were then entered into a primer selection program (www.cgr.ki.se/cgr/MEDUSA/). Primer pairs were selected to cross the G1/G3 domain splice junction for the V3 variant, the G1/ GAGß domain splice junction for the V1 variant, the GAGα/ G3 domain splice junction for the V2 and the GAGα/ GAGß domain splice junction for the V0 variant. The selected primer pairs were tested with the PCR simulation program (Amplify) to minimize non-specific products. Single-strand cDNA synthesis was performed in 40-µl reactions using 1–2

µg total RNA with random primers, 0.8 µl Rnase inhibitor (RNA-SIN; Ambion, Austin, TX, USA), and 2.6 µl ImProm-II Reverse Transcriptase (Promega, Madison, WI, USA) for 1 h at 42°C. Conditions for PCR amplification were optimized for $MgCl_2$ concentration and temperature using the PTC-200 gradient thermal cycler (MJ Research, Waltham, MA, USA). PCR reactions were performed using 1–2 µl single-strand synthesis product, 250 mM dNTP, 5x Dynazyme Optimized EXT buffer Buffer, 5 pmol of each primer, and 1.5 mM $MgCl_2$ in a 20-µl reaction volume. Data on the specific RT-PCR primers used in this study and on the corresponding RT-PCR conditions are described in Table 2. The table shows also a schematic picture of the location of these primers.

Real-Time RT-PCR Amplification and Relative Quantitative Analysis

Quantitative PCR was performed on a *Mx4000*® Multiplex QPCR System (Stratagene, La Jolla, CA, USA) with samples loaded in triplicate using 100 ng of total RNA. The RNA concentration of the samples was determined with the Ribogreen RNA quantification assay (Molecular Probes, Eugene, OR, USA). Quantitative PCR was run in a 25µl reaction using Stratagene Brilliant® Single-Step QRT-PCR Kit (2.5µl 10x core RT-PCR buffer, 5.0 mM $MgCl_2$, 800 nM each primer, 200 nM probe, 0.2 mM each dNTP, 75 nM passive reference dye, 12.5 units of Stratascript RT, 1.25 units of SureStart *Taq*DNA-polymerase) with PCR cycling conditions of 45°C for 30 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 30 seconds and 60°C for 1 minute. Pooled total RNA from SH15 and P95 cell lines was used for standard curves as serial dilutions.

Primer design- Sequence areas of domain splice junctions specific to dog versican V0, V1, and V3 splice variants (see above) and dog cyclophilin B (CYCB; Genbank NM000942) and TATA box binding protein (TBP)-associated factor (TAF10; NM020024) were used to design primers and probes with Primer Express 2.0 software (ABI, Foster City, CA, USA). Probes were also designed across exon boundaries to avoid gDNA detection. The total gene and splice variant specificity of the nucleotide sequences chosen for the primers and probes was confirmed by BLASTn searches. Primers were from Qiagen Operon (Valencia, CA, USA). Fluorescent labeled TaqMan® probes were from ABI or Integrated DNA Technologies (Skokie, IL, USA). The primers and probes for each splice variant and normalizing genes designed for Real-time PCR and the respective amplification efficiencies are listed in Table 3.

Table 2. Description of primers designed for the RT-PCR analysis of versican splice variants V0, V1, V2 and V3. The schematic picture shows the location of each primer

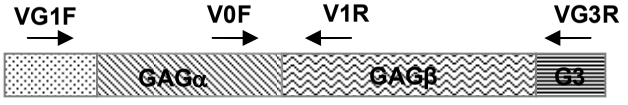
Primer name	Sequence (5'→ 3')	GenBank accession number/Location of primers	Amplicon name	Primer pairs (Forward-Reverse)	Amplicon size (bp)	PCR conditions
VG1F	GCTTTGACCAGTGCGATTATG	Predicted dog V0 seq	V0 variant	V0F-V1R	668	95°C, 50 sec;
V0F	GAAACCACCTTTGATTGACAGG	Predicted dog V0 seq	V1 variant	VG1F-V1R	528	57°C 30 sec;
V1R	TCGGCTACCACAAATGGATGT	Predicted dog V0 seq	V2 variant	V0F-VG3R	404	72°C, 30 sec
VG3R	CCTGGCACACAGGTACATACG	Predicted dog V0 seq				95°C, 50 sec;
GAPDHF	CTCAGTGTAGCCCAGGATGC	AB038240 (dog) F:828-809	V3 variant	VG1F-VG3R	264	55°C 30 sec;
GAPDHR	GCGTGAACCATGAGAAGTATGAC	R:395-417				72°C, 30 sec
			GAPDH	GAPDHF- GAPDHR	412	95°C, 50 sec; 57°C 30 sec; 72°C, 30 sec

Table 3. Description of the designed primers and probes for Real-Time PCR

CODE	SEQUENCE (5'→ 3')	AMPLICON SIZE (bp)	AMPLIFICATION EFFICIENCY
V0 splice-Qf	AAATGTTTTTGTTATTGAGGTCAGAGAA	73	92.3% R^2=0.999
V0 splice-Qr	TGGATGACCAATTACACTCAAATCA		
V0 splice-Qp	6FAM-ACAAGACAGGTCGAATGA-MGBNFQ		
V1 splice-Qf	CCCTCCCCCTGATAGCAGAT	76	93.9% R^2=0.998
V1 splice-Qr	TGGATGACCAATTACACTCAAATCAC		
V1 splice-Qp	6FAM-TACTGCTTTAAACGTCGAATG-MGBNFQ		
V3 splice-Qf	GGCACGGGTTCGTTTTACAA	64	96.5% R^2=1.000
V3 splice-Qr	CCTGATAGCAGATTTGATGCCTACT		
V3 splice-Qp	6FAM-ATCGGGTCGTTTAAAG-MGBNFQ		
CYCB-Qf	CATGATCCAGGGTGGAGACTTT	71	90.8% R^2=0.994
CYCB-Qr	GGAAGCGTTCACCATAGATGCT		
CYCB-Qp	6FAM-CCGAGGAGATGGCACTGGAGGAAA-BHQ1		
TAF10-Qf	CGCCTCTGGTGGACTTCCT	80	99.9% R^2=0.999
TAF10-Qr	GTTCAGGTAGTAACCAGTCACTGCAT		
TAF10-Qp	6FAM-AGCTGGAGGACTATACGCCTACGATCCCA-BHQ1		

Qf-foward primer; Qr-reverse primer; Qp-probe

The *Mx4000*® software performed a linear regression analysis for the standard dilutions and extrapolated and expressed the values for the experimental samples as corresponding nanogram of the standard RNA. The values in nanogram for each sample were divided by the value for the two normalizer genes CYCB and TAF10. The results were analysed by the relative standard curve method. In case of the cell lines, the normal mammary fibroblast (P217), and in case of the tumor tissues, the simple adenomas were used as a calibrator. Furthermore, to confirm that the assays were specific and not affected by the presence of any shared sequences within the other variants, we also applied the variant specific (V0, V1 and V3) RT-PCR products as a template in the assay of the respective variant. None of these templates interfered or generated signals in assays of each of the other variants. The corresponding variant specific templates in each assay

resulted in the same C_T value (data not shown) and the optimized reaction efficiencies of each assay were comparable to each other (<10% difference) and close to 100%. This allowed us to compare the relative expression levels of the splice variants (fold-increase) using the $2^{-\Delta Ct}$ formula (Applied Biosystem, Users Bulletin #2). For each sample, the average Ct value of the variants were substracted from each other in pairs to derive the respective ΔCt. Moreover, the similar and high (close to 100%) reaction efficiencies of each assay enabled us to calculate and compare the relative amount of total versican mRNA of the samples by adding the values in ng of each variant.

Statistical analysis- In case of the mammary cell lines all data of two separate experiments measured by Real-Time PCR amplification were assessed by analysis of variance (one-way ANOVA) followed by Bonferroni's multiple comparison tests. The level of significance was set as $p<0.05$.

Proteoglycan Isolation and Western Analysis

V0/V1 versican- Frozen tissues were first pulverized with a Bio-pulverizer™ (Research Products International Corp., Mt. Prospect, Illinois, USA) and extracted with 4 M guanine buffer (4 M guanidine, 0.5 M sodium acetate, pH 7.0) with protease inhibitors.[36] Proteoglycans in tumor homogenates and culture media were isolated by DEAE Sephacel chromatography, as described previously.[37] The GAG content of each sample was determined spectrophotometrically using dimethylmethylene blue dye and bovine chondroitin sulfate as a standard.[38] Equal amounts of DEAE-purified samples based on GAG content (30μg) were precipitated in 80% ethanol and 1.3% potassium acetate. The resulting pellets were digested with chondroitin ABC lyase (from *Proteus vulgaris*, Seikagaku, Tokyo, Japan) with protease inhibitors for 6 hours at 37ºC.

V3 versican- Cell layers were rinsed with phosphate-buffered saline and solubilized in 8 M urea buffer (8 M urea, 2 mM EDTA, 0.25 M NaCl, 50 mM Tris-HCl, and 0.5% Triton-X 100 detergent, pH 7.4) containing protease inhibitors (5 mM benzamidine, 100 mM 6-aminohexanoic acid, and 50 mM phenylmethylsulfonyl fluoride). The total protein concentration of harvested cell layers and full tissue extracts was determined with Pierce Protein Assay (BioRad, Hercules, CA, USA). Without enzymatic digestion equal amounts of samples (60μg total protein) were precipitated and dissolved in 8 M urea buffer without NaCl and Triton X-100.

Samples were separated on SDS-PAGE 4-12 % gradient gels with a 3% stacking gel and transferred to nitrocellulose membranes (Hybond ECL, Amersham Biosciences, Piscataway, NJ, USA) in a semidry electrophoretic transfer apparatus (Bio-Rad). After blocking the membranes with 2 % bovine serum albumin (BSA) in TBS/Tween 0.1%, the transferred versican proteins were detected with primary antibodies 12C5 (known to recognize the HA-binding domain [39]; DSHB, Iowa City, IA, USA; 1:500 dilution), 2B1 (known to recognize the G3 domain of versican [40]; Seikagaku Co., Tokyo, Japan; 1:2000 dilution) and with rabbit antibodies against bovine GAGα and GAGβ attachment domains (1:500 dilution each; kindely provided by Richard LeBaron /Division of Life Sciences, Cell and Molecular Biology, University of Texas at San Antonio, TX, USA/) using enhanced chemiluminescence (Western-Light Chemiluminescent Detection System with CSPD substrate; Tropix, Bedford, MA) and visualized by Hyperfilm ECL (Amersham Bioscience).

Protein mass prediction of dog versican isoforms- Predicted mRNA sequences for dog versican splice variants V0, V1, V2, and V3 were translated into protein sequence via the BCM SearchLauncher sequence utilities program (http://searchlauncher.bcm.tmc.edu/seq-util/seq-util.html). Protein sequence was then entered into the EXPASY mass prediction site (http://us.expasy.org/tools/peptide-mass.html).

ADAMTS generated versican products- Equal amounts of chondroitin ABC lyase digested protein from tumor homogenates were separated on 10% SDS polyacrylamide gel and transferred to Immobilon-P PVDF membrane (Millipore, Bedford, USA). The protein transfer was confirmed by staining the membrane with MEMcode^{tm} reversible protein stain (Pierce, Rockford, USA). After blocking with 5% ECL Blocking Agent (Amersham Biosciences, Buckinghamshire, England) in PBS/Tween 0.1% (PBST), membranes were incubated with the primary antibody Versican V0/V1 Neo (1:1000; Affinity BioReagents) in 0.5% ECL- blocking in PBST at room temperature. After washing with PBST the membrane was incubated for 1 hour in HRP-conjugated rabbit EnVision^{TM}+ reagent (1:50; DakoCytomation) in 0.5% ECL-blocking in PBST. Antibody binding was detected using chemiluminescence ECL^{TM} Western Blotting Detection Reagents (Amersham Biosciences) according to the manufacturer's recommendation and visualized by Hyperfilm ECL (Amersham Biosciences).

Results

Phenotypic Characterization of Canine Mammary Cell Lines

The canine mammary cell lines tested in this study have been previously well-characterized (Table 1), with the exception of the P95/168 (malignant spindle cells isolated from a mixed tumor). In order to determine the phenotype of the P96/168 cell line we stained these cells for cytokeratins, smooth muscle actin and vimentin. We found that 20-30% of these cells stained for broad-spectrum cytokeratin (Figure 1A) and 10-20% for smooth muscle actin (Figure 1B), whereas all cells were strongly stained for vimentin. These findings indicate that the P95/168 cell line consists of cells with mixed phenotype where a smaller percentage of the cells show myoepithelial characteristics and a larger percentage of the cells have fibroblast-like phenotype. The ultrastructural characteristics of the cell line are summerized in Table 1.

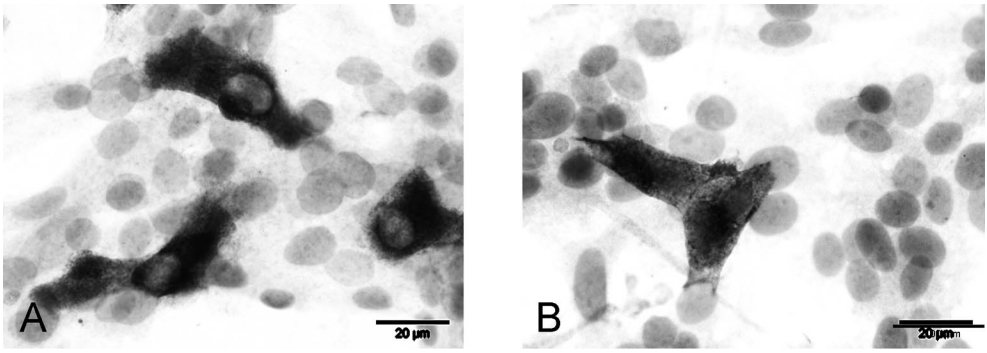


Figure 1. Immunolabelling of the P95/168 cell line revealed that 20-30% of these cells stained intensely for broad-spectrum cytokeratin (A) and 10-20% for smooth muscle actin (B). All cells stained strongly for vimentin (not shown).

Immunohistochemical Characterization of Canine Mammary Tumor Samples

Canine mammary tumors are well-known for their complexity in histological pattern. To establish the precise histological classification of our tumor samples, we have previously characterized the tumor cell populations within our tumor samples by immunohistochemical staining for broad-spectrum cytokeratin, CK14, SMA and vimentin.[24] One of the simple carcinomas of this study has not been previously characterized, thus we stained this tumor for the above markers. 100%

of the tumor cells stained strongly for vimentin and a large percentage of the tumor cells showed intense immunoreactivity to CK14 (Figure 2A) and moderate to intense immunoreactivity to SMA in this tumor (Figure 2B).

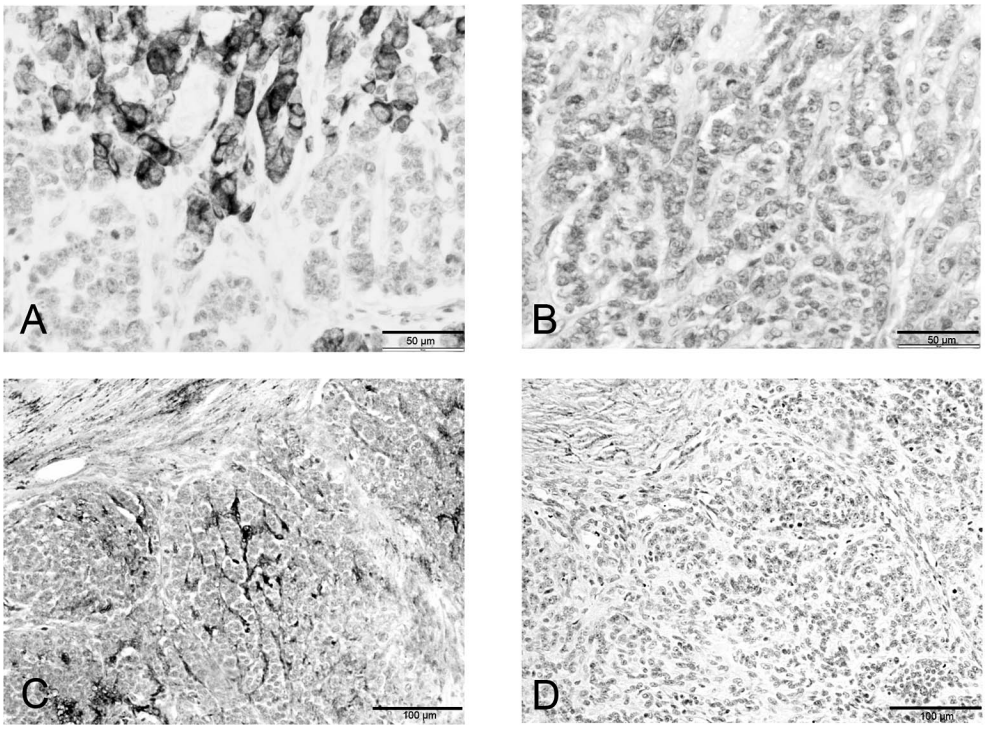


Figure 2. Immunolabelling of myoepitheloid carcinoma shows intense immunoreactivity to cytokeratin 14 (A) and moderate to intense reactivity to smooth muscle actin (B) in the cytoplasm of a large percentage of the tumor cells.

Based on these findings this tumor was subclassified as myoepitheloid carcinoma. Furthermore, we previously investigated the expression pattern of versican with antibody 12C5 (G1 domain) and antibody 2B1 (G3 domain) in our tumor samples.[24.] The two antibodies showed non overlapping staining in some areas, particularly in the ECM-rich myxoid tissues of complex and mixed tumors, suggesting that the protein core of versican was proteolytically processed in this tissues. Similarly, the myoepitheloid carcinoma tested in this study stained intensely for versican with antibody 12C5 both in peri-, and intratumoral

connective tissues (Figure 2C), whereas reactivity to versican with antibody 2B1 was restricted to the peritumoral tissues (Figure 2D). This finding indicates that versican may be cleaved by proteases also in the intratumoral stroma of this tumor.

V1/V0 are the Main Versican Variants in Canine Mammary Tumors and Cell Lines

Expression of mRNA splice variants

RT-PCR was performed to determine which mRNA splice variants of versican are present in canine mammary tumors and cell lines. Of the four versican splice variants we found detectable expression of V0, V1 and V3 variants, but not V2 both in the tumor tissues and in the mammary cell lines (data not shown). Therefore, we designed specific primers and probes for V0, V1 and V3 splice variants for Real-time PCR analysis. As a first step, we checked whether the normalizing genes, CYCB and TAF10, are expressed at equal level in all samples. Although the different type of tumors showed some heterogeneity in the expression of the normalizers, none of these differences proved to be significant. However, we found a significant ($p<0.001$) upregulation of both normalizers, CYCB and TAF10, in the tumor cell lines when compared to the non-tumorous mammary fibroblasts (P217). The validity of normalizing to total RNA has been reported.[41] Therefore, the relative quantitative comparisons of the expression of versican mRNA splice variants were performed with values normalized to total RNA measured with Ribogreen RNA quantification assay.

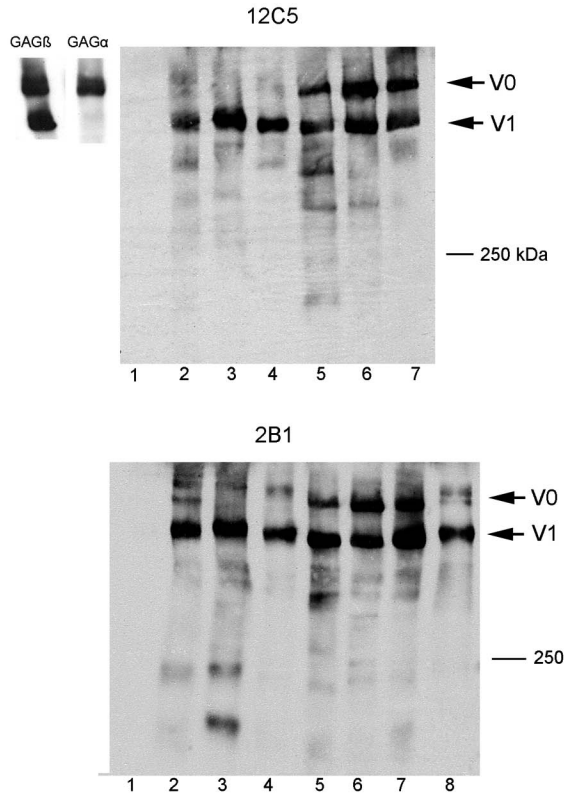
Cell lines- Comparison of the expression levels of splice variants within each cell lines revealed that the main variant was V1, followed by V0 with a similarly high expression in all the cell lines. We found that the expression of V1 variant was 1.1-12 fold higher than that of the V0 variant. The normal mammary fibroblasts (4.89-fold) and the P95/168 tumor cell line (11.92-fold) showed the highest fold increase in their V1 expression over that of the V0 variant. The V3 variant consistently represented the lowest expression level. Compared to V3 variant, the expression of V0 variant was in the range of 1.3-85 and the expression of V1 variant was in the range of 16-106. In both comparisons the P95/168 cell line showed the lowest fold-increase of V0 and V1 variant over V3, indicating a relatively high ratio of V3 versican in this tumor cell line.

Tumors- Tumor tissues displayed similar splice variant distribution compared to the cell lines, however, the difference in the expression level of V0 and V1

between the different tumor types was very small (1.03-1.57-fold). Moreover, some of the tumors, in particular the anaplastic carcinoma (1.57), showed higher V0 than V1 expression. Compared to V3 variant the average fold increase of V0 variant expression in the different histology groups of the tumor tissues was in the range 29-218 and the expression of V1 variant was in the range of 44-223. The lowest fold increase of V0 (29.38-fold) and V1 (44.53-fold) variant expression was in the benign mixed tumor indicating a relatively high V3 ratio in this tumor.
In summary these results showed the prevalence of V1 versican in all the cell lines followed by a similarly high V0 versican expression. In contrast to the cell lines some of the tumor tissues showed higher V0 than V1 versican levels. Out of the three mRNA splice variants the V3 variant was present in the lowest amount in both the mammary cell lines and mammary tumors. However, the different type of mammary cell lines and mammary tumors displayed different relative ratios of the three versican variants.

Expression of versican protein isoforms
In order to identify the high-molecular weight V0/ V1 versican proteins we performed western blot analysis of DEAE-purified and chondroitinase ABC digested proteoglycans of cell culture media and tumor homogenates (probed with antibodies 12C5 /G1 domain/ and 2B1 /G3 domain/). Culture medium of monkey arterial smooth muscle cells, known to contain the 455 kDa band that corresponds to V0 isoform and the 350 kDa band that corresponds to the V1 isoform, was used as a positive control.
Cell lines- Two major distinct bands were identified with both antibodies at approximately 455 kDa and 350 kDa in all the tumor cell lines. These bands however, could be detected only very weakly from the media of the normal mammary fibroblast (Figure 3A, lane-1); moreover, the bands were visible only after long exposure time. This finding suggests elevated V0/V1 versican protein expression in tumor cells compared to fibroblasts of the normal mammary tissue. Furthermore, in the tumor cell lines several prominent and faint bands appeared below 350 kDa ranging in size from approximately 70-320 kDa (Figure 3A, lane 2-7). These bands may represent various versican fragments (N-terminal versican fragments detected with antibody 12C5 and C-terminal fragments detected with antibody 2B1) generated by proteases secreted by the mammary tumor cells. In general, the multiple bands bellow 350 kDa appeared in all the tumor cell lines, but not in the normal mammary fibroblasts.

A. Cell lines



B. Tumor tissues

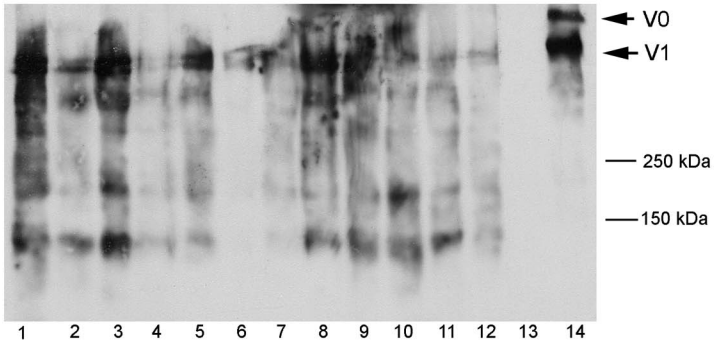


Figure 3. Western blot analysis of versican proteins after DEAE Sephacel chromatography and chondroitinase ABC treatment in canine mammary cell lines (A; lane 1 P217 normal mammary fibroblasts, lane 2 SH15, lane 3 P95/168, lane 4 CMT-3, lane 5 CMT-8, lane 6 CMT-U304, lane 7 CMT-U309, lane 8 /2B1/ positive control monkey smooth muscle cell line) and in canine mammary tumor tissues (B; lane 1-5 simple carcinomas, lane 6-7 simple adenomas, 8-10 benign complex-mixed tumors, 11-12 epitheloid myoepitheliomas, lane 13 negative control, lane 14 positive control monkey smooth muscle cell line). The two high molecular weight bands (shown by arrows) reacting with both antibody 12C5 and 2B1 represents the core protein of versican V0 and V1. As shown in the upper left corner of Figure 3A, the versican V0 reacted also with anti-GAG-α and anti-GAG-ß antibodies and versican V1 with anti-GAG-ß antibody. The bands that appeared below 350 kDa may represent versican fragments after proteolytic cleavage.

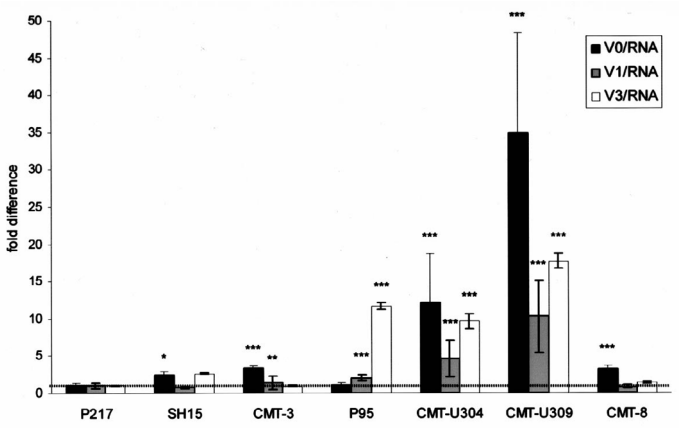
These bands appeared the most prominent in case of the myoepithelial-like (CMTU-304, CMTU-309) and myoepithelial/fibroblast-like (P95/168) tumor cell lines and they were also prominent in case of the malignant epithelial cell line (SH15). However, the atypical benign epithelial cell line (CMT-3) showed only few and faint bands in this range.

Tumors- The two high molecular weight bands at 455 kDa and at 350 kDa were present also in the tumor samples; however, particularly the 455 kDa band was less distinct than that of the cell lines (Figure 3B). In general, the 350 kDa band corresponding to V1 versican was more prominent in simple carcinomas and complex-mixed tumors than in simple adenomas or the epitheloid myoepitheliomas. Similar to what was observed in case of the tumor cell lines several additional bands appeared below 350 kDa. Moreover, in line with the findings in case of the benign epithelial cell line (CMT-3), simple adenomas displayed only few and faint bands in this range (Figure 3B, lane 6-7).

To confirm the specificity of these bands for V0 and V1 isoforms rabbit antibody to bovine α-GAG and ß-GAG subdomains of the protein core were also applied. As shown in Figure 3A, the 455 kDa band corresponding to versican V0 (containing both α-GAG and ß-GAG subdomains) reacted with both antibodies, whereas the 350 kDa representing versican V1 (containing only the ß-GAG subdomain) reacted with the anti-ß-GAG antibody, respectively.

In summary, the results confirm the expression of V1/V0 versican at protein levels in both the mammary cell lines and mammary tumors. However, in the tumor tissues the intact V0 protein appeared less abundant compared to the V1 protein. Furthermore, the tumor cell lines showed elevated levels of both V1/V0 proteins when compared to the normal mammary fibroblasts. The bands that appeared bellow 350 kDa may represent proteolytically processed core protein of versican, similar to those reported in other tissues or culture medium.[42]

In an attempt to detect versican V3 protein, full tissue extracts and cell lysates were electrophoresised and probed also with versican antibodies 12C5 and 2B1. Based on our calculations the predicted mass of dog V3 protein is 74.477 kDa. In accordance with this finding we detected an approximately 75 kDa band with both antibodies 12C5 and 2B1 in complex and mixed tumors that may correspond to versican V3 protein (not shown). Further investigation is necessary to confirm the expression of V3 protein in these tumors.



CELL LINE	V0/RNA	V1/RNA	V3/RNA
P217	1.00±0.31	1.00±0.41	1.00±0.06
SH15	2.38±0.48	0.79±0.13	2.66±0.12
CMT-3	3.45±0.28	1.40±0.88	0.99±0.08
P95/168	1.03±0.42	2.06±0.44	11.69±0.44
CMT-U304	12.04±6.67	4.60±2.44	9.60±1.04
CMT-U309	34.79±13.56	10.24±4.87	17.68±0.96
CMT-8	3.29±0.46	0.81±0.20	1.38±0.15

Figure 4. Versican splice variants, V0, V1, and V3 mRNA levels as measured by Real-time Taqman PCR in canine mammary cell lines. Results were analyzed using the relative standard curve method where total RNA measured with Ribogreen was used as a normalizer. Values are expressed as mean fold differences compared to the calibrator normal mammary fibroblast cell line (calibrator =1 as indicated by the dotted line). Comparisons were made using analysis of variance (one-way ANOVA) with Bonferroni's multiple comparison test. Values are mean ± std and significant differences compared to simple adenoma are shown *p<0.05, **p<0.01, *** <0.001.

Tumor cells express elevated levels of versican

Versican mRNA levels- When comparing the expression level of mRNA splice variants between the different type of mammary cell lines (Figure 4) the results showed that all tumor cell lines showed significantly higher V0 mRNA level ($p=0.043$ in CMT-3; $p<0.001$ in all other cell lines) compared to that of the normal mammary fibroblasts with the exception of the P95/168 cell line. V1 mRNA expression was significantly higher in the cell lines derived from spindle cell tumors and mixed tumors, namely CMT-U309, CMT-U304, P95/168 ($p=0.001$) and CMT-3 ($p=0.003$); however, it appeared lower, although not significantly, in the SH15 carcinoma cell line and in the CMT-8 osteosarcoma cell line. V3 mRNA level was also higher in all the tumor cell lines with the exception of the CMT-3 cell line that expressed approximately equal levels of V3 transcripts compared to the normal mammary fibroblasts. The higher V3 mRNA level proved to be significant in the malignant spindle cell line P95/168 ($p<0.001$) and in the two myoepithelial-like spindle cell lines CMT-U304 and CMT-U309 ($p<0.001$). In summary, the CMT-U309 (myoepithelial-like) cell line expressed the highest levels of all the three variants, followed by the highest V0 and V1 expression of the CMT-U304 (myoepithelial-like) cell line and the highest V3 expression of the P95/168 (myoepithelial/fibroblast-like) cell line.

The comparison of total versican mRNA expression (calculated as the sum of normalized values (ng) of V0, V1 and V3 mRNA levels) of the cell lines revealed the following relative frequency of versican expression: CMT-U309> CMT-U304> P95/168> CMT-3> CMT-8> SH15> P217. The result shows that all tumor cell lines express higher total versican mRNA compared to the non-tumorous mammary fibroblasts. The two myoepithelial-like cell lines expressed the highest levels of total versican mRNA levels followed by the myoepithelial/fibroblast-like P95/168 spindle cell line. When comparing the two myoepithelial-like spindle cell lines, CMT-U309 and CMT-U304, the results show a higher mRNA level (for all the three splice variants) in the CMT-U309 cell line that has a significantly higher growth rate and polymorphism compared to the CMT-U304 cell line. Moreover, the malignant P95/168 spindle cell line (isolated from an invasive malignant mixed tumor) also showed a higher total versican mRNA (higher V1 and V3, but lower V0 mRNA levels) compared to the CMT-3 cell line (originating from a non-invasive atypical mixed tumor) that shows a more differentiated morphology and lower growth rate.

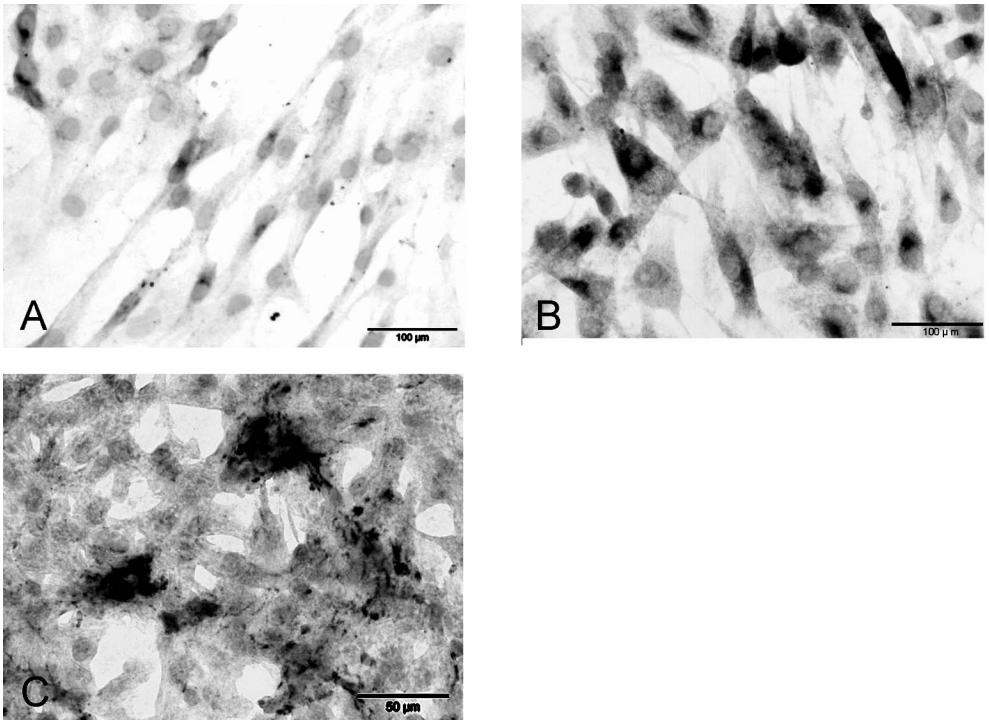


Figure 5. Immunolabelling of canine mammary cell lines. The normal mammary fibroblast cells (P217) showed weak (A) or no immunoreactivity to versican; whereas all the tumor cell lines stained intensely for versican (B - CMT-U304; C- P95/168; other tumor cell lines showed similar immunopattern for versican). Note that staining for versican appeared in the cytoplasm of the tumor cells and also in the intercellular spaces when cultures reached confluency.

Versican protein expression- As already indicated by the results of western analysis of DEAE-purified proteoglycans of cell culture media, we found an increased verican expression also at protein level in the tumor cell lines compared to the normal mammary fibroblasts. This finding was confirmed by immunohistochemical staining for versican on monolayer cultures of cells in medium that contained 10% fetal calf serum. We found that all the tumor cell lines stained strongly for versican, whereas the normal mammary fibroblast showed only weak to moderate (Figure 5A) or no immunoreactivity to versican. Intense versican immunoreactivity appeared in the cytoplasm of the tumor cells (Figure 5B-C) and also the intercellular areas of close cell contacts (Figure 5C). In serum free cultures the normal mammary fibroblast did not express any versican,

whereas all the tumor cell lines showed similarly intense versican immunoreactivity (data not shown).

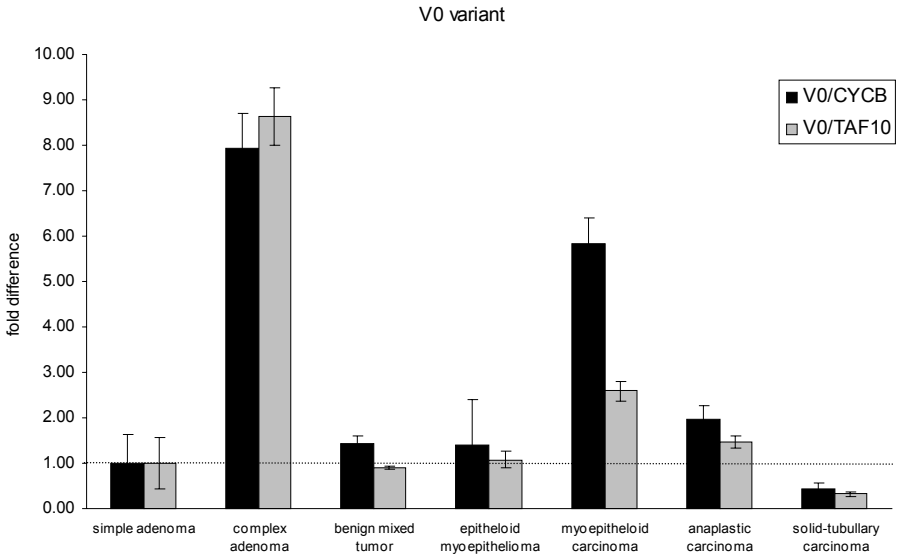
Differential expression of versican mRNA splice variants in canine mammary tumors

To compare the expression level of mRNA splice variants between the different types of mammary tumors the values measured by real-time PCR were analysed by the relative standard curve method. Results are shown as fold difference values compared to the calibrator (simple adenoma; Figure 6). The comparison showed similar results when values were normalized to CYCB and TAF10, although non-significant differences could be observed. The results revealed heterogeneity in the expression level of each variant between the different tumor types. The highest V0 and V1 mRNA levels appeared in complex adenomas, followed by the myoepitheloid carcinoma. V0 mRNA level was higher also in the anaplastic carcinoma and in the epitheloid myoepitheliomas, whereas the V1 mRNA level was higher also in the benign mixed tumor compared to simple adenomas. The two solid-tubulopapillary carcinomas displayed lower V0 and V1 mRNA levels compared to the simple adenomas. Interestingly, the V3 mRNA levels were several fold higher not only in complex adenomas and in the myoepitheloid carcinoma but also in the benign mixed tumor. The anaplastic carcinoma also showed higher V3 expression compared to simple adenomas.

In summary, these results indicate that versican mRNA splice variants are differently expressed in tumors with different histology. Complex adenomas and the myoepitheloid carcinoma consistently showed the highest levels of all the three mRNA splice variants. The expression level of V0 variant was considerable higher also in the anaplastic carcinoma, whereas the mRNA level of the V3 variant was higher also in the benign mixed tumor. There was no clear correlation between the expression level of versican transcripts and benign or malignant tumor behavior.

ADAMTS-generated versican fragments in canine mammary tumors

Immunohistochemistry- The DPEAAE neoepitope generated by ADAMTS cleavage of the versican core protein were present in the tumor tissues but not in the normal mammary tissues. They appeared to be most abundant in the myxoid tissues of complex and mixed tumors. Immunoreactivity to DPEAAE was consistently intense and mostly diffuse in these tissues (Figure 7A).


V0 variant
fold difference
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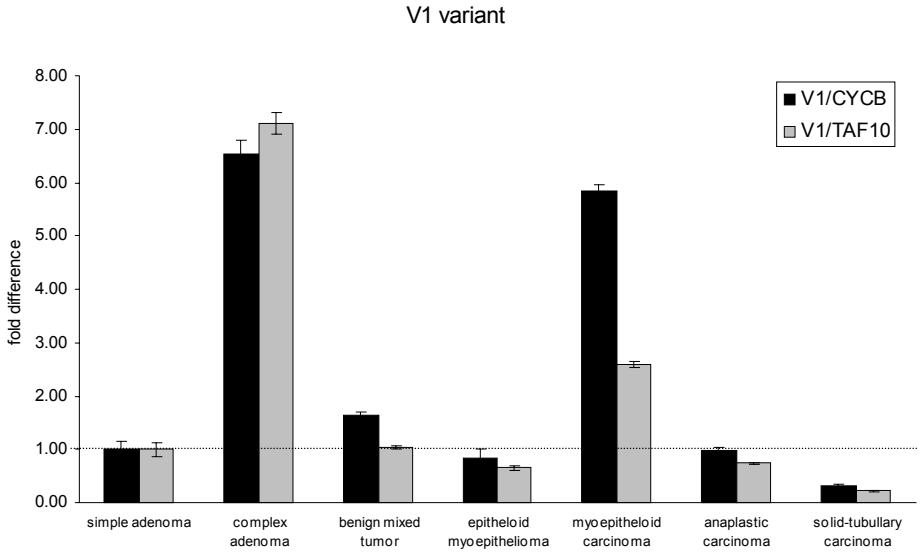
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
V0/CYCB
V0/TAF10
simple adenoma
complex adenoma
benign mixed tumor
epitheloid myoepithelioma
myoepitheloid carcinoma
anaplastic carcinoma
solid-tubullary carcinoma


V1 variant
fold difference
8.00
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V1/CYCB
V1/TAF10
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complex adenoma
benign mixed tumor
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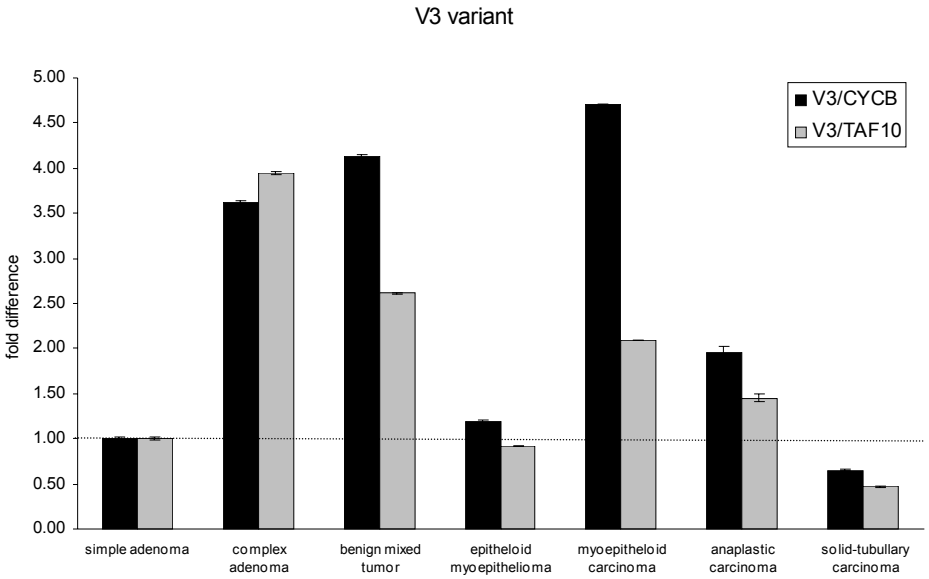
anaplastic carcinoma
solid-tubullary carcinoma



HISTOLOGY	V0/CYCB	V0/TAF10	V1/CYCB	V1/TAF10	V3/CYCB	V3/TAF10
Simple adenoma	1.00±0.64	1.00±0.56	1.00±0.14	1.00±0.12	1.00±0.0	1.00±0.02
Complex adenoma	7.94±0.76	8.62±0.63	6.54±0.23	7.11±0.20	3.63±0.02	3.94±0.01
Benign mixed tumor	1.43±0.16	0.90±0.02	1.64±0.07	1.04±0.03	4.14±0.02	2.61±0.004
Epitheloid myoepithelioma	1.40±1.01	1.08±0.18	0.85±0.16	0.65±0.04	1.19±0.03	0.91±0.003
Myoepitheloid carcinoma	5.83±0.59	2.59±0.22	5.84±0.10	2.59±0.06	4.71±0.01	2.09±0.00
Anaplastic carcinoma	1.98±0.28	1.47±0.13	0.99±0.03	0.74±0.02	1.95±0.07*	1.45±0.04
Simple carcinoma	0.44±0.13	0.32±0.04	0.31±0.03	0.22±0.01	0.65±0.01	0.47±0.003

Figure 6. Expression level of versican mRNA splice variants V0 (A), V1 (B) and V3 (C) as measured by Real-time Taqman PCR in canine mammary tumors. Results were analyzed using the relative standard curve method where CYCB and TAF10 were used as normalizing genes. Values are expressed as mean fold differences ± std compared to the simple adenomas (calibrator =1 as indicated by the dotted line).

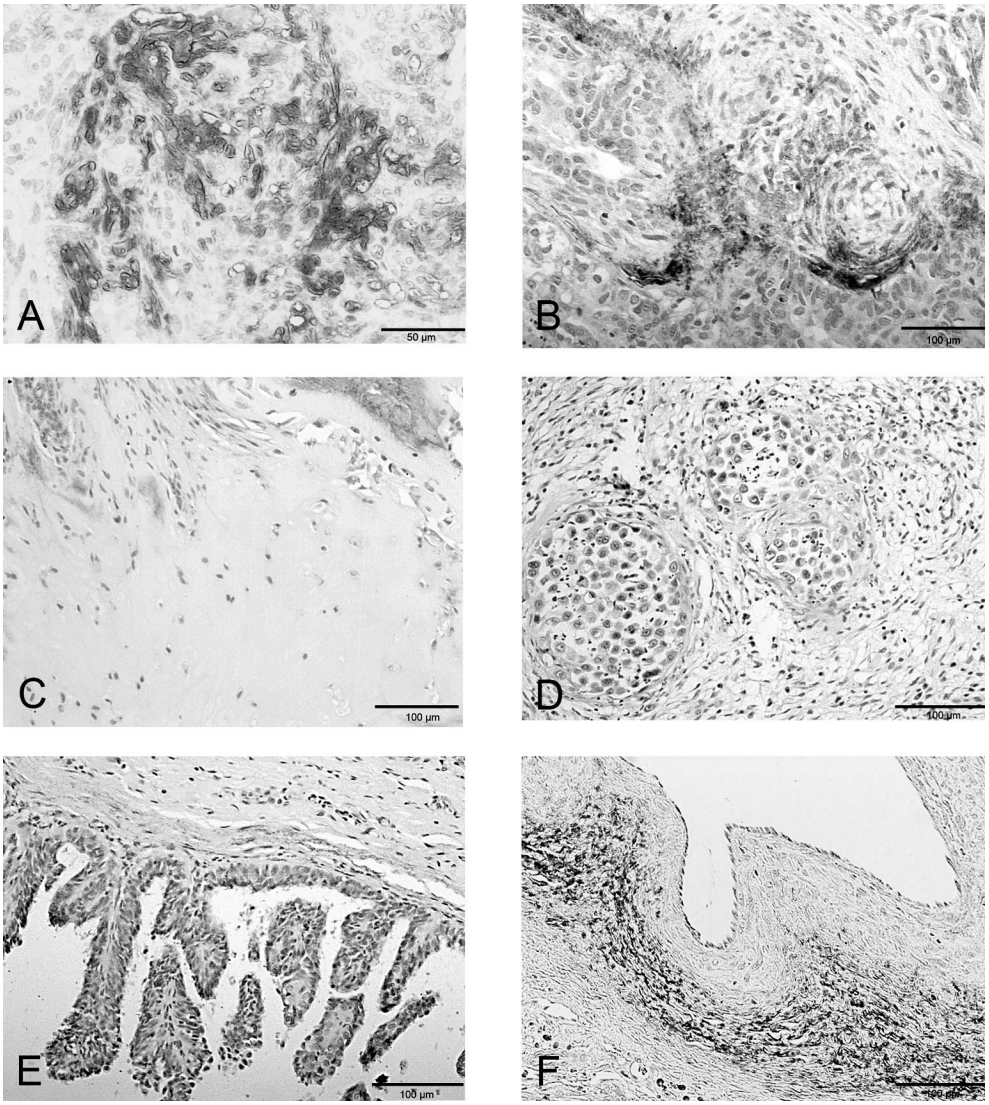


Figure 7. Immunolabelling of ADAMTS cleaved versican. Immunoreactivity to DPEAAE was intense and diffuse in the myxoid tissues (A) and moderate to intense, patchy or diffuse reactivity appeared in the ECM of myoepithelial-like spindle cell proliferations of complex and mixed tumors (B). Weak to moderate patchy immunoreactivity appeared in the ECM of early cartilage tissues; no reactivity could be seen in the more differentiated cartilage matrices or bone tissues (C). No reactivity occurred in the invasive zone of malignant tumors (D) or in he periductal tissues; only weak focal immunopositivity appeared in the periductal connective tissues of a complex adenoma (E). The wall of the intratumoral blood vessels was also often strongly immunopositive for DPEAAE (F).

Moderate to intense, patchy or diffuse reactivity often appeared also in the ECM of myoepithelial-like spindle cell proliferations of complex and mixed tumors (Figure 7B). In areas displaying histomorphological characteristics of early cartilage differentiation weak to moderate patchy immunoreactivity appeared in the ECM; however, no reactivity could be seen in the more differentiated cartilage matrices or bone tissues (Figure 7C). The described distribution of DPEAAE neoepitope is similar, although less extensive, to the versican distribution that we observed previously with the 12C5 antibody (G1 domain). However, in contrast to versican expression detected by 12C5 and 2B1 antibodies [5], no intense immunopositivity to DPEAAE occurred in the periductal tissues of any type of mammary tumors, nor in the invasive zone of malignant tumors (Figure 7D). Only weak and focal reactivity appeared in the periductal tissues in one of the complex adenomas (Figure 7E). In some of the tumors, both benign and malignant, moderate to intense reactivity could be observed in the fibrous tumor capsule. Interestingly, immunoreactivity appeared in myoepithelial cells surrounding small ductal structures of some of the tumor samples. The wall of the intratumoral blood vessels was also often immunopositive for DPEAAE (Figure 7F).

Western analysis- Tumor tissue extracts showed the presence of two major bands with a variable intensity at 70 kDa and approximately at 55 kDa in all the samples (Figure 8). The 70 kDa band appeared the most prominent in the complex-mixed tumors (Figure 8 lane 3-4) followed by myoepitheloid carcinoma (Figure 8 lane 6). The 55 kDa band was particularly intense in the anaplastic carcinoma (Figure 8 lane 7), and appeared as a strong band also in one of the simple adenomas (Figure 8 lane 2). A less prominent but distinct high molecular weight band approximately at 200-220 kDa appeared in the complex-mixed tumors (Figure 8 lane 5), and faintly in the myoepitheloid and anaplastic carcinoma. In addition a double band at 120-130 kDa could be seen in the anaplastic carcinoma (Figure 8 lane 7) and a low molecular weight band approximately at 40 kDa could be seen in the anaplastic carcinoma (Figure 8 lane 7) and in simple adenomas (Figure 8 lane 3-4). Moreover, the latter band appeared in most of the samples when 30 µg protein was electrophoresized and blotted instead of 10 µg. As a comparsion we also tested the cell culture media of the CMT-U309 cell line for the presence of ADAMTS truncated versican fragments. We found the same 70 kDa protein band (Figure 8 lane 9) as has been shown in the tumor tissues but none of the lower or higher molecular bands were seen in case of this cell line.

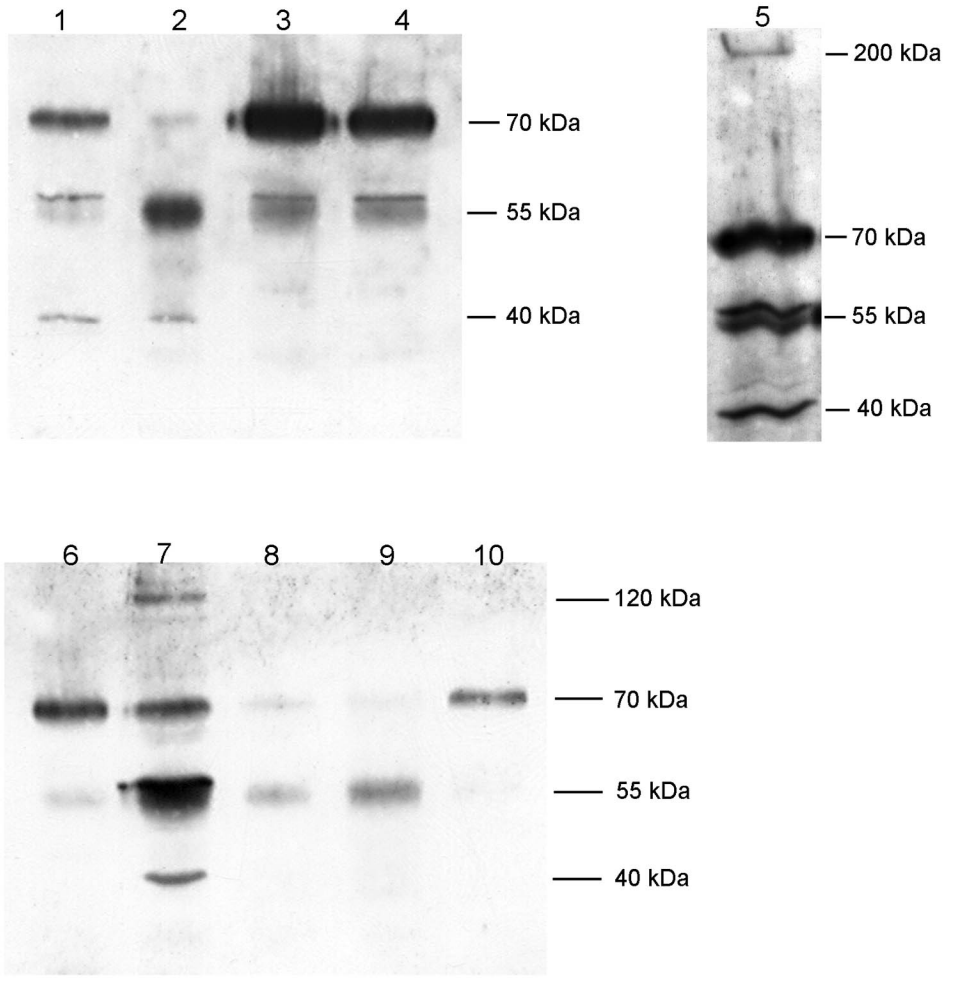


Figure 8. Western analysis of ADAMTS cleaved versican products in simple adenomas (lane 1-2); in complex and mixed tumors (lane 3-5); in myoepitheloid carcinoma (lane 6), anaplastic carcinoma (lane 7); in simple carcinomas (lane 8-9) and in the culture medium of the CMT-U309 cell line (lane 10).

In summary, these results show that versican is cleaved, at least partially, by ADAMTS proteases in canine mammary tumors and the truncated versican fragments are particularly abundant in complex and mixed tumors. The high molecular weight band at approximately 200-220 kDa is likely to correspond to

the truncated V0 versican protein and the 70 kDa band to the truncated V1 protein.[26] The bands that appeared at other molecular weights are likely to represent versican fragments generated by further N-terminal proteolytic cleavage of the 200-200kDa and 70 kDa ADAMTS-truncated versican products.

Discussion

We have recently described a marked production of versican in canine mammary tumors.[5] We now show that versican is present in multiple forms in canine mammary tumors and cell lines. In the following our present findings on the expression pattern of versican isoforms and on the presence of catabolic products are discussed separately.

Versican isoforms- Versican has been identified in several tissues/organs and its expression has been associated with tissues where cells are metabolically active and proliferating.[43,44] It is highly expressed in a number of tumor types, such as breast-, prostate and ovarian cancer.[2-4] However, due to the unavailability of antibodies that would clearly distinguish the four main versican isoforms V0, V1, V2 and V3, there is currently limited information about their relative tissue distribution and only few studies quantified their precise expression levels. Moreover, as far as the authors are aware, this is the first study that identifies and analyzes the expression of versican mRNA splice variants in canine tissues. The work presented in this paper shows that canine mammary tumors and canine mammary cell lines express at both the transcript and protein levels the full length versican isoform V0, and express also the V1 variant containing the βGAG but not the αGAG region. Moreover, the relatively small and rare V3 variant, lacking both GAG subdomains, is also expressed at least at mRNA level, albeit in a much lower amount than the two large variants. The V2 isoform could not be demonstrated in any of the canine mammary tumors or cell lines.

The versican splice variants differ dramatically in length and in their glycosaminoglycan and oligosaccharide profile, moreover a tissue-, developmental stage-dependent expression has been demonstrated suggesting that each plays specific roles.[45,46] Characteristically, mesenchymal tissues/ fibroblastic cells are believed to express predominantly the V1 variant. The brain tissues of several species have been shown to be particularly rich in the V2 isoform after it has replaced versican V1, which is the principal expressed form

during prenatal brain development.[47] The V0 and especially the V3 variant are known to be expressed only in a limited number of tissues. In general, our results show a higher level of versican expression at both the mRNA and protein levels in the mammary tumor cell lines compared to the normal mammary fibroblasts supporting the importance of this proteoglycan in tumor cell biology. We also show that the expression levels of the three versican splice variants and their ratio within the different cell types and tumors showed heterogeneity. We found that the normal mammary fibroblasts express approximately 5-fold higher V1 transcript level over that of the V0 variant, whereas in the tumor cell lines and in the tumor tissues the expression level of V0 and V1 is rather similar. The only exception was the P95/165 spindle cell line that expressed an approximately 11-fold increase of V1 over V0. The predominance of V1 versican in the normal mammary fibroblasts is consistent with several other previous studies indicating the V1 isoform as the characteristic versican variant of the connective tissues/fibroblasts. Similarly this study showed that in the P95/168 spindle cell line most of the cells display a fibroblast-like phenotype. Therefore, the relative overexpression of V1 variant over that of the V0 may be related to the predominantely fibroblast-like phenotype of this cell line. However, based on the findings of this study it seems that in the canine mammary tumor cell lines and in particular in the tumor tissues there is a relative overexpression of V0 mRNA. This is consistent with findings of others showing the V1 variant as the most widespread versican variant in the normal adult tissues, whereas the expression of V0 variant is only very limited or not expressed in normal tissues.[46] Moreover, we found that the highest V0/V1 ratio among all the tumors appeared in the anaplastic carcinoma that gave metastases to several distant organs. Since the V0 isoform carries the highest number of negatively charged CS side chains, this variant has the highest hydrophilic nature; therefore it is likely to contribute most efficiently to the increased viscoelastic properties of the tumor ECM. This highly hydrated ECM surrounding the tumor cells provides an anti-adhesive environment which is optimal for cell shape changes necessary for tumor cell proliferation, migration and also (de)differentiation.

Although versican was originally identified as the fibroblast CSPG [48], it has been shown that arterial smooth muscle cells (ASMCs) secrete much more of this molecule in culture.[49] Here we show that among the canine mammary tumor cell lines tested, including cell lines with epithelial, mesenchymal and myoepithelial phenotype, the myoepithelial-like spindle cells express the highest level of

versican, followed by the myoepithelial/fibroblast-like cell line. The lowest expression appeared in the malignant epithelial and malignant osteosarcoma cell line. In line with these findings we detected the highest level of versican expression, particularly that of the V1 and V0 variants, in complex adenomas that are characterized by the proliferation of myoepithelial-like spindle cells and ECM rich myxoid matrices. These data confirm also our previous immunohistochemical findings in canine mammary tumors showing the most intense and widespread immunoreactivity to versican in myoepithelial-like spindle cell proliferations and myxoid tissues. Moreover, in accordance with findings in human breast and prostate cancer [3,50], we have previously shown that in simple carcinomas versican immunoreactivity is mostly rescricted to the connective tissues at the invasion zone and the malignant epithelial cells are mostly negative.[5] Therefore, the relatively low expression of versican variants in the SH15 carcinoma cell line compared to the myoepithelial cell lines is not surprising. In accordance with the literature showing no or low levels of versican expression in cartilage and bone tissues [51,52] , we have previously demonstrated that versican expression was decreased in the differentiating cartilage tissues of the cartilaginous mammary mixed tumors. This may explain our current finding that the CMT-8 osteosarcoma cell line expresses relatively low amounts of versican variants.

Another interesting finding of this study was the relative overexpression of V3 variant in the P95/168 malignant spindle cells (isolated from a cartilaginous-osseous malignant mixed tumor) and although to a lesser extent, in the two myoepithelial-like spindle cell lines CMT-U304 and CMT-U309. Consistent with these findings, the benign mixed tumor containing a cartilaginous ECM showed the highest relative increase in V3 mRNA, and the complex adenomas and myoepitheloid carcinoma also expressed high levels of V3 transcripts. We previously suggested that versican may play a role in ectopic cartilage formation of canine mammary mixed tumors and that the cartilage originates from the myoepithelial-like spindle cells in these tumors.[24] During this process the myoepithelial-like spindle cells undergo an epithelial to mesenchymal transition. As the cells differentiate into a chondroid phenotype they downregulate their cytokeratin and smooth muscle actin expression. Taken our present and previous findings together it is tempting to speculate that the high expression of versican V3 is associated with cells showing at least partial myoepithelial phenotype (chondroprogenitor cells) and may have a specific importance in ectopic cartilage development in canine mammary tumors. This is in line with previous reports

showing versican as one of the major matrix molecules in prechondrogenesis [51,53] promoting chondrocyte proliferation.[54]

When comparing cell lines with the same tumor type of origin (e.g. CMT-U309 compared to CMT-U304) the cells with a higher growing capacity express higher levels of versican transcripts. However, taken all the observations together, an important message of this study is that the level of versican (splice variant) expression seems to depend not only on tumor behaviour, but also on the tissue/cell type of origin of the tumor. There are conflicting observations in the literature on the strength of increased versican expression as a prognostic factor in different types of tumors. [3,4,50] As it has been previously suggested the variation in the results might be influenced by the different methods applied (e.g. different antibodies).[4] However, it is more likely that each of the splice variants and catabolic products of versican (see below) exerts, at least partially, different or even opposite influences on cell behaviour. On the other hand, cells with different tissue of origin or different phenotypes might be differentially regulated by the same versican variant or versican fragment. Therefore, our present findings strongly suggest that caution is necessary when one evaluates the biological significance of overall tumoral versican levels. Instead, precise detection of expression of all types of versican forms should be performed.

Catabolic products- Despite the relatively well described degradation pathways of aggrecan [55], little is known regarding versican turnover. The ADAMTS are a relatively newly described branch of the metzincin family of proteases that contain metalloproteinase, disintegrin, and thrombospondin motifs. They have been implicated in various cellular events, including cleavage of proteoglycans and extracellular matrix degradation.[25-27,56] It is known that ADAMTS-1, -4, -5 and -9 are capable of cleaving versican.[25-27] In fact, ADAMTS-1 and -4 have been recenly shown to cleave V0/V1 versican in the GAGß domain in vivo. The 70 kDa N-terminal fragment of V1, which represents the G1-domain (G1-DPEAAE^{441}), has been identified from extracts of aorta intima as the in vivo cleavage product by ADAMTS-4. In addition, a low abundance 220 kDa protein has been detected and indicated as the corresponding cleavage product of V0 isoform.[26] Furthermore, the cleavage product of V1 versican by ADAMTS-1 in the expanded cumulus oocyte matrix during ovulation and an extensive degradation of versican by ADAMTS in the bovine tendon has been also reported.[57,58] However, as far as the authors are aware, there is no available literature on ADAMTS generated versican degradation in tumors.

Previously we described a difference in the expression pattern of the 12C5 (G1 domain) and 2B1 epitope (G3 domain) of versican in the ECM-rich myxoid tissues of complex and mixed tumors. The intense and diffuse pattern of 12C5 immunoreactivity in contrast to the patchy or absent immunoreactivity to 2B1 and to CS indicated a proteolytic cleavage of the versican core protein.[24] Here we show the presence of several in vivo versican fragments in canine mammary tumors, among which we identified the ADAMTS generated truncated form of V0 (approximately 200-220 kDa) and V1 (70 kDa). The DPEAAE- immunoreactive bands smaller than 70 kDa can be explained by further proteolysis of the N-terminal products from the N-terminus. The truncated (DPEAAE-immunoreactive) versican fragments contain the G1 domain but not the G3 domain and they are most abundant in the myxoid tissues of complex and mixed tumors. Therefore, this finding compliments our previous suggestion that due to proteolysis of the core protein the G1 domain accumulates in the myxoid tissues of complex and mixed tumor. The versican V1/V0 Neo antibody did not react with any other of the 12C5-, or 2B1-reactive fragments that migrated approximately between 90-320 kDa, suggesting that they are generated by cleavage at other sites than the characteristic Glu^{441}-Ala^{442}site in V1 and Glu^{1428}-Ala^{1429} in V0 for ADAMTS-1/4. Therefore, the presence of a series of versican fragments in the wide range of 70-320 kDa and the apparent relative paucity of full-length versican in the tumor extracts, particularly that of the V0 isoform, suggest an excessive in vivo proteolysis at multiple sites possibly by different proteases. A cleavage site of V0 versican at the Glu^{405}-Gln^{406} in the GAGα region has already been shown in the human brain and the ability of MMP-2 (gelatinase-A), MMP-7 (matrilysin) [59] and MMP-3 (stromelysin-1) [60] to cleave versican has been also demonstrated. Identification of potential proteases other than members of the ADAMTS family that may actively cleave versican in vivo and the elucidation of the potential bioactivity of the generated fragments in canine mammary tumors awaits further studies. Furthermore, which ADAMTS are responsible of versican degradation in canine mammary tumors needs to be further investigated. In our opinion, the ADAMTS-4 is the most likely candiate since it is upregulated in human breast cancer, whereas ADAMTS-1, -5, and -9 are down-regulated [61], and seems to cleave versican more actively than ADAMTS-1.[26]

We show that all types of mammary tumors and the culture media of all the tumor cell lines contain several similar versican fragments but with different abundance. We found the least versican degradation in simple adenomas and in

the benign epithelial cell line (CMT-3) and a more extensive degradation in complex-mixed and simple carcinomas in line with the intense and hight number of versican fragments in the culture media of the myoepithelial-like and malignant epithelial cell lines. These findings suggest regulated versican degradation and support a biological role for the truncated versican forms in canine mammary tumors. Similarly, a developmental regulation of the proteolytic cleavage of versican in the human skin [61] and proteolytic cleavage of neurocan in the rat cerebrum have been described [62].

In conclusion, we proposed that the different hydrophilic properties of the splice variants V1, V0 and probably V3 and the high degree of versican degradation together contribute to the formation of the highly versatile extracellular environment in the canine mammary tumors. This matrix has a high capacity of altering its viscoelastic nature thus providing the tumor cells with the proper environment for their active proliferation, migration and differentiation. Importantly, we showed that besides the expression of versican splice variants, considerable versican cleavage occurs in canine mammary tumors by ADAMTS proteases and that these versican fragments are most abundant in the precartilagenous myxoid tissues of complex and mixed mammary tumors. We suggest that the proteolytic fragments of versican generated in tumor tissues may posses similarly important and distinct biological roles in tumor cell-matrix interactions as its splice variants. Therefore, in our opinion, besides determining the precise expression pattern of versican isoforms in tumors, it is becoming important to investigate the presence of versican fragments also in human breast cancer and in other tumor types. Future investigations are warranted to explore the pathways involved in versican catabolism and the biological significance of the resultant versican fragments to better understand the importance and complex role of this PG in tumor biology.

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CHAPTER 5

Localization and activity of matrix metalloproteinases and cathepsin B in canine mammary tumors

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In preparation

Abstract

Matrix metalloproteinases and cathepsin B have been implicated in matrix degradation, including proteoglycan cleavage, during tumor development. We previously reported an extensive degradation of versican, a large chondroitin-sulfate proteoglycan, in canine mammary tumors. In this study, the localization (in 52 mammary tissues) and activity (in 18 mammary tissues) of MMP-2, -3, -9, -11, 13 and cathepsin B, potential candidates to cleave versican, was determined by multiple methods in normal and neoplastic canine mammary tissues.

Immunohistochemistry showed that all MMPs were present in both the tumor cells and stromal fibroblasts and indicated enhanced expression of MMPs in tumors compared to the normal mammary tissues. Zymography and quenched fluorescent substrate hydrolysis confirmed an increased amounts and activity of MMP and cathepsin B in tumors. There was also a trend for increased protease activity particularly that of MMP-2, MMP-13 and cathepsin B, in simple carcinomas compared to simple adenomas. Furthermore, *in situ* zymography showed that gelatinases exert their activity primarily in the tumor stroma, particularly surrounding ductal structures, rather than in the tumor cells themselves.

In conclusion, we show that tumor tissues display enhanced *in vivo* activity of MMP and cathepsin B, confirming the role of these proteases in tumor progression. The results indicate that gelatinases play an important role in basement membrane degradation in tumors, thus in the conversion of *in situ* carcinomas to invasive lesions. Future studies are necessary to identify which of these active proteases may contribute to versican degradation in canine mammary tumors.

Introduction

The active role of the extracellular matrix (ECM) in many developmental and pathological processes has been recognized. The ECM is a biological reservoir of a variety of cell binding proteins and growth factors that affect cell behavior. In tumors it is also substantially modified by proteases produced by tumor cells, stroma cells and inflammatory cells. ECM remodeling/degradation is, to a large extent, mediated by matrix metalloproteinases (MMPs), a large family of calcium-dependent, zinc-containing endopeptidases that are structurally and functionally related.[1] MMPs are classified into subgroups of collagenases (MMP-1, MMP-8 and MMP-13), gelatinases (MMP-2, MMP-9), stromelysins (MMP-3, MMP-10, MMP-12), matrilysins (MMP-7, MMP-26), membrane-type MMPs (MMP-14, MMP-15, MMP-16, MMP-17) and others (MMP-11, MMP-19, MMP-20). These enzymes are capable of cleaving and digesting various extracellular proteins, but have also other substrates such as cell-cell and cell-matrix adhesion molecules, cytokines and growth factor receptors, which may also promote the tumor cell proliferation and invasion process.[2] The degradation of ECM molecules and cell adhesion molecules may release signaling sequences from these ECM molecules by the proteolytic action that in turn may modulate cell behavior (for review see reference 3). MMPs are secreted in an inactive (latent) form. These latent MMPs require an activation step before they are able to cleave extracellular matrix (ECM) components.[1] Furthermore, the activity of MMPs is regulated by several types of inhibitors, of which the tissue inhibitors of metalloproteinases (TIMPs) are the most important.[4] The balance between MMPs and TIMPs is largely responsible for the control of degradation of ECM proteins.[5] Disruption of this balance is a characteristic of diverse pathological conditions involving extensive tissue degradation, such as tumor progression.[6,7] However, many different MMPs are involved also in normal tissue remodeling and resorption. In almost all human cancers, the MMP expression and activity are increased. The increased levels of MMPs seem to correlate with invasiveness and poor prognosis.[8] To prevent tissue degradation by a disturbed MMP-TIMP balance, it is important to know which MMPs and TIMPs are involved in the development of specific diseases. Thus, the analysis of MMPs in biological samples can contribute to the characterization of certain diseases involving tissue destruction and possibly to the development of new therapies.

Little is known about the expression and *in vivo* activity of MMPs and other ECM degrading enzymes in canine tumors, such as mammary tumors. Besides MMPs the cysteine proteases, e.g. cathepsin B, have been shown to degrade extracellular proteins, including proteoglycans.[9] Similarly to several MMPs the expression and activity of cathepsin B is increased in many different tumors, such as in human breast cancer, and it might be involved in the development, invasion and metastasis of a number of tumor types (for recent review, see reference 10). We previously demonstrated that versican, a large extracellular chondroitin-sulfate proteoglycan, is degraded *in vivo* in canine mammary tumors and versican fragments generated by ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) proteases have been identified.[11] However, the broad range of versican fragments detected in canine mammary tumors indicates multiple cleavage of this proteoglycan possibly by different proteases. Therefore, in this study we were particularly interested to identify and measure the activity of proteases, including several MMPs and cathepsin B, which potentially may contribute to versican degradation besides the ADAMTS proteases in canine mammary tumors.

The expression of MMPs can be analyzed with several techniques.[12-14] A widely used method is substrate zymography, which identifies MMPs by the degradation of their preferential substrate and by their molecular mass. Using this technique, one can determine whether the MMP is in an active or latent form. In this study we used gelatin zymography, which is a sensitive, quantifiable assay to analyze the pro-, and active forms of MMP-2 and MMP-9. Casein zymography was performed in an attempt to detect MMP-3 and MMP-7 (gelatinases can be also detected when present in high concentrations). To localize MMPs and their activites in tissue sections, immunohistochemistry and *in situ* zymography with quenched DQ-gelatin was performed. The actual MMP activity in the tumor tissues was finally measured by quenched fluorescent substrate assays. Cathepsin B activity was localized with an enzyme histochemical fluorescent method using 5-nitrosalicylaldehyde and measured by a quenched fluorescent substrate assay.

Materials and Methods

Tissues

2 normal mammary tissues and 16 mammary tumors were resected surgically at a private veterinary clinic in Utrecht (NL). A part of the tissues was fixed in 10% neutral-buffered formalin and a part of them was snap frozen in liquid nitrogen under RNase free conditions and stored at – 80°C before use. After 24 hours formalin fixation the tissues were embedded in paraffin, after which 5μm sections were cut and stained with haematoxylin and eosin (H&E). Tumors were classified from the H&E-stained sections according to the recent WHO classification.[15]

Samples included different types of mammary tumors, including simple adenomas and carcinomas, benign mixed tumors and myoepitheliomas. The 8 benign tumors were classified as *simple adenoma* (n=3) and *benign mixed tumor* (n=5). The 5 benign mixed tumors showed heterogeneity in their histological characteristics. Two of them consisted of large areas of myoepithelial-like spindle cell proliferations and myxoid tissues with abundant ECM and few small tumor cell foci displaying histomorphological characteristics of early cartilaginous differentiation. The other three benign mixed tumors contained large areas of well-differentiated ectopic cartilage and bone tissue. In two of the 16 surgically resected tumors the epithelial tumors cells expressed cytokeratin 14 and smooth muscle actin indicating a myoepithelial differentiation[16]; therefore, these tumors were classified as *epitheloid myoepitheliomas (n=2)*. Both of these tumors were well-circumscribed but showed atypia at cellular level and early stages of stromal invasion. All the malignant tumors were classified as *simple carcinomas (n=6)*; however, they showed some heterogeneity in their histomorphology, degree of malignancy and in metastatic characteristics. One of the simple carcinomas was a myoepitheloid carcinoma (invasive solid carcinoma characterized by cytokeratin 14 and smooth muscle actin expressing epithelial tumor cells)[11] and one of them was an anaplastic carcinoma with metastases to lymph nodes and several organs. Two of the six simple carcinomas showed solid and/or tubular growth pattern with invasive growth into the tumor stroma and two of them displayed invasive tubular growth accompanied by focal proliferation of myoepithelial-like spindle cells. None of the latter 4 tumors gave metastasis to lymph nodes or organs.

For immunohistochemical analysis an additional 35 mammary tissues were obtained from the archives of necropsies of the Department of Veterinary Pathology, Utrecht University. The histological type and number of all the tissues examined are summarized in Table 1. The latest WHO classification describes the benign myoepithelioma as a subgroup of simple adenomas. However, due to their different MMP expression and activity, we discuss these tumors as a different subgroup of benign tumors. Furthermore, the mixed tumors with malignant epithelial components are described as malignant mixed tumors.

Table 1. Histological classification of mammary tissues tested

HISTOLOGY	NUMBER OF SAMPLES
Normal mammary tissues	5
Benign tumors (n=28)	
Simple adenoma	
Tubular/tubulopapillary	5
Myoepithelioma	5
Complex adenoma	3
Benign mixed tumor	15
Malignant tumors (n=19)	
Simple carcinoma (n=10)	
Tubular/Tubulopapillary	5
Solid	3
Anaplastic	2
Malignant myoepithelioma	4
Complex carcinoma	2
Mixed tumor with malignant epithelium	3

WHO classification [15]

Immunohistochemistry

Immunohistochemistry was performed using an indirect immunoperoxidase staining procedure, applying the streptavidin-biotin based technique (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA), as described previously.[16,17] Prior to the incubation with antibodies for MMP-3, -7, -9 a ten minute microwave

heating step was performed in 0.01M citrate buffer (pH 6.0). Sections were incubated overnight at 4°C with the primary mouse monoclonal antibodies (Oncogene, San Diego, CA; 1:50) that recognize both the latent and active forms of MMP-2 (clone 75-7F7), MMP-9 (clone IA5), MMP-3 (clone SL-1 IIIC4), MMP-11 (clone SL3-05) and MMP-13 (clone 181-15A12). As negative control, Mouse IgG1 serum (X0931, DAKO) was employed for the reaction instead of primary antibodies in the same protein dilution as the monoclonal primary antibodies.
Sections were analyzed by two experienced observers simultaneously (IE and HN) for staining intensity and distribution of immunoreactivity (f- focal reactivity; d-diffuse reactivity) within the tissues. The intensity of staining was graded using an empirical semi-quantitative system: (–) no reaction; (+) weak; (++) moderate; (+++) intense reaction.

Gelatin and Casein Zymography

Zymography was performed as described previously.[12,19] Total protein concentration of each homogenized sample (10 mg of tissues wet weight) was determined (Pierce, BSA protein assay) and a volume of samples corresponding to 20 µg protein (gelatin zymography) and 100 µg protein (casein zymography) were electrophoresed on a 10% precast zymogram gel (containing 0.1% (w/v) of either gelatin or casein as substrates) together with prestained SDS-PAGE Standards (BioRad). To control that the gelatinolytic bands appeared due to MMP activity, control gels were incubated and washed with the same buffers with 50 mM EDTA.
Densitometric and statistical analysis of gelatin zymograms*- The electrophoresed gelatin zymogram gels were photographed with ImageMaster®VDS (Pharmacia Biotech) connected to LISCAP Capture Application Software (Version 1.0). Image analysis of the detected bands was performed using Molecular Analyst® PC Software (Bio-Rad Laboratories). All data of two measurements of the gelatinolytic activity of pro-, and active forms of MMP2 and MMP-9 were assessed by analysis of variance (one-way ANOVA) followed by Bonferroni's multiple comparison tests. The level of significance was set as $p<0.05$.

In situ zymography/enzyme histochemistry with quenched fluorogenic DQ-gelatin/substrate

In situ gelatinolytic activity was demonstrated in unfixed cryostat sections (4µm thick) from mammary tissues using DQ-gelatin as a substrate (ENZ-Chek;

Molecular Probes, Eugene, OE) as described previously.[19] DQ-gelatin was dissolved in a concentration of 1 mg/ml in distilled H_20 and then diluted (1:10) in 1% (w/v) low gelling temperature agarose (Sigma Chemical Co., St. Louis, MO, USA) in PBS containing DAPI (1.0 µg/ml) to counterstain nuclei. 50 µl of the mixture was applied on top of the air-dried tissue sections and covered with coverslips. After gelling of the agar at 4°C, the incubation was performed for 1 hr at room temperature (RT). Fluorescence of FITC was detected with excitation at 460-500 nm and emission at 512-542 nm. DAPI was detected with excitation at 340-380 nm and emission at 425-∞ nm. To control that the gelatinolytic activity corresponds to MMP activity control sections were preincubated with 20 mM EDTA for 1hour at RT and negative control slides were prepared without DQ-gelatin as substrate. Furthermore, to compare the localization of gelatinolytic activity and MMP-2 and MMP-9 protein, immunohistochemistry was performed on serial cryosections with monoclonal antibodies for MMP-2 (clone 75-7F7) and MMP-9 (clone IA5).

In situ cathepsin B activity was demonstrated by enzyme histochemistry as described previously.[20-21] Unfixed cryosections were air-dried (5-10 min, at RT) and subsequently covered with 50 µl of incubation medium [100 mM PBS, (pH 6.0), 1.3 mM EDTA, 1 mM dithiothreitol, 2.67 mM L-cysteine, 1 mM 2-hydroxy-5-nitrobenzaldehyde as coupling agent] and 1 mg/ml N-CBZ-Ala-Arg-Arg-4-methoxy-2-naphthylamide (Bachem, Bubendorf, Switzerland) as substrate. The specificity of the reaction was verified by incubation in the absence of the substrate. The incubation was performed at constant RT on the stage of a fluorescent microscope and images were taken as soon as fluorescence emission appeared (usually after 5-10 minutes). Fluorescence of the final reaction product was detected with excitation at 460-500 nm and emission at 512-542 nm.

Quenched fluorescent substrate assay

This technique apply quenched fluorescent substrates specific for a certain enzyme (e.g. MMP or groups of MMPs). In general, the activated enzyme (MMP) cleaves the substrate at a specific sequence, releasing the fluorescent group e.g. Mca or Dnp from the internal quenching group e.g. Dpa. After homogenization the total protein concentration of each sample was determined with BSA protein assay (Pierce). The enzyme activity was determined by incubating 10 µl tissue sample lysate (corresponding to 100 and 200 µg protein) with 40 µl mixture of the assay buffer (in case of the MMP substrates 50 mM Tis-HCl pH 7.6, 150 mM NaCl,

0.01% Triton X-100, 5 mM $CaCl_2$, 1μM $ZnCl_2$; in case of the cathepsin B substrate 100mM PBS pH 5.7, 1mM dithithreitol, 2.67 mM L-cysteine, 1.3 mM EDTA) with the substrate (0.1 μg/μl final concentration). From each snap frozen tissue two separate homogenates were prepared from two different areas. The enzyme activity was measured in duplicate of each homogenate. The sequences and sources of the substrates are shown in Table 2.

Table 2. Quenched fluorescent substrate assay. Description of substrate sequences and sources

ENZYME	SUBSTRATE E	SOURCE
MMP-2	MCA-Pro-Leu-Ala-Nva-Dpa-Ala-Arg-NH_2	AnaSpec
MMP-3	DNP-Pro-Tyr-Ala-Tyr-Trp-Met-Arg-OH	AnaSpec
MMP-7	DNP-Arg-Pro-Leu-Ala-Leu-Trp-Arg-Ser-OH	AnaSpec
MMP-9/MMP-1	DNP-Pro-ß-cyclohexyl-Ala-Gly-Cys(Me)-His-Ala-Lys(N-Me-Abz)-NH_2	Bachem
MMP-11/MMP-14	MCA-Pro-Leu-Ala-Cys(Mob)-Trp-Ala-Arg-Dap(Dnp)-NH_2	Bachem
MMP-13	MCA-Pro-Cha-Gly-Nva-His-Ala-Dpa-NH_2	AnaSpec
Cathepsin B,L,S	N-CB2-L alanyl-L argynil-L arginine-4 methoxy-ßnaphtylamide acetate 2 ½ H_20)	Bachem

The samples were incubated for 1 hour at RT and the enzyme activity was determined with a fluorimeter (WallacVictor2; 300sec/per plate; 20 repeat) running the Wallac 1420 Manager, Workout software (λ_{ex} 355 nm and λ_{em} 430 nm). In case of the MMP-9/MMP-1 substrate additional samples were incubated for 1 hour at RT with the gelatinase inhibitor H-Cys-Thr-Thr-His-Trp-Gly-Phe-Thr-Leu-Cys-OH (Bachem; 1 μg/μl final concentration) in addition to the substrate. This allowed us to calculate the activity of MMP-9 in the samples by subtracting the values measured after inhibition from the total activity. In the cathepsin (B, L, S) assay the cysteine protease inhibitor E64 (trans-epoxy, succinyl-L leucylamido-/4-guanidino/-butane; Sigma) and the cathepsin B inhibitor CAO74 (L-trans-epoxysuccinyl-Ile-Pro-OH propylamide; Bachem) was added to additional samples at a 10 μM final concentration to control the specificity of cathepsin B activity.

Table 3. Immunoreactivity to MMPs in canine mammary tissues

TUMOR TYPE	MMP-2	MMP-9	MMP-3	MMP-11	MMP-7	MMP-13
Normal mammary tissue						
Luminal epithelial cells	+f	+/++f	+/++f	+/++f-d	++f-d	+/++f
Resting myoepithelial cells	++/+++f	+/++f	+f	++f	++f	+/++f
Stroma						
fibroblasts	++/+++f	+/++f	+f	++f	++f	+/++f
ECM	-	-	-	-	-	-
Simple adenoma						
Neoplastic epithelial cells	+/++f	+/+++f	+/++f	+/+++f	+/+++d	+/++f
Stroma						
fibroblasts	+/++f	+/+++f	+f	+/+++f	+/+++f	+/++f
ECM	+f	+/++f	-	+/++f	+/++f	-
Complex adenoma/ benign mixed tumor						
Epithelial tumor cells	+/+++f	+/+++d	+/+++f	++/+++d	++/+++d	+f
Myoepithelial-like spindle cells	++/+++f	++/+++f	+/++f	++/+++f	++/+++f	++/+++f
Myxoid tissues						
spindle cells	+/+++f	+/+++f	+/+++f	++/+++d	++/+++d	+/++f
ECM	+/++f	+/++f	+/++f	+/++f	+/++f	+/++f
Early cartilage tissues						
tumor cells	+/++f	+/++f	++/+++f	++/+++f	++/+++f	++/+++f
ECM	+/++f	+/++f	+/++f	+/++f	+/++f	+/++f
Cartilage						
chondroblasts	+/++f	+/++f	+/++f	+/++f	+/++f	+/++f
ECM	-	-	-	-	-	-
Bone						
Osteoclasts	+++d	+++d	-	+++d	+++d	+++d
Osteoblasts	++/+++d	++/+++d	-	++/+++d	++/+++d	++/+++d
ECM	-	-	-	-	-	-
Stroma						
fibroblasts	+/+++d	++/+++d	+f	++/+++d	++/+++d	+/+++d
ECM	+/++f	+/+++f	-	+/++f	+/++f	+/++f

Table 3. continued

TUMOR TYPE	MMP-2	MMP-9	MMP-3	MMP-11	MMP-7	MMP-13
Myoepithelioma						
Tumor cells						
Epitheloid						
Well-differentiated	+/++d	+/++d	+f	++/+++d	++d	+/++d
Poorly differentiated	+++d	++/+++d	++d	++/+++d	+++d	+++d
Spindle						
Well-differentiated	+/+++f-d	+/+++f-d	+/+++f	++/+++d	+/+++f-d	+/+++d
Poorly differentiated	+++d	++/+++d	+/+++f	+++d	+/+++d	+++d
Stroma						
fibroblasts	+/+++f-d	+/+++f-d	+f	++/+++d	++/+++d	++/+++d
ECM	+/+++f	+/+++f	-	+f	+f	+f
Simple carcinoma						
Tumor cells	+/+++f-d	+/+++f-d	+/++f	+++d	+/+++d	+/++f
Stroma						
fibroblasts	+/+++f-d	+/+++f-d	++f	+++d	+/+++d	+/+++f-d
ECM	+/++f	+/++f	-	-	-	-

– no staining; + weak staining; ++ moderate staining; +++ intense staining; f-focal staining/ only certain percentage of the cells are stained); d-diffuse staining/ all of the cells are stained

*Statistical analysis**- All data of two measurements of each enzyme activity in the different histological groups were assessed by one-way ANOVA followed by Bonferroni's multiple comparison tests. The level of significance was set as $p<0.05$.

* We acknowledge that due to the low sample number in some of the groups, the homogeneity of variance could not be evaluated in the statistical analysis, thus our uses of statistical tests that assume homogeneity of variances may results in more false positive results than would normally be expected. Therefore, the results must be confirmed by analyzing larger groups in the future. However, this study used a combination of relevant techniques (immunohistochemistry, zymography, quenched fluorescent substrate assay) that increase the validity of the presented data.

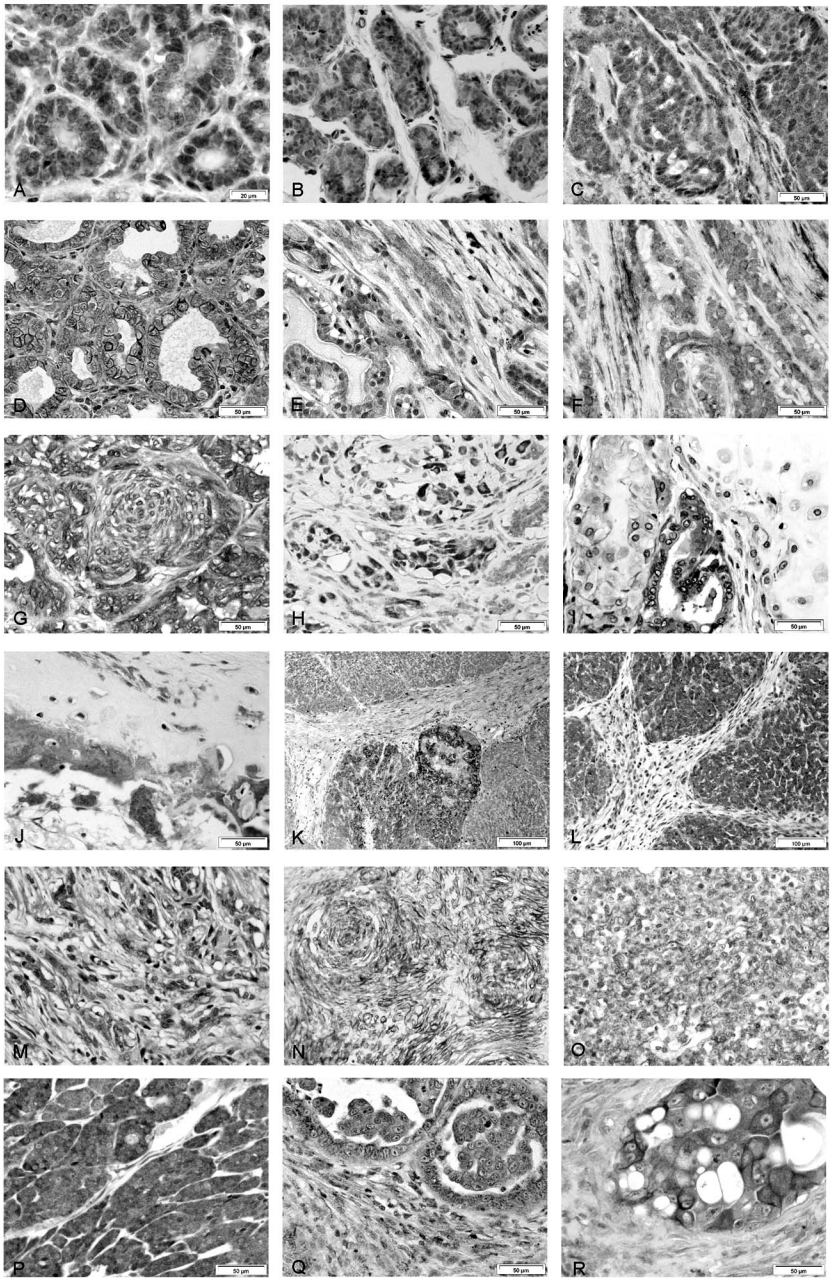
Results

Localization and activity of MMP-2 and MMP-9

Immunohistochemistry- The results of immunohistochemical analysis are shown in Table 3.

The *normal mammary tissues* displayed an uneven immunoreactivity to gelatinases. In some lobules all or most of the luminal epithelial cells displayed weak, fine granular reactivity to MMP-2 and weak to moderate reactivity to MMP-9 (Figure 1A), whereas other lobules did not show any reactivity.

Figure 1. *Localization of gelatinases (MMP-2 and MMP-9) detected by immunohistochemisty.*
Weak to moderate reactivity to MMP-9 in the luminal epithelial cells of *normal mammary tissues* (A) and prominent staining for MMP-2 in the myoepithelial cells surrounding acini (B). Immunoreactivity to MMP-2 (C) and MMP-9 (D) in the epithelial tumor cells and stromal fibroblasts in *simple adenomas*. In c*omplex/mixed tumors* reactivity to gelatinases appeared in the epithelial tumor cells and stromal tissues (E: MMP-2; F: MMP-9), in the myoepithelial-like spindle cell proliferations (G: MMP-2), in the myxoid tissues (H: MMP-9), in the early cartilagenous foci and at the peripheral parts of the ectopic cartilage (I: MMP-2), in the osteoclasts and osteoblast-like tumor cells of the bone tissues in mixed tumors (J: MMP-2). Intense MMP-2 reactivity in the atypical, polymorph tumor cells in *epitheloid myoepitheliomas* (K) and moderate MMP-9 reactivity throughout the tumor tissue (L). Weak to intense immunoreactivity to MMP-2 in a *spindle cell myoepithelioma* (M). Intense immunoreactivity to MMP-2 in the malignant spindle cells (N) and undifferentiated tumor cells (O) of *malignant myoepitheliomas*. Intense MMP-9 reactivity in a s*imple carcinomas* (P) and granular apical MMP-9 reactivity in the cytoplasm of the luminal epithelial tumor cells (Q). Intense reactivity to MMP-2 in the invading and highly undifferentiated tumor cells in a simple carcinoma (R).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The resting myoepithelial cells surrounding epithelial structures and the stromal fibroblasts displayed moderate to intense immunoreactivity to MMP-2 (Figure 1B), and weak to moderate immunoreactivity to MMP-9. In general, the tumor tissues, including benign tumors (Figure 1C-D, *simple adenomas*) displayed a more intense and widespread immunoreactivity to gelatinases, both in the tumor cells and in the stroma. Reactivity to gelatinases appeared particularly intense and widespread in *complex and mixed tumors*, including the epithelial tumors cells (Figure 1E-F,) the myoepithelial-like spindle cell proliferations (Figure 1G) and myxoid tissues (Figure 1H). However, the reactivity in the myoepithelial-like spindle cells proliferations showed a relatively great heterogeneity.
Furthermore, immunoreactivity to both enzymes was seen in the undifferentiated tumor cells of early cartilagenous tissues (Figure 1I), in the peripheral chondroblast-like tumor cells of the more differentiated ectopic cartilage tissues (Figure 1I), in the osteoclasts and osteoblast-like tumor cells of the bone tissues in mixed tumors (Figure 1J) and throughout the tumor stroma (Figure 1E-F). In complex carcinomas and malignant mixed tumors the pattern of immunoreactivity was similar to their benign counterparts but it appeared more intense in the fibrous stroma. An interesting finding was that the less differentiated, polymorph tumor cells in *myoepitheliomas* displayed increased immunoreactivity to gelatinases compared to the more differentiated tumor cells (Figure 1K, M-O). In the epitheloid myoepitheliomas MMP-2 immunoreactivity appeared more intense in the tumor cells compared to that of MMP-9; however, MMP-9 reactivity showed a more even distribution (Figure 1L). In s*imple carcinomas* the immunoreactivity to gelatinases showed similar localization; however, it was more intense, particularly that of MMP-9 (Figure 1P), compared to their benign counterparts (simple adenomas). The luminal epithelial tumor cells often displayed a characteristic granular apical MMP-9 reactivity in the cytoplasm (Figure 1Q). Immunoreactivity to MMP-2 largely varied within the same tumor tissue and between the tumor samples. Similar to what was observed in myoepitheliomas, the highly undifferentiated, invading tumor cells consistently displayed intense immunoreactivity to gelatinases (Figure 1R). Moreover, immunoreactivity to gelatinases appeared also in the metastatic tumor cells in the regional lymph node (not shown). The wall of blood vessels and the reactive inflammatory cells in the stroma were moderately to intensely stained for gelatinases.

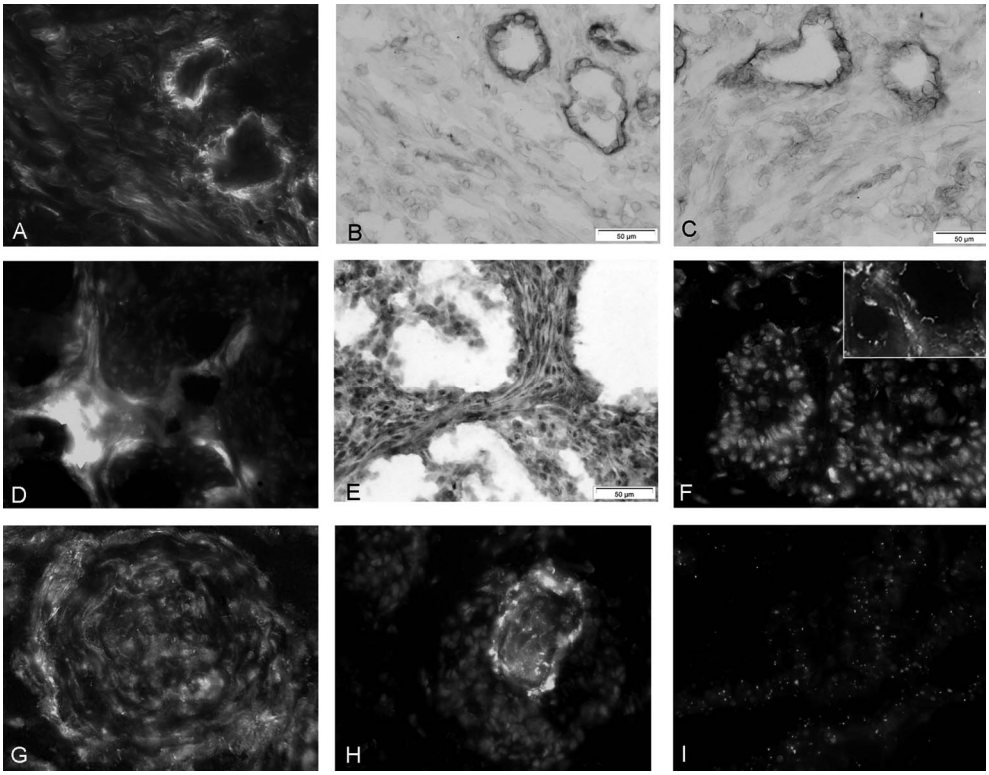

Figure 2. *In situ zymography and immunohistochemistry of gelatinases and in situ activity of cathepsin B.* In tumor tissues intense fluorescence (A) and immunoreactivity to MMP-2 (B) and MMP-9 (C) appeared surrounding ductal structures. Intense fluorescence (D) and immunoreactivity (E: MMP-9) was detected in the tumor stroma of epitheloid myoepithelioma. In few tumors fluorescence appeared in the cytoplasm of tumor cells (F), often in the apical cytoplasm (F inset). In complex/mixed tumors intense fluorescence could be seen also in the myoepithelial-like spindle cell proliferations (G) and myxoid ECM (H). Cathepsin B activity was detected in the cytoplasm of tumor cells and stromal fibroblasts in all the tumor tissues (I).

In situ zymography- Gelatinolytic activity was demonstrated in unfixed cryostat sections of the surgically resected mammary samples using DQ-gelatin as an overlay substrate. To confirm that the localization of gelatinolytic activity in the above areas corresponds to MMP-2 and/or MMP-9 activity immunohistochemistry for MMP-2 and MMP-9 was performed on serial cryostat sections. In the normal mammary tissue we could not detect any fluorescence. However, in both benign

and malignant tumors fluorescence was consistently localized in the intratumoral stroma and showed mostly a fibrillar appearance in the fibrous ECM. Intense fluorescence consistently appeared surrounding ductal structures, particularly surrounding invading clusters of tumor cells in malignant tumors (Figure 2A). In line with this finding, immunoreactivity to both MMP-2 and MMP-9 was intense surrounding the neoplastic ductal structures (Figure 2B-C). Interestingly, fluorescence in the tumor stroma was particularly bright in the epitheloid myoepithelioma (Figure 2D). In this tumor immunoreactivity to both MMP-2 and MMP-9 appeared in the fibrous ECM and in the tumor cells (see Figure 1K-L), but immunoreactivity to MMP-9 was particularly intense in some areas of the fibrous stroma (Figure 2E). In few of the tumor samples, both benign and malignant, fluorescence was also seen in the cytoplasm of tumor cells (Figure 2F), often in the apical cytoplasm (Figure 2F inset). In accordance with this finding, as already described and shown in Figure 1, immunoreactivity to both MMP-2 and MMP-9 could be detected in many of the tumor cells and particularly that of MMP-9 often displayed an apical localization in the cytoplasm. Furthermore, bright fluorescence appeared in peritumoral fibrous tissues of both benign and malignant tumors and immunoreactivity to both MMP-2 and MMP-9 could be detected in these areas (not shown). In general, among all the tumor types the complex and mixed tumors showed the most widespread fluorescence. In these tumors besides the periductal pattern moderate to intense fluorescence could be seen in the myoepithelial-like spindle cell proliferations (Figure 2G) and in the myxoid ECM (Figure 2H). As described above (see Immunohistochemistry, Figure 1G-H) both MMP-2 and MMP-9 immunoreactivity was present in these areas. EDTA, a general MMP inhibitor diminished fluorescence in all areas generated by the breakdown of the DQ-gelatin.

Gelatin zymography- Gelatin zymography identifies and separates the gelatinases MMP-2 and MMP-9 in both latent (pro) and active forms due to differences in their molecular mass. In the canine mammary tissue homogenates the following main four lysis bands were observed: 92 kDa corresponding to pro-MMP-9; 82 kDa active MMP-9; 72 kDa pro-MMP-2, and 64 kDa active MMP-2 (Figure 3). However, not all the above forms could be detected in all samples and the intensity of the bands varied between the different tumor types. Furthermore, faint high molecular weight bands approximately at 130 and 220 kDa were often detected in the tumor samples. These bands are likely to represent complexed forms of

MMP-2 and MMP- 9. Moreover, in some of the samples faint lysis bands appeared also below 50 kDa but were not further analyzed in this study. The result of densitometric analysis is shown in Figure 4. As mentioned above, the group of benign mixed tumors showed heterogeneity in their histological characteristics. We found considerable difference in the amount of pro-, and active forms of gelatinases within this group.

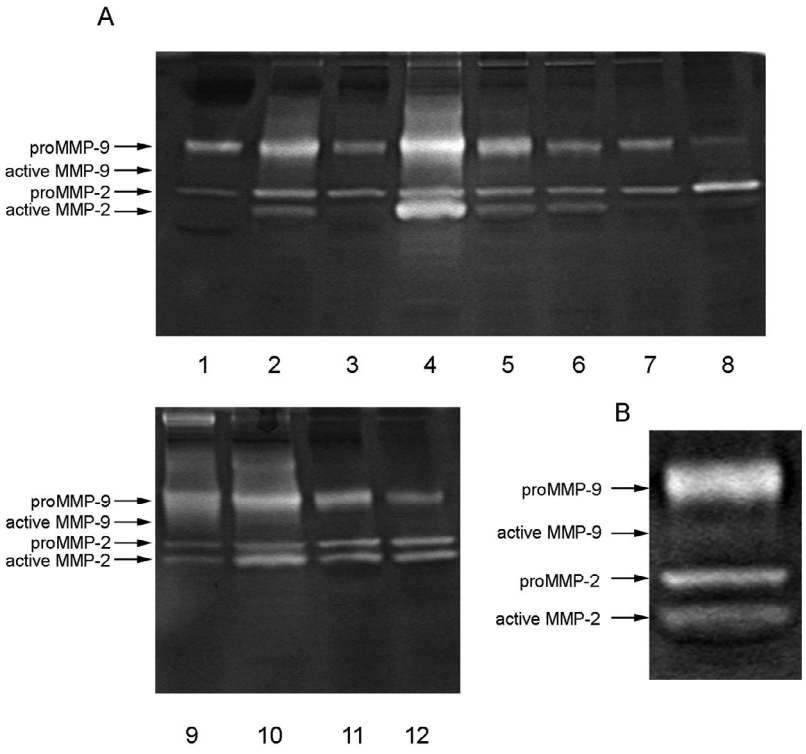

Figure 3. *Gelatin zymography* showed the presence of four main lysis bands (A-B), which appeared with variable intensity among the different tumor types, corresponding to pro-MMP-9 (92 kDa), active MMP-9 (82 kDa), pro-MMP-2 (72 kDa), and active MMP-2 (64 kDa). A lane 1: normal mammary tissue; lane 2-3: simple adenoma; lane 4-5: complex adenoma; lane 6: benign mixed tumor; lane 7-8: myoepitheliomas; lane 9-12: simple carcinoma.

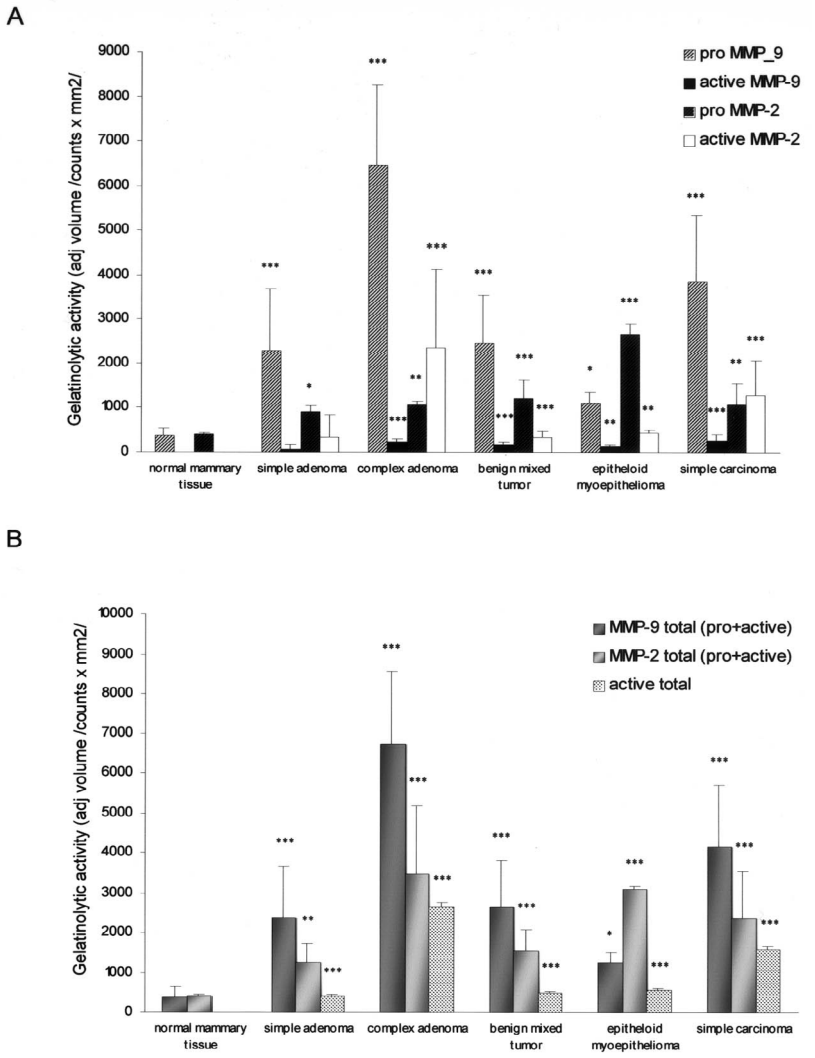

Figure 4. G*elatinase activity detected by gelatin zymography:* levels of pro-, and active forms (A) and levels of total MMP-2, -MMP-9 and total amounts of active gelatinases (B) in normal and neoplastic canine mammary tumors. There was considerable difference in the amount of pro-, and active forms of gelatinases within the main group of benign mixed tumors. Therefore, comparison is shown separately for the two subgroups, complex adenomas (large areas of spindle cell proliferations and myxoid tissues and few early cartilaginous foci) and benign mixed tumors (large areas of ectopic cartilage and/or bone). Comparisons were made by one-way ANOVA followed by Bonferroni's multiple comparison. Values are mean ± std. (*p<0.05, **p<0.01, ***p<0.001)

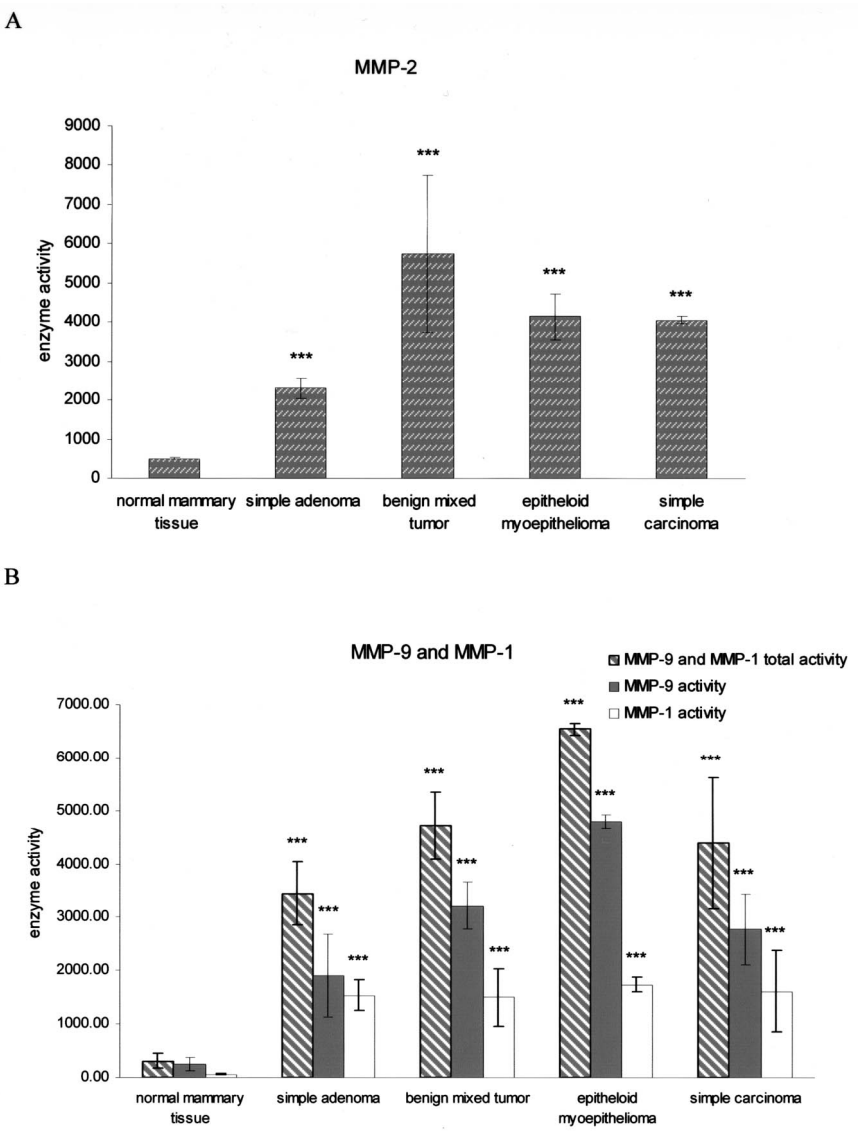

Figure 5. *In vivo activity of MMP-2 (A) and MMP-9/1(B) detected by quenched fluorescent substrate hydrolysis.* Data were analyzed by one-way ANOVA with Bonferroni's multiple comparison. Values are mean ± std. (***p<0.001)

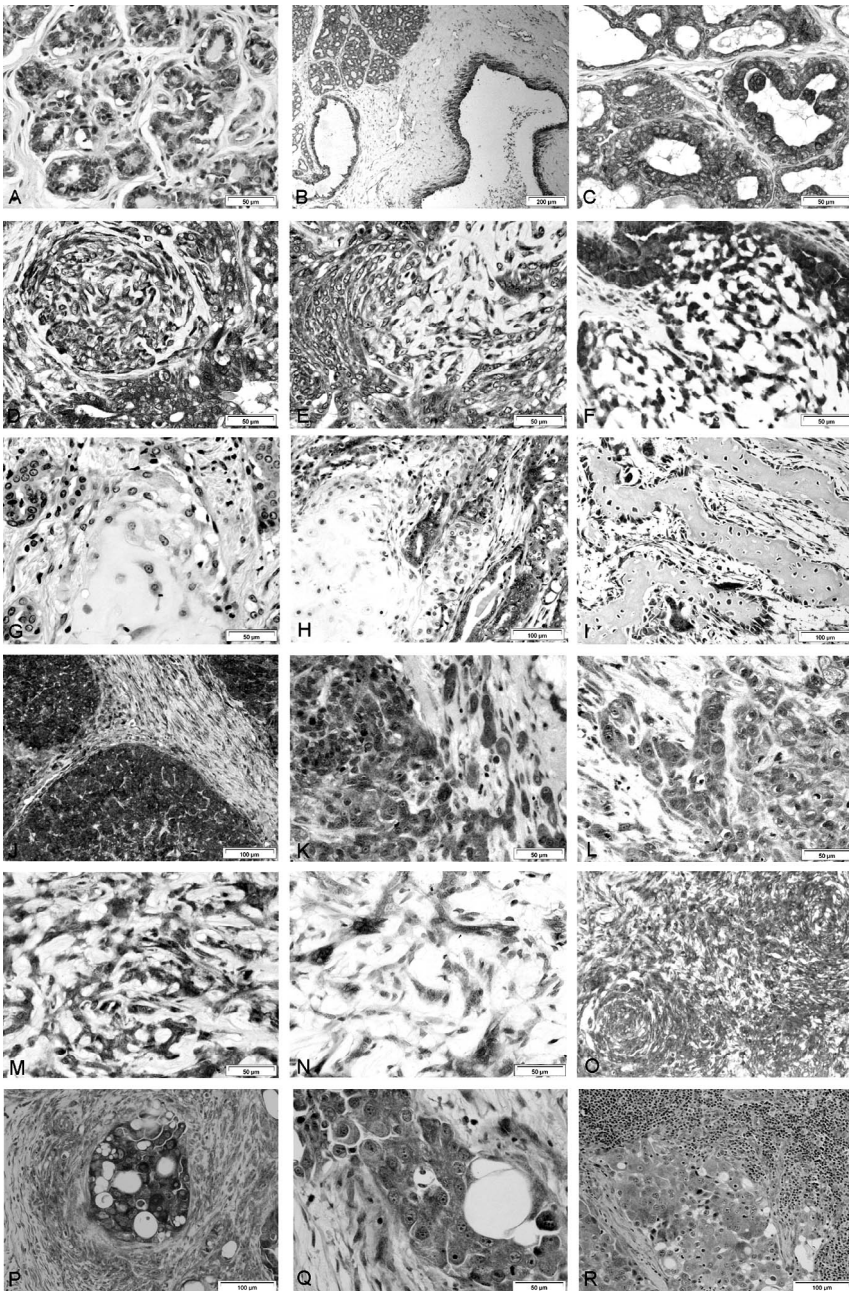
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Figure 6. *Localization of MMP-3, -11, -7, and -13 detected by immunohistochemisty.* Immunoreactivity to MMP-11 in high numbers of the luminal epithelial cells (A), surrounding large extralobular ducts (B) in a *normal mammary tissue* and increased MMP-11 reactivity in a *simple adenoma* (C). Intense and widespread immunoreactivity were seen in c*omplex/mixed tumors* (D: MMP-7). Immunoreactivities in the myxoid tissues (E: MMP-3, F: MMP-11), in early cartilagenous tissues (G: MMP-13, H: MMP-11), in the peripheral chondroblast-like tumor cells of the ectopic cartilage (H: MMP-11), in the osteoclasts and osteoblast-like tumor cells of the bone tissues in mixed tumors (I: MMP-11). Intense reactivity in the polymorph and/or invading tumor cells in epitheloid and spindle cell *myoepitheliomas* (J: MMP-7, K: MMP-13) and moderate to intense immunoreactivity to MMP-11 throughout the tumor tissues (L-M). Intense immunoreactivity to MMP-7 and MMP-3 in the multinuclear polygonal tumor cells (N: MMP-7) of a spindle cell myoepithelioma. Intense immunoreactivity to MMP-11in the poorly differentiated spindle cells of a malignant myoepithelioma (O). Intense MMP-7 reactivity in the invading, undifferentiated tumor cells of a s*imple carcinoma* (P) and intense MMP-11 reactivity in the malignant epithelial tumor cells and in the tumor stroma (Q). Immunoreactivity to MMP-11 (R) in metastatic tumor cells of a simple carcinoma.

Therefore, the benign mixed tumors characterized by large areas of myoepithelial-like spindle cell proliferations and myxoid tissues and few early cartilaginous foci were further sub-classified as complex adenomas (n=2) and shown as separate groups (Figure 4). Multiple comparison of gelatinase activity showed significantly higher ($p<0.05$) pro-MMP-2 and pro-MMP-9 levels in tumors compared to the normal mammary tissues. Furthermore, with the exception of simple adenomas all the other tumor types showed significantly higher ($p<0.05$) active MMP-2 and active MMP-9 levels (Figure 4). In fact, no bands for active gelatinases were detected in the normal mammary tissues (Figure 3A, lane 1). The highest level of pro-MMP-9 appeared in complex adenomas (Figure 3A, lane 4-5) and this difference proved to be significant ($p<0.01$) compared to the other tumor types with the exception of the simple carcinomas. However, active MMP-9 level was the highest in simple carcinomas followed by complex adenomas and these differences were significant compared to the normal mammary tissues and simple adenomas ($p<0.01$). Interestingly, the epitheloid myoepitheliomas showed the highest level of pro-MMP-2 (Figure 3, lane 7-8) and the difference proved to be significant when compared to the normal mammary tissues ($p<0.001$), simple adenomas ($p=0.01$) and simple carcinomas ($p=0.013$). Relatively high level of pro-MMP-2 appeared also in benign mixed tumors, followed by complex adenomas and simple carcinomas; but these differences were significant only compared to the normal mammary tissues. Active MMP-2 level was the highest in complex adenomas followed by simple carcinomas and the differences were significant compared to the normal mammary tissues and simple adenomas ($p<0.01$). In general, the levels of pro-gelatinases were higher than that of the active forms in

all groups. However, complex adenomas and simple carcinomas showed higher levels of active than pro-MMP-2.

Finally, we compared the total amount of MMP-9, MMP-2 (sum of pro-, and active forms) and total amount of active forms (sum of active MMP-9 and active MMP-2) between groups. All tumor types showed significantly higher amounts of total MMP-9 and MMP-2, and also significantly higher levels ($p<0.05$) of active gelatinases (Figure 4B). Complex adenomas showed the highest levels of total MMP-9 and MMP-2, and total amounts of active forms. The latter was significant compared to the normal mammary tissues ($p<0.01$) and simple adenomas ($p=0.001$). The relatively high total amount of active forms in simple carcinomas was significant compared to the normal mammary tissues ($p<0.01$).

In summary, the tumor tissues showed higher levels of both latent and active forms of MMP-2 and MMP-9. Among all the tumor types, complex adenomas, followed by simple carcinomas displayed the highest gelatinase activity. By comparing the malignant tumors (simple carcinomas) to their benign counterparts (simple adenomas) both active MMP-2 and active MMP-9 levels were significantly higher ($p<0.001$) in the malignant tumors.

Quenched fluorescent substrate assay- The measurement of the *in vivo* activity of MMP-2 and MMP-9 revealed a very low enzyme activity in the normal mammary tissues and showed increased activity in all types of mammary tumors. In the following the results of multiple comparisons are described in the main 5 histological groups: (1) normal mammary tissues, (2) simple adenomas, (3) benign mixed tumors, (4) epitheloid myoepitheliomas and (5) simple carcinomas.

MMP-2 activity was significantly higher ($p<0.001$) in all tumor types compared to the normal mammary tissue. The highest activity appeared in benign mixed tumors, followed by the epitheloid myoepitheliomas and simple carcinomas. These tumors showed significantly higher activity also compared to the simple adenomas ($p<0.001$; $p=0.048$; $p=0.001$, respectively). There were no significant differences between the other tumor types (Figure 5A). Within the main group of benign mixed tumors the subgroup of complex adenomas showed considerable higher MMP-2 activity than the other benign mixed tumors (not shown).

Activity of total MMP-9/MMP-1 was significantly higher ($p<0.001$) in all tumor types compared to the normal mammary tissue. *Activity of MMP-9* was inhibited with a gelatinase inhibitor (see Materials and Methods) and calculated by subtracting the values measured after inhibition from the total MMP-9/MMP-1 activity. The values detected after gelatinase inhibition were assumed to

represent *MMP-1 activity*. MMP-9 activity and also that of MMP-1 was significantly higher ($p<0.001$) in all tumor types compared to the normal mammary tissue. The highest MMP-9 activity was detected in epitheloid myoepitheliomas, followed by benign mixed tumors and simple carcinomas. The activity in the epitheloid myoepithelioma ($p=0.002$) and benign mixed tumors ($p=0.005$) was significantly higher compared to the simple adenomas. There was no significant difference between the other tumor types (Figure 5B). Within the main group of benign mixed tumors MMP-9 activity was only a little higher in complex adenomas than in other benign mixed tumors (not shown).

Localization and activity of MMP-3, -7, -11 and, -13

Immunohistochemistry- All the mammary tissues showed immunoreactivity to all or most of the stromelysins (MMP-3 /stromelysin-1/, MMP-11 /stromelysin-3/), matrilysin (MMP-7) and to collagenase-3 (MMP-13). The immunohistochemical results are summarized in Table 3. Similar to gelatinases, immunoreactivity to these enzymes showed uneven distribution in the *normal mammary tissues*. Among all the enzymes tested, immunoreactivity to MMP-11 and MMP-7 (Figure 6A) appeared in the highest number of the normal luminal epithelial cells. Intense MMP-11 immunoreactivity appeared surrounding some of the large extralobular ducts (Figure 6B). In *simple adenomas* immunoreactivity to MMP-7 and MMP-11 (Figure 6C) was more intense and widespread compared to the normal mammary tissues. Immunoreactivity to MMP-11 appeared more intense in the stromal fibroblast and ECM compared to the epithelial tumor cells. In c*omplex/mixed tumors,* similar to gelatinases, immunoreactivities to stromelysins, matrilysin and collagenase-3 were more widespread and intense compared to the normal mammary tissues and simple adenomas (Table 3, Figure D). Although MMP-3 immunoreactivity was mostly weak in the myoepithelial-like spindle cell proliferations a large percentage of the spindle cells of myxoid areas stained moderately or intensely to MMP-3 (Figure 6E). All these cells stained intensely to MMP-7, MMP-11 (Figure 6F) and MMP-13. The undifferentiated tumor cells of early cartilagenous tissues showed moderate to intense reactivity to MMP-13 (Figure 6G) and stromelysins (Figure 3H). The chondroblast-like tumor cells of the ectopic cartilage tissues displayed weak to moderate immunoreactivity to all the MMPs tested, in particular at the less differentiated peripheral part (Figure 6H). The osteoclasts and osteoblast-like tumor cells of the bone tissues in mixed tumors displayed moderate to intense immunoreactivity to

each MMP (Figure 6I) with the exception to MMP-3. Immunoreactivity to MMP-11 and MMP-13 was intense both in the epithelial and the stromal components of malignant complex/mixed tumors and immunoreactivity to MMP-7 was particularly intense in the malignant epithelial tumor cells. Similar to gelatinases, the polymorph, invading tumor cells in epitheloid and spindle cell *myoepitheliomas* showed more intense reactivity to these enzymes (Figure 6J-K) compared to the more differentiated tumor cells; however, immunoreactivity to MMP-11 was moderate to intense throughout the tumor tissues (Figure 6L-M). Immunoreactivity to MMP-7 and MMP-3 appeared the most intense in the multinuclear polygonal tumor cells when present (Figure 6N). In malignant myoepitheliomas the poorly differentiated spindle cells displayed intense immunoreactivity to all the enzymes, particularly to MMP-11 (Figure 6O) and MMP-13. In general, s*imple carcinomas* showed increased immunoreactivity to all the enzymes tested compared to simple adenomas, especially in the invading, undifferentiated tumor cells (Figure 6P). The most intense and even reactivity appeared to MMP-11 both in the malignant epithelial tumor cells and in the stroma (Figure 6Q). The metastatic tumor cells in the regional lymph nodes showed the most intense reactivity to MMP-11 and MMP-7 (Figure 6R). MMP-3 reactivity appeared in the walls of intratumoral blood vessels. Many of the reactive inflammatory cells in the tumor stroma when present were moderately stained for all the enzymes. Immunohistochemical staining of the negative control sections did not show immunoreactivity to any MMPs tested.

Casein zymography- A major band appeared in tumors at approximately 28-30 kDa that is likely to correspond to pro-MMP-7 based on the molecular mass. This band appeared the most intense in simple carcinomas followed by benign mixed tumors (Figure 7), whereas it was only faint in simple adenomas and could not be detected in the epitheloid myoepitheliomas. A faint but distinct band appeared in simple carcinomas also at approximately 18-20 kDa that may represent the active form of MMP-7. Another distinct band migrated approximately at 55/59 kDa that is likely to correspond to pro-MMP-3 based on the molecular mass of the band. The intensity of the band varied between samples. All the bands could be prevented by incubation and washing the gels with EDTA-containing buffers indicating that casein degradation was due to MMP activity.

Quenched fluorescent substrate assay- Similar to gelatinases, fluorimetric measurement of the *in vivo* activity of MMP-11/MMP-14 and MMP-13 showed low

enzyme activity in the normal mammary tissues and increased activity in mammary tumors.

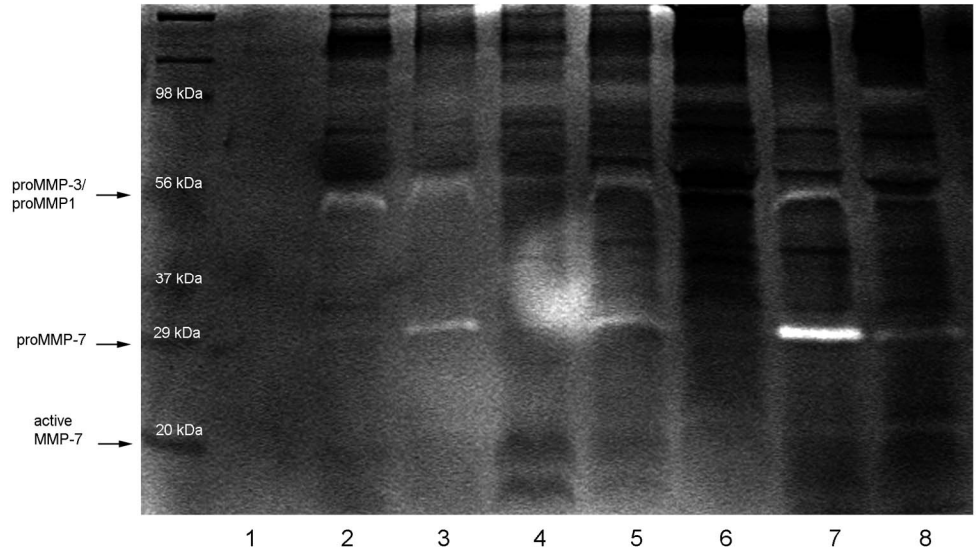
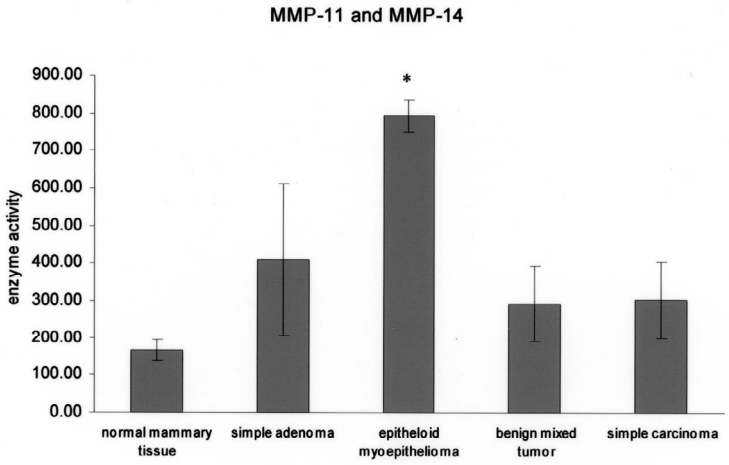


Figure 7. *Casein zymography* detected three lysis bands: a major band at 28-30 kDa (pro-MMP-7), a faint band at 18-20 kDa in simple carcinomas (active MMP-7), and another distinct band at 55/59 kDa (pro-MMP-3). Lane 1: normal mammary tissue; lane 2: simple adenoma; lane 3-5: complex/mixed tumors; lane 6: myoepithelioma; lane 7-8: simple carcinoma.

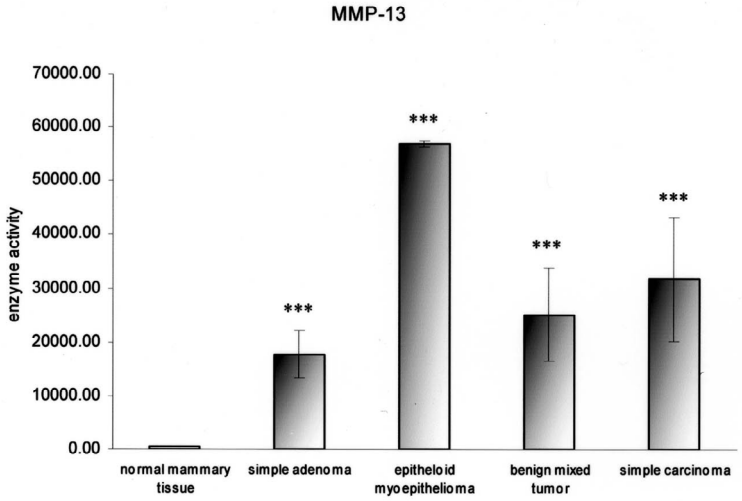
Activity of MMP-11/MMP-14 was higher in tumor tissues compared to the normal mammary tissue but the difference proved to be significant only in the epitheloid myoepithelioma ($p=0.018$). Interestingly, the epitheloid myoepithelioma showed relatively high activity when compared to the other tumor types; however, there was no significant difference between the different tumor types (Figure 8A). Within the main group of benign mixed tumors complex adenomas showed a very similar activity compared to other benign mixed tumors. MMP-11/MMP-14 activity did not prove to be higher in simple carcinomas compared to simple adenomas.
MMP-13 activity was significantly increased ($p<0.001$) in all types of mammary tumors compared to the normal mammary tissues. The highest activity was detected in epitheloid myoepitheliomas and the difference was significant

compared to the simple adenomas (p=0.002) and benign mixed tumors (p=0.026). The second highest activity appeared in simple carcinomas and the difference was significant compared to simple adenomas (p=0.027) (Figure 8B).

A



B



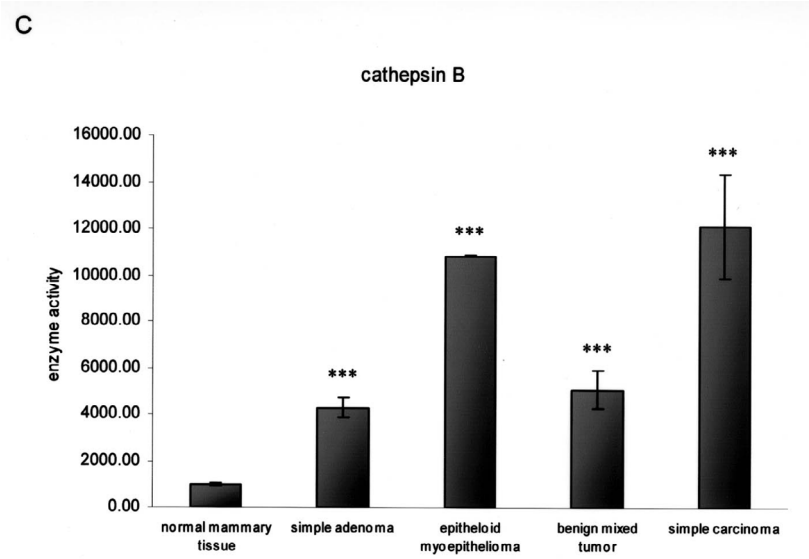


Figure 8. *In vivo activity of MMP-3, -7, -11 and, -1.* Data were analyzed by one-way ANOVA with Bonferroni's multiple comparison. Values are mean ± std. (*p<0.05, ***p<0.001)

Within the main group of benign mixed tumors complex adenomas showed higher activity than the benign mixed tumors.

Localization and activity of cathepsin B

Enzyme histochemistry- Cathepsin B activity was detected in the cytoplasm of tumor cells and stromal fibroblasts of all the tumor tissues tested. The most widespread and intense activity appeared in the tumor cells of complex and mixed tumors and in the epithelial tumor cells of malignant tumors. In the normal mammary tissues no or only very few weak fluorescent spots were seen in the intralobular connective tissue. In accordance with previous reports, after incubating the samples for 3-5 minutes, non-specific, green and diffuse background fluorescence occurred in the cytoplasm of the cells.[20-21] After 5 minutes, small bright fluorescent particles started to appear in the cytoplasm (Figure 2I). The number of the bright fluorescent particles increased between 5 and 10 minutes of the incubation time. The negative control samples incubated for 1 hour in the absence of substrate did not show formation of any bright

fluorescent particles, whereas the background fluorescence in the cells was present.

Quenched fluorescent substrate assay- The cathepsin substrate used in this study is specific for cathepsin B, -L, and -S. However, the enzyme activity detected in the samples could be completely inhibited by both the general cathepsin inhibitor E64 and the cathepsin B inhibitor CAO74. This indicates that the cleavage of the substrate can be entirely attributed to cathepsin B activity in our samples.

Cathepsin B activity was significantly higher in all tumor types ($p<0.001$) compared to the normal mammary tissues. The highest activity was detected in simple carcinomas, followed by the epitheloid myoepitheliomas and the differences proved to be significant in both tumor types when compared to the simple adenomas ($p<0.001$) and benign mixed tumors ($p<0.001$) (Figure 8C). Complex adenomas showed higher activity than other benign mixed tumors.

Briefly summarizing the main result, all the methods used in this study indicate increased expression and activity of MMPs and cathepsin B in tumors compared with the normal mammary tissue. In general, myoepitheliomas and complex adenomas showed high enzyme expression and activity. Furthermore, simple carcimomas displayed increased enzyme expression and activity compared to their benign counterparts (simple adenomas).

Discussion

Although the expression, activity and specific role of distinct MMPs and other ECM degrading proteases have been extensively studied in human malignancies, little is known about these enzymes in canine tumors, such as the mammary tumors. It is generally assumed that the primary mechanism by which MMPs promote cancer spread is the degradation of the ECM. However, we have now become aware that the role of proteases in tumor biology, in particular that of MMPs, is far more complex. The active proteases extensively modify and remodel the tumor microenvironment that leads to important changes in cell-cell and cell-ECM interactions, and generate new signals from the cell surface. These signals affect gene expression and ultimately influence cell behaviors such as proliferation, survival, differentiation, and motility. Moreover, these proteases

can target many non-ECM proteins, such as growth factors and their receptors, cytokines and various cell-associated molecules.

We recently reported that versican, a large extracellular proteoglycan, is extensively expressed and proteolytically cleaved in canine mammary tumors.[11,19] A broad range of versican fragments were present in these tumors indicating multiple cleavage of this proteoglycan possibly by different proteases. Moreover, we identified the ADAMTS cleaved versican fragments, which were particularly abundant in the myxoid tissues of complex and mixed mammary tumors.[11] In the present study our main objective was to identify the localization and activity of proteases, including several MMPs and cathepsin B, which are known to degrade a variety of ECM proteins; therefore they may contribute to the ECM remodeling in canine mammary tumors. Whether these proteases are differentially expressed in the different histological types of tumors or in benign and malignant mammary tumors was also a question of interest.

The few previous studies on MMPs in canine mammary tumors tend to use individual techniques to determine the synthesis of proteases and none of these studies examined a large spectrum of proteases nor measured the *in situ* or *in vivo* enzyme activity in these tumors.[22-25] The study presented here used a combination of techniques to determine the cellular location and *in vivo* activity of a number of proteases. Our findings reveal the presence of multiple proteases, including gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3, MMP-7 and MMP-11), collagenases-3 (MMP-13) and cathepsin B in canine mammary tumors. We show that both the production of these proteases tend to be greater in tumors than in the normal breast tissue. However, the most important determinant for proteolysis *in vivo* is the balance between the production and activation of proteases and the production of their inhibitors. Quenched fluorescent substrate hydrolysis determined the amount of free active MMPs present within the tissue samples. This is indicative of proteolysis occurring *in vivo* and has not been determined previously in canine mammary tissues. In general, the increased levels of MMPs, including the MMPs investigated in this study, seem to correlate with invasiveness and poor prognosis in several tumor types. [26-29] However, the generally proposed role of MMPs in tumor invasion is based mainly on observation of high-level expression of distinct MMPs in invasive malignant tumors [26-31] and the measurement of the activity of distinct MMPs in tumor tissues *in vivo* is very limited. Up to now, to our best knowledge, only the total MMP activity was measured in the human breast cancer by quenched fluorescent substrate

hydrolysis and no reports are available on the activity of distinct proteases. In general, we found a positive correlation of increased enzyme production with enhanced *in vivo* activity. We showed that in canine mammary tumors besides the increased presence of MMPs, detected by immunohistochemistry and zymography, the activity of MMPs and also that of cathepsin B was significantly greater than in the normal mammary tissues. Quenched fluorescent substrate hydrolysis revealed a particularly high enzyme activity in myoepitheliomas, complex adenomas and simple carcinomas.

The presence of the active forms of MMP-2 and MMP-9 was greater in mammary tumors and would therefore contribute to the increased gelatinase activity. The increased activity of these proteases in canine mammary tumors may be relevant for increased proteolysis and therefore tumor progression and metastasis *in vivo*. This was also supported by other observations of this study. First, there was a trend for increased MMP and cathepsin B activity to correlate with malignancy (simple carcinomas versus simple adenoma). Furthermore, most of the MMPs, including the gelatinases, matrilysin and stromelysin-3, were present also in the lymph node metastasis. Second, in accordance with the literature indicating MMP-2 and MMP-9 as the main MMPs responsible for the degradation of basement membrane components, we show that gelatinase activity is increased in canine mammary tumors and this activity is particularly intense surrounding ductal structures in tumors. This suggests a critical role for MMP-2 and MMP-9 in the conversion of *in situ* carcinomas to invasive lesions, similar as it has been indicated in human breast cancer.[32,33] The significantly increased cathepsin B activity in malignant canine mammary tumors is consistent with findings in human breast cancer. The cysteinee protease cathepsin B was the first lysosomal protease associated with malignancy of human breast cancer.[34,35] A more recent study measured cathepsin B activity in a large number of human invasive breast carcinomas and cathepsin B proved to be a useful prognostic marker for recurrence and overall survival of node-negative patients.[36] However, we detected expression and activity of all the MMPs and cathepsin B not only in malignant but also in benign mammary tumors suggesting that they play a role in other events in tumors than invasion and metastasis. Moreover, the normal mammary tissues did have some MMP activity, implying that MMPs are involved in normal tissue remodeling of the canine mammary gland.

During invasion of tumor cells, the connective tissue stroma is believed to be degraded primarily by the interstitial collagenases and some of the stromelysins.

Initially it has been suggested that cancer cells themselves produce the MMPs required for degradation of ECM, but more recent studies have demonstrated that stromal cells were the principal source of MMPs within human tumors (e.g. breast, GI, lung), rather than the cancer cells themselves.[37-41] In line with this suggestion our present results of *in situ* zymography confirmed that activity of gelatinases are mainly localized in the stromal ECM and fibroblasts of canine mammary tumors, although immunohistochemistry showed their presence equally in tumor cells and stromal fibroblasts. Similar to gelatinases, MMP-11 (stromelysin-3) was highly expressed by both tumor cells and stromal fibroblasts, whereas MMP-13 (collagenase-3) was more prominently expressed in the tumor stroma. In human breast carcinomas both of the latter proteases are expressed primarily by stromal fibroblasts.[37,41] Stromelysin-1 (MMP-3) is infrequently identified in human tumors, but in human breast cancer it has been demonstrated in the ECM surrounding tumor cells and adjacent to blood vessels.[42] Although it was the least abundant MMP tested in this work, we found a relatively prominent expression in the spindle cells of myxoid tissues in complex/mixed tumors and in the spindle-cell myoepitheliomas, suggesting that it is produced predominantly by spindle cells with myoepithelial or myoepithelial-like differentiation and may have a specific role in these tissues. Regarding matrilysin (MMP-7) it is generally accepted that in contrast to other MMPs it is predominantly produced by carcinoma cells.[43-45] Our study shows that although matrilysin is more prominently expressed in the epithelial tumors cells, its expression in stromal fibroblasts is surprisingly prominent in canine mammary tumors. An explanation of the distinct role of stromal cells in tumors originate from the observation that carcinoma cells produce a stimulatory factor, Extracellular Matrix Metalloproteinase Inducer (EMMPRIN), which induces stromal fibroblasts to produce MMPs.[46] Furthermore, complex activation cascades have been revealed that ensure cooperation between tumor and stromal cells, but the discussion of such interaction is beyond the scope of this study. On the other hand, increasing data demonstrated that, in association with a process of epithelial-to-mesenchymal transition (EMT; loss of epithelial characteristics and expression of mesenchymal markers) as tumors cells differentiate into an invasive phenotype, many MMPs can be expressed by tumor cell themselves.[47,48] In fact, a number of MMPs (e.g. MMP-3 and MMP-7 in mammary epithelial cells) have been implicated in cleavage of cell adhesion molecules, such as E-cadherin, leading to epithelial-to mesenchymal transition, thus to invasive phenotype. [48,49]

This may explain the relatively high expression of MMPs in tumor cells of canine mammary tumors and the increased immunoreactivity in undifferentiated, invading tumors cells compared with the more differentiated tumors cell within the same tumor tissue.

Our finding that the reactive inflammatory cells within the tumor tissues were also reactive to MMPs is not surprising. Recent studies have indicated that besides MMP produced by the tumor or stroma cells lymphocyte-derived MMPs may be also advantageous to developing tumors by promoting angiogenesis, tumor cell proliferation, and progression to malignancy.[50]

In summary, this study showed increased production and *in vivo* activity of multiple proteases that are known to cleave various ECM components, including proteoglycans, in canine mammary tumors. The increased protease activities in malignant tumors underline the importance of the *in vivo* proteolytic activity of MMPs and cathepsin B in progression of canine mammary tumors, but the distinct role of individual proteinases remains to be further established. If there is a correlation of increased protease activity with tumor grade and/or stages and clinical outcome of canine mammary tumors large numbers of dogs with mammary tumors at different stage must be further investigated. Furthermore, to clarify which of these active proteases may contribute to versican degradation in canine mammary tumors awaits further studies.

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CHAPTER 6

General discussion: summary, conclusions and future directions

Over the last 10-15 years, a revolution has taken place that has put the extracellular matrix (ECM) research at the heart of cell biology, and has had extensive implications in many fields, including cancer research. A major impetus behind this revolution was the rapid development of techniques, particularly in molecular biology. This has lead to the recognition that the role of ECM is much more than only to serve as a passive scaffold for the cells in tissues but rather has an active influence on many crucial biological events. In addition, the fast development in the generation of a wide-range of antibodies that recognize extracellular epitopes, has largely facilitated the identification of ECM components in several tissues and the spatial- and temporal distributions of extracellular molecules in developmental and pathological processes. The recent advances of ECM research in the field of tumor biology are briefly reviewed in *Chapter 1* of this thesis.

Significant changes in the composition of the ECM during tumor development, including altered concentrations and distributions of glycosaminoglycans (GAGs) and proteoglycans (PGs), have been long recognized and relatively well-documented in humans. However, little work has been done in this field in tumors of animals, such as the mammary tumor of the dog. Several controversies surround the complex histogenesis of canine mammary tumors. Up to now, most of the approaches and experimental attempts to understand the origin of spindle cells, cartilage and bone of complex and mixed tumors was based on the identification of intracellular and cell surface molecules. Recognizing the importance of the ECM in morphogenesis and tumor development prompted us to place the primary focus of this thesis on the exploration of ECM components in canine mammary tumors. It has been previously shown that complex and mixed tumors are particularly rich in chondroitin sulfate. Therefore, in *Chapter 2* we investigated by immunohistochemistry the expression pattern of versican, which was the most likely candidate among the chondroitin-sulfate (CS) PGs to be expressed in canine mammary tumors. We showed that despite the lack of versican immunoreactivity in the normal mammary tissues, versican was expressed in all types of mammary tumors, indicating the importance of this PG in canine mammary tumor development. There were three characteristic localizations of versican in the tumor tissues:

(1) In both benign and malignant tumors versican deposition consistently occurred around ductal structures. We concluded that versican deposition is associated with the formation of tumor basement membranes; however, what

role versican plays in this process awaits further studies. In this context, it may be relevant to investigate whether the deposition of versican in the periductal tissues is associated with elastosis, a process that frequently occurs in the periductal spaces of some neoplastic tissues especially in breast cancer.[1] It is known that versican is closely associated with elastic networks, *in vivo*.[2] In fact, the lectin-like region of its G3 domain binds fibulin-1 and fibulin-2, that are modular ECM proteins expressed in elastic fibers and basement membranes.[3-5] In addition, versican interacts with fibrillin-1, which is a major component of the elastic microfibrillar network.[6] Fibulin may serve as a bridge between versican and fibrillin, forming highly ordered multimolecular structures important in the assembly of elastic fibers. (The versican motifs and their interaction with other molecules are shown in the Figure 1, Appendix). The group of Wight and colleagues has special interest in the role of versican in elastogenesis, particularly in certain cardiovascular diseases. They have shown that overexpression of the small versican variant V3 by rat arterial smooth muscle cells (ASMCs) significantly increases the expression of tropoelastin and the formation of elastic fibers *in vitro* and *in vivo*.[7] They suggested that the elastin-binding protein (EBP) plays an important role in regulating the V3 elastogenic effect; however V3 may act also as a structural component in elastic fiber assembly.[8]

(2) Another location of versican accumulation was the peripheral invasive areas of malignant tumors. Similar to their benign counterpart, simple carcinomas showed no or only low amounts of versican in the intratumoral stroma, other than the periductal fibrous elements or blood vessel walls. However, the periepithelial mesenchymal tissues around nests and clusters of invading tumor cells displayed high amounts of versican. Versican is involved in diverse cell functions, such as adhesion, migration, proliferation, and apoptosis. Reports sometimes provide seemingly contradictory observations. This may be reconciled by an appreciation of the structure and expression diversity of versican. Moreover, versican does not act by itself. It has several binding partners that play important roles in determining the effect of versican on cell behavior (Fig 1, Appendix). Therefore, in order to better understand the function of this complex proteoglycan it is especially important to analyze its molecular interactors, which also vary spatially and temporally. In this regard, in *Chapter 3* we investigated the co-localization of versican with hyaluronan (HA) and link protein (LP), two molecules that bind to the G1 domain of versican, in canine mammary

tumors. Versican expression has been long associated with proliferative cell phenotype. One of the ways versican is believed to facilitate cell proliferation and migration is the binding of HA via the G1-domain and this interaction is often stabilized by LP. HA production is also increased at sites of cell proliferation and tumor cell invasion (for recent review see reference 9). This molecule is thought to modulate cell behavior by binding to specific cell surface receptors, most importantly CD44.[10] Consistent with this notion, hyaluronan- CD44 interaction has been observed to enhance growth of certain tumors *in vivo*.[10-12] Hyaluronan-CD44 signaling can activate several pathways through activation of specific intermediates [13], including the Rho and Rac1 GTPases, whose activities lead to subsequent reorganization of the actin cytoskeleton [14-16]; erbB2 tyrosine kinase [17], which leads to cell proliferation; src-related tyrosine kinases [18], and nuclear factor-kB [NF-kB] [19-20]. On the other hand hyaluronan-CD44 interaction is also known to affect cell adhesion and migration.[21] Since versican binds HA with high affinity and HA is linked to the cell surface via CD44, it is likely that versican stabilizes a large supramolecular complex at the cell surface, contributing towards the formation and integrity of a specialized, highly viscoelastic pericellular matrix. As a result, this matrix provides the tumor cells with an optimal microenviroment for both proliferation and migration. In accordance with this we show (*Chapter 3*) that besides versican, HA and LP is also expressed in canine mammary tumors and the three molecules co-localize in the peripheral invasive zones of malignant tumors. Therefore, it is likely that surrounding invading clusters of tumor cells in malignant canine mammary tumors versican exerts its function by forming complexes with HA, and this interaction is stabilized by link protein. Based on our findings, future studies are warranted to confirm that versican in fact enhances tumor cell proliferation and migration *in vivo* in mammary tumors, possibly through interaction with hyaluronan and subsequent activation of CD44. However, as discussed in the previous chapters, versican may influence cell proliferation, migration and survival by several other mechanism, e.g. through the epidermal growth factor receptor (EGFR) pathway and/or through cell cycle-related proteins. It is important to consider, that different versican variants may have different or even opposite effects on cell behavior. In fact, it has been recently shown that the GAGß domain activates, whereas the GAGα suppress the expression of EGFR and its downstream signal pathway. This explains that V1 versican (containing the GAGß subdomain) enhances, whereas the V2 variant (containing the GAGα subdomain) inhibits cell

proliferation *in vitro*. Furthermore, V3 versican that lacks CS chains down-regulates migration and proliferation of rat arterial smooth muscle cells and this effect is likely to occur via the displacement of versican V0/V1 from the cell surface.[22] Regarding the largest V0 variant of versican, we know that its expression is limited in the adult tissues but it is highly expressed during fetal development and in several malignancies where cells are metabolically active and intensely proliferating. In line with these findings, in *Chapter 4* we show that the main versican variants expressed in canine mammary tumors and cell lines are the large V0 and V1variants, whereas the V2 variant could not be detected. We also detected the unique and rarely expressed short V3 variant, although at a much lower levels than the V0/V1 variants. In conclusion, it seems that the large V0/V1 versican variants play a particularly important role in highly proliferating tissues, such as canine mammary tumors. However, much need to be done to clarify how exactly these molecules influence tumor cell behavior and what molecular mechanisms are involved in those processes. The biological significance of the presence of the short V3 versican is open to speculation.

(3) The third and most prominent location of versican accumulation was the myoepithelial-like spindle cell proliferations, and especially the ECM-rich myxoid tissues in complex and mixed tumors. The origin of cartilage in mixed tumors has been a long-standing question and source of debate for veterinary pathologists. Since versican is one of the major molecules expressed during mesenchymal cell condensation in the early phase of chondrogenesis, we postulated that accumulation of versican in the above tissues reflects early events of chondrogenesis; thus, the myxoid tissues and/or spindle cell proliferations are the precursor tissues of ectopic cartilage in mixed tumors. In *Chapter 3*, we provide evidence that these tissues, indeed, display chondroid differentiation, shown by the expression of cartilage markers, such as collagen type II and aggrecan both at mRNA and protein levels. Interestingly, myoepitheliomas also expressed these cartilage markers. By using a panel of antibodies we also showed that the spindle cells embedded in the cartilagenous matrix have, at least partially, a myoepithelial phenotype and express the 3B3 neoepitope (a neopitope of chondroitin-6- sulfate that is highly expressed in the developing cartilage). All these findings point to a chondroprogenitor role of myoepithelial cells in canine mammary tumors. Last, but not least, an interesting observation was that malignant epithelial cells in chondrogenic carcinomas (metaplastic carcinomas) exhibited at least a partial myoepithelial differentiation. In

summary, the findings strongly suggest that in canine mammary tumors the cartilage formation is a result of an (epithelial→) myoepithelial→ mesenchymal (chondroid) transition process. The molecular mechanism by which versican influences cartilage differentiation is not known and neither did our study directly address this question. However, recent findings by Kamiya and colleagues (2005) suggest that the versican affects the gene expression and deposition of aggrecan into the matrix and the chondroitin-sulfate chains of versican are functional sites for modification of cell-matrix interactions during mesenchymal condensation and the subsequent steps of chondrogenesis.[23]

Matrix biology expanded its sphere of interest over the recent decades from its original morphological functions to incorporate control of cell phenotype and behavior during development, maturation and also tumor development. Tumor growth is accompanied by a vigorous matrix remodeling and loss of original phenotypic traits of the cells from which the tumor arises. Most of these functions are related to signaling by matrix components to cells through cell-membrane receptors. Some of the signaling molecules are produced by proteolytic degradation of macromolecules of the extracellular matrix. Such peptides (matrikins or matricryptins) exhibit biological functions absent in the native molecule from which these peptides were derived. Some of these novel activities are potentially harmful and appear to be involved in tumor initiation as well as progression. In *Chapter 4* we show that besides being expressed as multiple splice variants, versican is present in several short fragments in canine mammary tumors. In that study we identified the 70 kDa N-terminal, G1-bearing V1 fragment generated by ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) cleavage at the ß-GAG region. In some of the tumors, a corresponding fragment of V0 versican was also present at lower levels. The highest amounts of ADAMTS generated versican fragments appeared in the myxoid tissues of complex and mixed tumors. This is in accordance with our immunohistochemical finding in *Chapter 2*, indicating the accumulation of an N-terminal (12C5-reactive) versican fragment in myxoid tissues. Degradation of aggrecan by ADAMTS in the cartilage matrix has been long recognized as the "cardinal feature" of joint diseases such as osteoarthritis. The ADAMTS-mediated aggrecanolysis is destructive to cartilage function via the release of the complete GAG-rich region from the tissue. The resulting decrease in the charge density of the cartilage tissue is thought to initiate a cascade of events that leads to irreversible tissue disruption. In contrary, aggrecanolysis by MMPs has been

suggested to be beneficial rather than destructive and may be required for cartilage matrix homeostasis.[24] Although increasing evidence is accumulating also for the presence and importance of *in vivo* versican cleavage in some tissues, especially by ADAMTS proteases in vascular disease [25], further studies will be necessary to determine the effects of specific fragments of versican. Our study, described in *Chapter 4*, provides the first evidence of *in vivo* catabolic processing of versican. At this phase we could only speculate on the possible effects of versican breakdown in mammary tumors based on the current knowledge of versican biology. Nevertheless, based on the available information on versican degradation, it is likely that proteolysis of versican both in normal and pathological turnover pathways, modifies its biological activity and consequently tissue properties and cell activities.

The broad range of versican fragments present in canine mammary tumors indicates multiple cleavage of this proteoglycan by different proteases. Therefore, in *Chapter 5* we aimed to identify the expression and *in vivo* activity of several MMPs and cathepsin B that have the capability to cleave proteoglycans and/or a large spectrum of ECM molecules; thus they may contribute to versican degradation besides the ADAMTS proteases in canine mammary tumors. On the other hand this study has special importance in the veterinary field, since little is known about these enzymes in canine tumors. Up to now no data are available on the *in vivo* activity of these proteases in canine tumors. We demonstrated the expression and elevated *in vivo* activity of MMP-2, -3, -7, -9, -11 and -13 and cathepsin B in all types of mammary tumors. Gelatinase (MMP-2 and MMP-9) activity was particularly intense along the basement membranes of neoplastic ductal structures, suggesting a critical role for these enzymes in the conversion of *in situ* carcinomas to invasive lesions. In our opinion, among the active enzymes identified in canine mammary tumors, MMP-2, MMP-3 and MMP-7 are the most likely candidates to contribute to versican degradation since their ability to cleave recombinant and/or native versican has been already demonstrated.[26,27] However, cathepsin B and most of the MMPs tested in this study have been shown to cleave aggrecan in cartilage.[28,29] Therefore, future studies are warranted to test all these active proteases in their ability to cleave native versican in canine mammary tumors.

The importance of the work presented in this thesis resides in its novelty considering that it provides the first extensive and systematic data on the ECM

composition and organization in canine mammary tumors. It is apparent that questions remain, in fact, we have just started. However, our results confirm that performing fundamental research in matrix biology is essential to understand tumor biology and deserves much more attention in veterinary research.

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CHAPTER 7

Summary/ Samenvatting/ Magyarnyelvű összefoglaló

Summary

The extracellular matrix (ECM) research has become fundamental to understand cancer. It is apparent that the interactions of cells with the ECM have an active influence on cell signaling and behavior. Up to now, little attention has been paid to the ECM of canine mammary tumors. Therefore, the experimental work presented in this thesis focuses on the exploration of ECM composition and organization in canine mammary tumors, with a special interest in the large chondroitin-sulfate proteoglycan (PG), versican.

Chapter 1 gives a general overview of the most recent concepts in matrix biology, particularly in tumors, and shortly introduces the ECM molecules investigated in the following chapters. This chapter includes schematic figures showing the domain structure of versican (G1-domain, GAG-domains, G3-domain) and the isoforms generated by alternative splicing of the mRNA transcript for versican, namely V0, V1, V2 and V3.

Chapter 2 describes the immunohistochemical analysis of versican in canine mammary tumors. We show that versican protein is not present in the normal mammary tissues; however, there is an increased expression in both benign and malignant tumors. Versican accumulates at three main locations in tumors:
(1) In periductal tissues, probably in association with basement membranes of ducts.
(2) In peripheral invasive areas of malignant tumors, suggesting the involvement of versican in tumor cell invasion.
(3) In the myoepithelial-like spindle cell proliferations and myxoid areas of complex and mixed tumors. The immunopattern of versican epitopes (12C5 and 2B1) revealed that the G1-domain of versican accumulates in complex and mixed tumors, particularly in the myxoid tissues.

In **Chapter 3** we investigated whether the high expression of versican in the myoepithelial-like cell proliferations and myxoid areas of complex and mixed tumors is associated with cartilagenous differentiation. We show that mRNA of versican, collagen type II and aggrecan is up-regulated in tumors, the latter two

being characteristic cartilage components, especially in complex and mixed tumors. Immunohistochemistry revealed the expression of cartilage biomarkers (collagen type II, aggrecan, 3B3 neoepitope) not only in the cartilagenous tissues of mixed tumors, but also in myoepitheliomas and in the myoepithelial-like cell proliferations and myxoid tissues of complex and mixed tumors. The results indicate that the myxoid tissues and myoepithelial-like cell proliferations are the precursor tissues of the ectopic cartilage in mixed tumors. Furthermore, it is likely that cartilage formation in canine mammary tumors is a result of a (myo)epithelial to mesenchymal transition.
In addition, the expression of hyaluronan (HA) and link protein (LP) was demonstrated in canine mammary tumors, suggesting that versican-HA complexes may be formed and stabilized by LP. The co-localization of the three molecules surrounding invading clusters of tumor cells indicates the formation of a highly viscoelastic microenvironment that may promote tumor cell proliferation and migration.

In **Chapter 4** we show that versican is present in multiple forms, including splice variants and catabolic products. We identified the precise expression pattern of the mRNA splice variants and their protein products to be V1, V0 and V3 versican, with a prevalence of V1/V0 variants in canine mammary tumors and cell lines. The V2 variant was not detectable. It was shown that tumor cell lines express increased mRNA and protein of V0 and V1 versican compared to the normal mammary fibroblasts and the myoepithelial-like tumor cells express the highest levels of all mRNA splice variants. It is suggested that the high expression of the hydrophilic V0/V1 variants contributes towards the formation of a highly hydrated microenvironment that is optimal for tumor cell proliferation, migration and differentiation in canine mammary tumors.
Furthermore, a broad range of versican fragments are present in canine mammary tumors, among which ADAMTS generated fragments were identified. These fragments represent N-terminal G1-bearing V1/V0 versican cleaved within the ß-GAG subdomain and appear the most abundant in the myxoid tissues of complex and mixed tumors. This confirms our previous suggestion (Chapter 2) that the G1-domain of versican accumulates in these tissues. These findings indicate regulated versican degradation and a distinct biological role for the truncated versican forms in canine mammary tumors.

In **Chapter 5** our main objective was to identify and measure the *in vivo* activity of proteases, including several MMPs and cathepsin B that may participate in the ECM turnover and versican degradation in canine mammary tumors. We used a combination of techniques, including immunohistochemistry, gelatine and casein zymography, *in situ* zymography with quenched fluorogenic DQ-gelatin/substrate and quenched fluorescent substrate assay. MMPs were secreted by both tumor cells and stromal fibroblasts; however, *in situ* zymography showed that gelatinases exert their activity primarily in the tumor stroma, particularly surrounding ductal structures. All methods indicated elevated enzyme expression and activity in tumors and their metastases compared to the normal mammary tissues. There was a trend for increased protease activity particularly that of MMP-2, MMP-13 and cathepsin B, in simple carcinomas compared to simple adenomas. This study has a special importance in veterinary research, because the *in vivo* activity of these enzymes has not been demonstrated previously in canine tumors. The enhanced enzyme activity in tumor tissues and their metastases, confirms the role of these proteases in tumor development and progression. Gelatinases seem to play an important role in converting *in situ* carcinomas to invasive lesions. Future studies are necessary to identify which of these active proteases may contribute to versican degradation in canine mammary tumors.

In **Chapter 6** we give an overview of the major findings and conclusions of this thesis and discuss the future directions of research.

Samenvatting

Onderzoek naar de extracellulaire matrix (ECM) is van fundamenteel belang geworden voor het inzicht in kanker. Het is gebleken dat de interactie van cellen met de ECM een actieve bijdrage levert aan processen zoals cellulaire signalering en celgedrag. Tot op heden is er weinig aandacht besteed aan de ECM van mammatumoren van honden. Daarom concentreert het experimentele werk dat hier wordt gepresenteerd zich op een onderzoek naar de samenstelling en organisatie van de ECM in mammatumoren van honden, waarbij vooral aandacht is besteed aan versican, een chondroitin sulfaat-proteoglycan.

In **Hoofdstuk 1** wordt een algemeen overzicht gegeven van de meeste recente concepten die in de matrixbiologie worden gebruikt, met name de matrixbiologie van tumoren; in het hoofdstuk worden kort de ECM moleculen geintroduceerd die in de erna volgende hoofdstukken worden onderzocht. Ook zijn een aantal schema's opgenomen waarin de domein structuur van versican (G1-domein, GAG-domeinen, G3-domein) aan de orde komen, evenals de iso-vormen die tot stand komen door alternatieve *splicing* van het mRNA transcript voor versican namelijk de V0, V1, V2 en V3 varianten.

Hoofdstuk 2 beschrijft de resultaten van een immunohistochemische analyse van versican in mammatumoren van honden. We laten hierin zien dat het versican eiwit niet aanwezig is in normale melkklierweefsels maar dat er een toename optreedt in de expressie van versican in zowel goedaardige als kwaadaardige tumoren. Versican stapelt in belangrijke mate op de volgende drie verschillende plaatsen in de tumoren:
1. In periductale weefsels, waarschijnlijk in een associatie met de basaalmembranen van de mekkliergangen.
2. In de periferie van de invasieve gebieden van kwaadaardige tumoren, wat suggereert dat versican betrokken is bij invasie van de tumor.
3. In de gebieden waarin myoepitheelachtige spoelvormige cellen prolifereren en waarin myxoide gebieden van complexe en gemengde tumoren aanwezig zijn. Het patroon van de immunokleuring van de versican epitopen die met de

antilichamen 12C5 en 2B1 reageren laat zien dat het G1 domein van versican stapelt in complexe en gemengde tumoren, met name in de myxoide weefsels.

In **hoofdstuk 3** hebben we onderzocht of in de gebieden waarin de myoepitheelachtige spoelvormige cellen prolifereren en in de myxoide gebieden van complexe en gemengde tumoren de verhoogde expressie van versican daarin geasssocieerd is met met een differentatie van het weefsel tot kraakbeen. We laten zien dat mRNA van versican, collageen type II en aggrecan opgereguleerd is in de tumoren; de laatste twee eiwitten zijn karakteristieke kraakbeen componenten die met name in de complexe en gemengde tumoren voorkomen. Immunohistochemisch onderzoek liet zien dat de expressie van kraakbeen biomarkers (collageen type II, aggrecan, de 3B3 neoepitope) niet alleen plaats vindt in de kraakbeen-achtige weefsels van gemengde tumoren, maar ook in myoepitheliomas en in de myoepihteel-achtige celwoekeringen en in de myxoide weefsels van complexe en gemengde tumoren. De resultaten wijzen erop dat de myxoide weefsels en de myoepitheel-achtige celwoekeringen de voorloper weefsels zijn van het ectopische kraakbeen in de gemengde tumoren. Bovendien lijkt het waarschijnlijk dat de vorming van kraakbeen in gemengde mammatumoren van honden het resultaat is van een (myo)epitheel-mesenchym transformatie.
Daarnaast konden we laten zien dat hyaluronzuur (HA) en linkprotein (LP) in honden mammatumoren tot expressie komen, wat suggereert dat er een versican-HA complex wordt gevormd dat gestabiliseerd wordt dor LP. De co-localisattie van de drie moleculen in de omgeving van invaderende clusters van tumorcellen wijst erop dat er een sterk viscoelastische micro-omgeving wordt gevormd dat van belang kan zijn voor het stimuleren van proliferatie en migratie.

In **hoofdstuk 4** laten we zien dat versican aanwezig is in een veelheid van varianten, zowel in de vorm van splice-varianten als van afbraakproducten. We stelden het preciese expressiepatroon vast van de mRNA splice-varianten en hun eiwitten en identificeerden V0, V1 en V3 versican, met als meest voorkomende vorm in honden mammatumoren en mammatumor cellijnen de V1/V0 variant. De V2 variant was niet aantoonbaar. Tumorcellijnen brachten verhoogde concentraties van de mRNA en de eiwitten van V0 en V1 versican tot expressie, vergeleken met normale melkklier fibroblasten, en de myoepitheliale tumor cellen brengen de hoogste niveaus van alle mRNA splice-varianten tot expressie.

Dit suggereert dat de verhoogde expressie van de hydrofiele V0/V1 varianten bijdraagt aan de vorming van een sterk gehydrateerde micro-omgeving, die optimaal is voor tumorcel proliferatie, migratie en differentiatie in honden mammatumoren.
Daarnaast waren er een groot aantal afbraak fragmenten van versican aanwezig in honden mammatumoren, en daarin waren door ADAMTS gegenereerde fragmenten aantoonbaar. Deze fragmenten vertegenwoordigen N-terminaal G1-domein bevattende V1/V0 versican dat is gesplitst in het ß-GAG domein; deze fragmenten zijn overvloedig aanwezig in de myxoide weefsels van complexe en gemengde tumoren. Dit bevestigt onze eerdere suggestie (Hoofdstuk 2) dat het G1-domein van versican in deze weefsels stapelt. Deze bevindingen wijzen op een gereguleerde versican afbraak en een speciale biologische rol voor de getrunceerde versican vormen in honden mammatumoren.

Het belangrijkste doel van het in **hoofdstuk 5** beschreven onderzoek was het identificeren en meten van de *in vivo* activiteit van proteases, waaronder een aantal MMPs en cathepsine B, die mogelijk participeren in de ECM ombouw en versican afbraak in honden mammatumoren. Daarvoor werd een combinatie van technieken gebruikt waaronder immunohistochemie, gelatine- en caseinezymografie, *in situ* zymografie met een gequencht fluorogeen DQ-gelatine substraat en een assay voor gequencht fluorescerend substraat. Gevonden werd dat MMPs werden uitgescheiden door zowel de tumorcellen als de stromale fibroblasten; *in situ* zymografie liet echter zien dat gelatinases hun activiteit voornamelijk uitoefenen in het tumorstroma, en dan vooral rond ductale structuren. Alle gebruikte methoden toonden aan dat de enzymen toegenomen waren in expressieniveau's en activiteit in de tumoren en hun metatstases, vergeleken met normale melkklier weefsels. Er was een tendens voor toegenomen protease activiteit, met name die van MMP-2, MMP-13 en cathepsine B, in eenvoudige carcinomen vergeleken met eenvoudige adenomen. Dit onderzoek is met name van belang voor veterinair onderzoek, omdat de *in vivo* activiteit van deze enzymen nooit eerder is aangetoond in tumoren van honden. De toename van de activiteit van deze enzymen in tumor weefsels en hun metatstases bevestigt de rol van deze enzymen in de ontwikkeling en de progressie van tumoren. Gelatinases lijken een belangrijke rol te spelen in de ombouw van *in situ* carcinomen naar invasieve lesies. Verdere studies zijn nodig

om er achter te komen welke van deze actieve proteases een bijdrage leveren aan de afbraak van versican in mammatumoren van honden.

In **hoofdstuk 6** wordt een overzicht gegeven van de belangrijkste bevindingen en conclusies van dit proefschrift en worden de verdere gewenste richtingen van onderzoek bediscussieerd.

Magyar összefoglaló

Az extracelluláris mátrix (ECM) kutatása kulcsfontosságúvá vált a daganatok kialakulásának és viselkedésének megértéséhez. Ma már tudjuk, hogy a sejtek és az ECM közötti kapcsolat aktívan befolyásolja a sejtek közötti jelátvitelt, valamint a sejtek viselkedését.
Az ECM vizsgálatára mindmáig kevés figyelmet szenteltek a kutyák emlődaganatainak kutatása során. Ezért a jelen PhD munka kísérletei az ECM összetételének és elrendeződésének a megismerésére irányultak, különös tekintettel a kondroitin-szulfát proteoglikánok egyik tagjára, a verzikánra.

Az **első fejezetben** áttekintést adunk a mátrix biológia jelenlegi nézeteiről, különös tekintettel a daganatokra vonatkozólag. Röviden ismertetjük azokat a mátrix molekulákat, amelyekkel a következő fejezetekben foglalkozunk. E fejezetben két sematikus ábrán keresztül bemutatjuk a verzikán doménszerkezetét (G1-domén, glikózaminoglikán (GAG)-domén, G3-domén) és az alternatív hasítása révén létrejött verzikán izoformákat (V0, V1, V2, V3).

A **második fejezet** a verzikán immunhisztokémiai vizsgálatát mutatja be a kutyák emlődaganataiban. Megmutatjuk, hogy míg a normál emlőszövetekben a verzikán nem jelenik meg fehérje szinten, addig a daganatok, mind a jó és a rosszindulatúak, megnövekedett mennyiségben tartalmazzák ezt a proteoglikánt. A verzikán három fő helyen expresszálódik a kutya emlődaganataiban:
1.: a periduktális szövetekben, feltehetően a duktusok bazális membránjával kapcsolatban;
2.: a rosszindulatú daganatok perifériás invazív területein, amely arra utal, hogy a verzikán szerepet játszhat a daganat sejtek inváziójában;
3.: a komplex és vegyes daganatok mioepithel-szerű sejtproliferációiban és mixoid szöveteiben. A verzikán 12C5 és 2B1 epitópjának expressziós mintázata a G1-es domén akkumulációjára utal, különösen a mixoid szövetekben.

A **harmadik fejezetben** arra kerestük a választ, vajon a verzikán nagymértékű expressziója a mioepithel-szerű sejtproliferációkban és mixoid szövetekben összefüggésben áll-e ezen szövetek esetleges porcirányú differenciálódásával.

Megmutatjuk, hogy a verzikánon kívül, két porcszövetre jellemző összetevő, a kollagén II és az aggrekán is magasabb szinten expresszálódik a komplex és vegyes típusú daganatokban. Továbbá, immunhisztokémiai vizsgálataink szerint ezen molekulák és a 3B3 neoepitóp, amely szintén a porcszövetekre jellemző, jelen van nemcsak a porcszöveteket tartalmazó vegyes daganatokban, hanem a mioepitheliómákban és a komplex és vegyes daganatok mioepithel-szerű sejtproliferációiban és mixoid szöveteiben. Ezek az eredmények azt mutatják, hogy a mioepithel-szerű sejtproliferációk és mixoid szövetek a komplex és vegyes típusú emlődaganatokban a vegyes daganatok ektopikus porcszöveteinek prekurzor szövetei. Továbbá, e fejezet eredményei arra utalnak, hogy az ektopikus porcszövet a (mio)epitheliális sejtek kötőszöveti (porc) sejtekké való átalakulásából származik. Ezen felül kimutattuk a hyaluronsav és a link protein (LP) expresszióját a kutyák emlődaganataiban, utalva arra, hogy a verzikán nagy valószínűséggel komplexet alkot a hyaluronsavval amelyet a link protein stabilizál. E három molekula kolokalizációja a daganatos sejtek klaszterei körül egy rendkívül viszkoelasztikus mikrokörnyezet kialakulására utal, amely elősegítheti a daganatos sejtek proliferációját és migrációját.

A **negyedik fejezetben** megmutatjuk, hogy a verzikán számos formában van jelen a kutya emlődaganataiban és ezek sejtvonalaiban, beleértve a különféle hasítási variánsokat és katabolikus termékeket. Meghatároztuk a mRNS hasítási variánsainak és azok fehérje termékeinek pontos expressziós mintázatát. A V1, V0 és V3 verzikán típusokat azonosítottuk, melyek közül túlnyomórészt a V1/V0 variánsok jelentek meg. A V2 variáns nem volt kimutatható. Megmutattuk, hogy a daganatos sejtvonalakban több mRNS, ill. V0 és V1 verzikán fehérje termelődik, mint a normál emlő fibroblasztokban, valamint a mioepithel-szerű daganatsejtek expresszálják a legnagyobb mértékben mindegyik mRNS hasítási variánst. Gyanítható, hogy a nagymértékben hidrofil V0/V1 variánsok magas szintű kifejeződése hozzájárul egy erősen hidratált mikrokörnyezet kialakításához a kutyák emlődaganataiban, ami optimális feltételeket nyújt a daganatsejtek proliferációja, migrációja és differenciálódása számára.
Ezen felül, számos verzikán fragmentum is jelen van kutyák emlődaganataiban, melyek közül az ADAMTS (A Disintegrin-like And Metalloprotease domain with ThromboSpondin type I motifs) által létrehozott fragmenseket azonosítottunk. Ezek az N-terminális G1-domént hordozó fragmentumok a V0/V1 verzikán ßGAG-aldoménjének hasításával jönnek létre, és a legnagyobb mennyiségben a komplex

és vegyes daganatok mixoid szöveteiben mutathatók ki. Ez megerősíti korábbi feltevésünket (2. fejezet), miszerint a verzikán G1 doménje akkumulálódik ezen szövetekben. Ezek az eredmények a verzikán szabályozott degradációját, és a csonkított verzikán formák megkülönböztetett biológiai szerepét jelzik kutyák emlődaganataiban.

Az **ötödik fejezetben** fő célunk azon proteázok azonosítása és *in vivo* aktivitásának mérése volt, melyek szerepet játszhatnak az ECM átalakításában és a verzikán degradációjában a kutyák emlődaganataiban, úgymint számos mátrix metalloproteináz valamint katepszin-B. Többféle technika kombinációját alkalmaztuk: immunhisztokémiai elemzéseket, zselatin és kazein zimográfiát, *in situ* zimográfiát fluorogén kioltásos D/Q-zselatin/szubsztrát, és fluoreszcens kioltásos szubsztrát vizsgálatokkal. A mátrix metalloproteinázokat mind a daganatsejtek, mind a stroma kötőszöveti sejtjei szekretálják, habár az *in situ* zimográfia kimutatta, hogy a zselatinázok aktivitásukat elsősorban a tumor stromában fejtik ki, különösen a duktális struktúrákat körül. Valamennyi módszer megemelkedett enzim expressziót és aktivitást mutatott ki a daganatokban és áttétjeikben a normál emlőszövetekhez képest. Emelkedett proteáz aktivitási tendencia mutatakozott különösen a MMP-2, MMP-13 és katepszin-B esetében az egyszerű karcinómákban az egyszerű adenómákhoz képest.
Ennek a tanulmánynak különös jelentősége van az állatorvosi kutatások terén, mivel ezeknek az enzimeknek az *in vivo* aktivitása korábban még nem került bemutatásra a kutyák daganataiban. A fokozott enzimaktivitás a daganatos emlőszövetekben és áttétjeikben megerősíti ezen enzimeknek a daganatok kialakulásában és progressziójában játszott szerepét. Eredményeink arra utalnak hogy a zselatinázok fontos szerepet játszhatnak az *in situ* karcinómák invazív folyamatokká alakításában. További vizsgálatok szükségesek annak azonosítására, melyek azok az említett aktív proteázok közül, amelyek hozzájárulnak a verzikán degradációjához a kutyák emlődaganataiban.

A **hatodik fejezetben** áttekintést adunk a tézisben leírt főbb eredményeinkről és következtetéseinkről, valamint megvitatjuk a kutatás jövőbeni folytatásának irányvonalait.

Appendix

WHO Histological Classification of Mammary Tumors of the Dog

Benign Tumors

Adenoma
- Simple adenoma
- Complex adenoma
- Basaloid adenoma

Fibroadenoma
- Low-cellularity fibroadenoma
- High-cellularity fibroadenoma

Benign mixed tumor

Duct papilloma

Malignant Tumors

Noninfiltrating (in situ) carcinoma

Complex carcinoma

Simple carcinoma
- Tubulopapillary carcinoma
- Solid carcinoma
- Anaplastic carcinoma

Special types of carcinomas
- Spindle cell carcinoma
- Squamous cell carcinoma
- Mucinous carcinoma
- Lipid-rich carcinoma

Sarcoma
- Fibrosarcoma
- Osteosarcoma
- Other sarcomas

Carcinosarcoma

Carcinoma or sarcoma in benign tumor

Unclassified Tumors

Table 1. Matrix metalloproteinases in mammalian. Classification, members and substrate specificity

MATRIX METALLOPROTEINASES (MMPs)				
Main groups	**MMP designation***	**Common names**	**Substrates**	**Bioactive substrates****
Collagenases	MMP-1	collagenase-1 fibroblast collagenase interstitial collagenase	collagen I-III, VII, VIII, X, gelatin, aggrecan, perlecan	L-selectin, α1-proteinase inhibitor, α1-antichymotrypsin, α2-macroglobulin, pro-IL-1ß, pro-TNFα, SDF-1, IGFBP-2,-3,-5
	MMP-8	collagenase-2 neutrophil collagenase	collagen I-III, VII, VIII, X, gelatin, aggrecan	L-selectin, α1-proteinase inhibitor, α2-macroglobulin, α2-antiplasmin, IGFBP, MCP-1, IP-10, MIG
	MMP-13	collagenase-3, rat collagenase	collagen I-IV, IX, X, XIV, gelatin, aggrecan	Endostatin, α1-antichymotrypsin, α2-macroglobulin, pro-TNFα, SDF-1, MCP-3
Gelatinases	MMP-2	gelatinase-A 72 kDa gelatinase 72 kDa type IV collagenase	gelatin, collagen I-IV, VII, X, aggrecan, versican, decorin	MCP-3, α1-proteinase inhibitor, α2-macroglobulin, KiSS-1/metastin, endothelin-1, CC-chemokine ligand 7, CXC-chemokine ligand 12, 12pro-IL-1ß, pro-TNFα, pro-TGFß, FGFR-1, SDF-1, IGFBP-3,-5
	MMP-9	gelatinase-B 92 kDa gelatinase 92 kDa type IV collagenase	gelatin, collagen IV, V, aggrecan	Zona occludens 1, IP-10, MIG, GCP-2, fibrin, NG2 PG, ENA-78, tumstatin, endostatin, plasminogen, α1-proteinase inhibitor, α2-macroglobulin, KiSS-1/metastin, IFN- ß, IL-2Rα, pro-IL-8,pro-VEGF, pro-TNF-α, pro-TGFß, IFN-ß, FGFR-1, SDF-1, GROα, CTAP-III

Table 1. continued

MATRIX METALLOPROTEINASES (MMPs)				
Main groups	**MMP designation***	**Common names**	**Substrates**	**Bioactive substrates****
Stromelysins	MMP-3	stromelysin-1, transin-1	collagen II, IV, IX-XI, gelatin, aggrecan, versican, perlecan, decorin, laminin	endostatin, plasminogen, α1-proteinase inhibitor, α1-antichymotrypsin, α2-macroglobulin, antithrombin III, pro-IL-1ß, pro-TNFα, pro-TGF ß1, pro-HB-EGF, IGFBP-3, SDF-1,E-cadherin, L-selectin, MCP-1,-2,-3,-4
	MMP-10	stromelysin-2, transin-2	collagen IV, laminin, fibronectin, elastin, aggrecan	unknown
	MMP-11	stromelysin-3	collagen IV, fibronectin, laminin, aggrecan	α1-proteinase inhibitor, α2-macroglobulin, IGFBP-1, α1-antitrypsin
Matrilysins	MMP-7	matrilysin, PUMP	fibronectin, laminin, elastin collagen IV, gelatin, aggrecan, versican, decorin, syndecan	endostatin, plasminogen, α1-proteinase inhibitor, α2-macroglobulin, pro-TNFα, pro-α-defensin, pro-HB-EGF, FasL, E-cadherin, ß4 integrin
	MMP-26	endometase, matrilysin-2	fibrinogen, fibronectin, gelatin	α1-proteinase inhibitor IGFBP-1
MT-MMPs	MMP-14	MT1-MMP, Membrane type MMP	fibronectin, laminin, gelatin, syndecan, aggrecan	tissue transglutaminase, α1-proteinase inhibitor, α2-macroglobulin, KiSS-1/metastin, fibrin αv ß3 integrin, CD44, SDF-1, MCP-3
	MMP-15	MT2-MMP	fibronectin, laminin, gelatin	tissue transglutaminase, fibrin, pro-TNFα

Table 1. continued

MATRIX METALLOPROTEINASES (MMPs)				
Main groups	**MMP designation***	**Common names**	**Substrates**	**Bioactive substrates****
	MMP-16	MT3-MMP	fibronectin, laminin, gelatin, syndecan	tissue transglutaminase, fibrin, KiSS-1/metastin, pro-TNFα
	MMP-17	MT-4MMP	fibrinogen, fibrin	pro-TNFα
	MMP-24	MT5-MMP	fibronectin, laminin, gelatin	KiSS-1/metastin
	MMP-25	MT6-MMP, leukolysin	gelatin	α1-proteinase inhibitor
Others	MMP-12	Macrophage metalloelastase	elastin, fibronectin, collagen IV	plasminogen, α1-proteinase inhibitor, pro-TNFα, endostatin
	MMP-19		aggrecan, elastin, fibrillin, collagen IV, gelatin	unknown
	MMP-20	Enamelysin	Aggrecan, amelogenin	unknown
	MMP-21	XMMP	aggrecan	unknown
	MMP-22			unknown
	MMP-23	CA-MMP	gelatin, casein, fibronectin	unknown
	MMP-27	CMMP	unknown	unknown
	MMP-28	Epilysin	unknown	unknown

*There is no MMP-4, MMP-5, or MMP-6. After matrilysin was discovered and designated as MMP-7, MMP-4 to MMP-6 were determined to be either MMP-2 or MMP-3. MMP-18 (collagenase-4) was isolated from Xenopus and no mammalian homolog has been identified.
** Substrates of MMPs distinct from typical ECM components

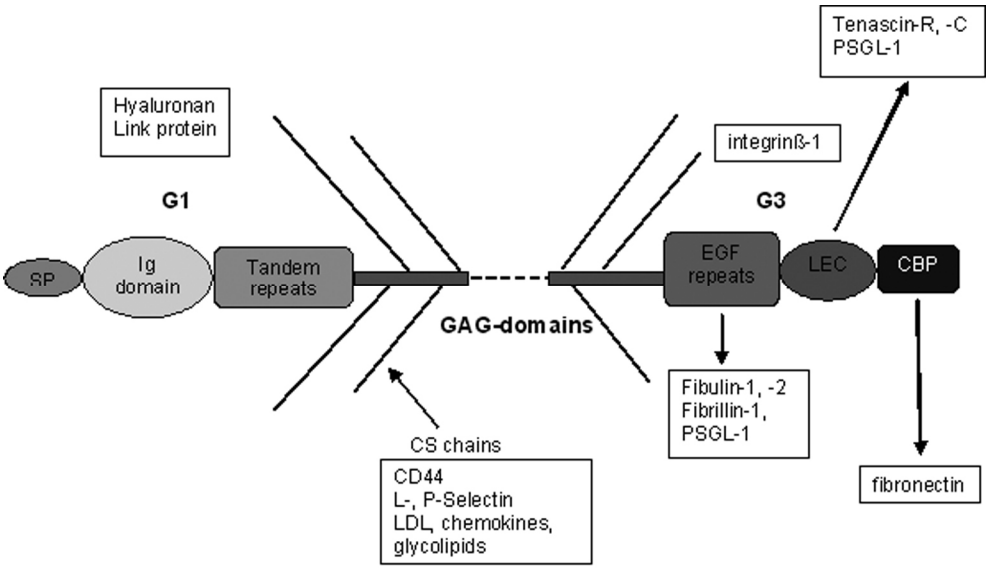

Figure 1. *Location of versican motifs and their interactions with other molecules* Figure 1.
SP- signal peptide; G1domain: immunoglobulin(Ig)-like domain, two tandem repeats (also known as HA-binding repeats); GAG-domains carrying the chondroitin sulfate (CS) chains; G3-domain: two epidermal growth factor (EGF)-like motifs; lectin (LEC)-like motif (also called the CRD- carbohydrate recognition domain); CBP- complement binding protein-like module; LDL - low density lipoproteins; PSGL-1 - P-selectin glycoprotein ligand.

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Anyu, ezt a kis könyvet bár én irtam, de neked köszönhetem. Kiskorom óta úgy neveltél, hogy a tanulás sohasem kényszert vagy kötelességet jelentett számomra, hanem élvezetet. Köszönök mindent, a szeretetet, a törődésedet és nem utolsó sorban a kimeríthetetlen türelmedet, amire sokszor szükséged lehetett az elmúlt 32 év során. Köszönöm mindannyiatoknak.

Publications

(in referred journals)

Erdelyi I, Meszaros A, Jansen IDC, Frederiks WM, Creemers LB, Everts V, van Dijk JE, Nederbragt H*: Localization and activity of matrix metalloproteinases and cathepsin B in canine mammary tumors.* In preparation

Erdelyi I, Braun KR, van Liere EA, van Ederen AM, Rutledge EA, van Yserloo B, van Dijk JE, Hellmen E, Wight TN, Nederbragt H: *Multiple forms of versican in canine mammary tumors and cell lines.* Submitted for publication

Erdelyi I, van Asten AJ, van Dijk JE, Nederbragt H: *Expression of versican in relation to chondrogenesis-related extracellular components in canine mammary tumor*s. Histochem Cell Biol 2005, 124: 139-149

Erdelyi I, Nieskens DHM, van Dijk JE, Vass L, Nederbragt H: *Immunohistochemical evaluation of versican, in relation to chondroitin sulphate, in canine mammary tumors.* Histol Histopath 2003, 18:1067-1080

Szell Z, Erdelyi I, Sreter T, Albert M, Varga I: *Canine ocular onchocercosis in Hungary.* Vet Parasitol 2001, 97:245-251

Szell Z, Sreter T, Erdelyi I, Varga I: *Ocular onchocercosis in dogs: aberrant infection in an accidental host or lupi onchocercosis?* Vet Parasitol 2001, 101:115-125

Széll Z, Máthé Á, Erdélyi I, Deim Z, Bende Z, Varga I.: *Spirocercosis and Alariosis in dogs - two rare case reports and a short review of the literature.* Magyar Állatorvosok Lapja, 2001, 123:421-428

CONTRIBUTION/SUPERVISION TO UNIVERSITY UNDERGRADUATE THESES

2000 *Comparison of the value of cytology, histopathology and immunohistochemistry in the diagnostics of canine non-Hodgkin lymphomas*, Veterinary Faculty, Szent István University, Budapest

2000 *Use of the Agnor technique in the diagnostics of canine non-Hodgkin lymphomas*, Veterinary Faculty of Szent István University, Budapest

2001 *Immunohistochemical examination of canine round cell tumours*, Veterinary Faculty of Szent István University, Budapest

Curriculum Vitae

PERSONAL DATA

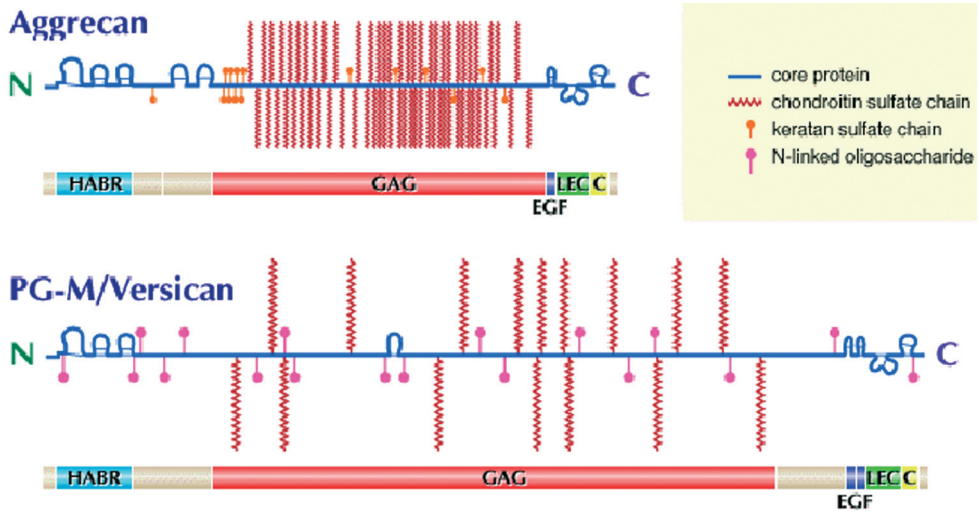
Name : Ildikó Erdélyi (Ms)
Nationality : Hungarian
Date of birth : 31/10/1973
Place of birth : Budapest, Hungary

PROFESSIONAL HISTORY

July 2005 - present **Research Associate/Part-time resident in veterinary pathology**
Strang Cancer Prevention Center, Chemoprevention Laboratory/ The Rockefeller University, New York
October 2002 - April 2005 **PhD candidate,** Department of Pathobiology, Pathology Divison, Faculty of Veterinary Medicine Utrecht University. PhD subject: Extracellular Matrix in canine mammary tumours, with special focus on versican, a versatile extracellular proteoglycan
April 2001 - September 2002 Marie Curie Research Fellow (contract No: HPMT-CT-2000-00005), Hungarian State Eötvös Scholarship fellow (417-1/2202. MÖB), Department of Pathobiology, Pathology Divison, Faculty of Veterinary Medicine Utrecht University.
Project: "Immunohistochemistry of epithelial cell-stroma interaction in mammary tumours"
October 1998 - March 2001 **Assistant lecturer**, Department of Pathology, University of Veterinary Science, Budapest
September 1997 - September 1998 **Trainee,** Department of Pathology, University of Veterinary Science, Budapest
2000-2003 **Editor** of a bimonthly Hungarian veterinary journal Kisállatpraxis

EDUCATION

January 2000 **Certificate of Laboratory Animal Science and Experimental Design** postgraduate course, Budapest
December 1997 **Doctor of Veterinary Medicine, University of Veterinary Science,** Budapest 1992-1997, cum laude, Thesis topic: Immunohistochemical evaluation of tumours of dogs
1988-1992 Eötvös József Secondary School, Italian Language Class, Budapest



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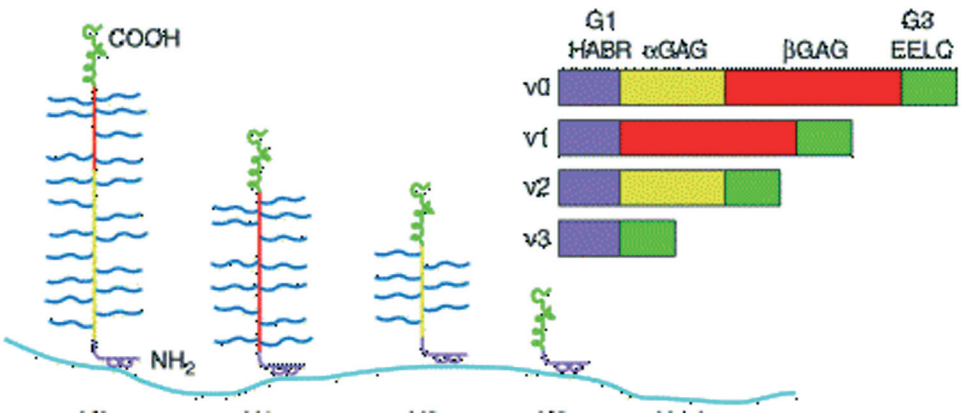

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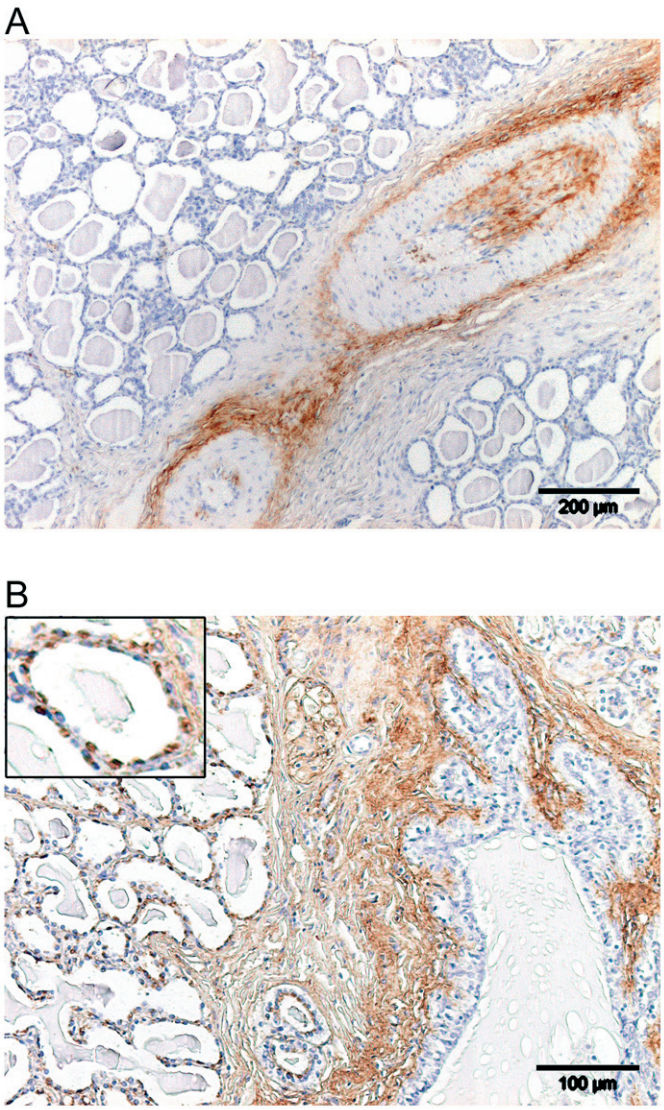
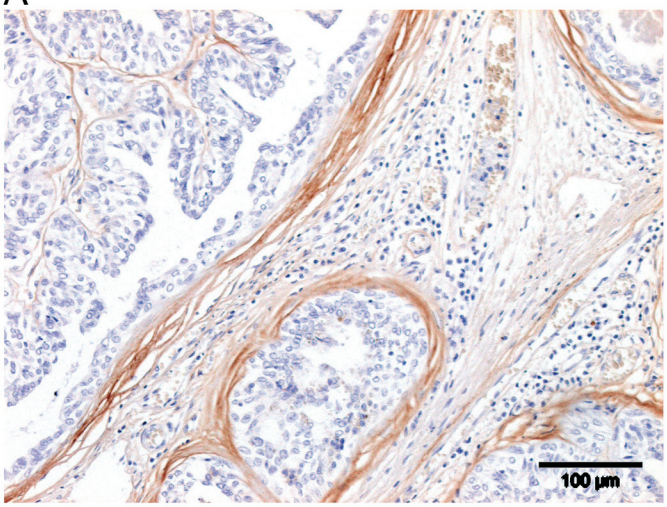


Chapter 2 - Figure 1

A



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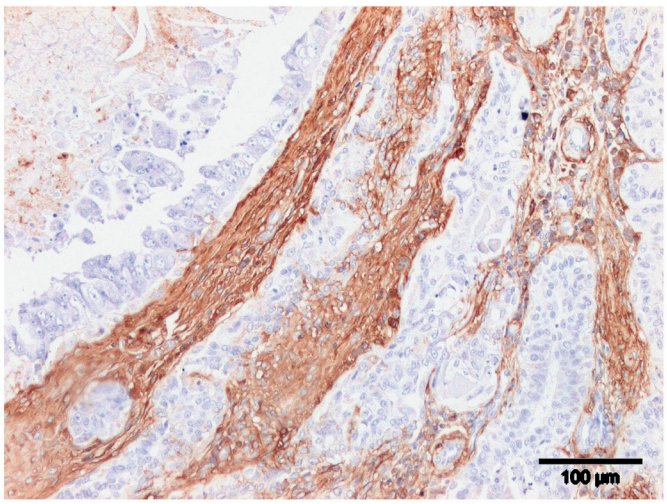

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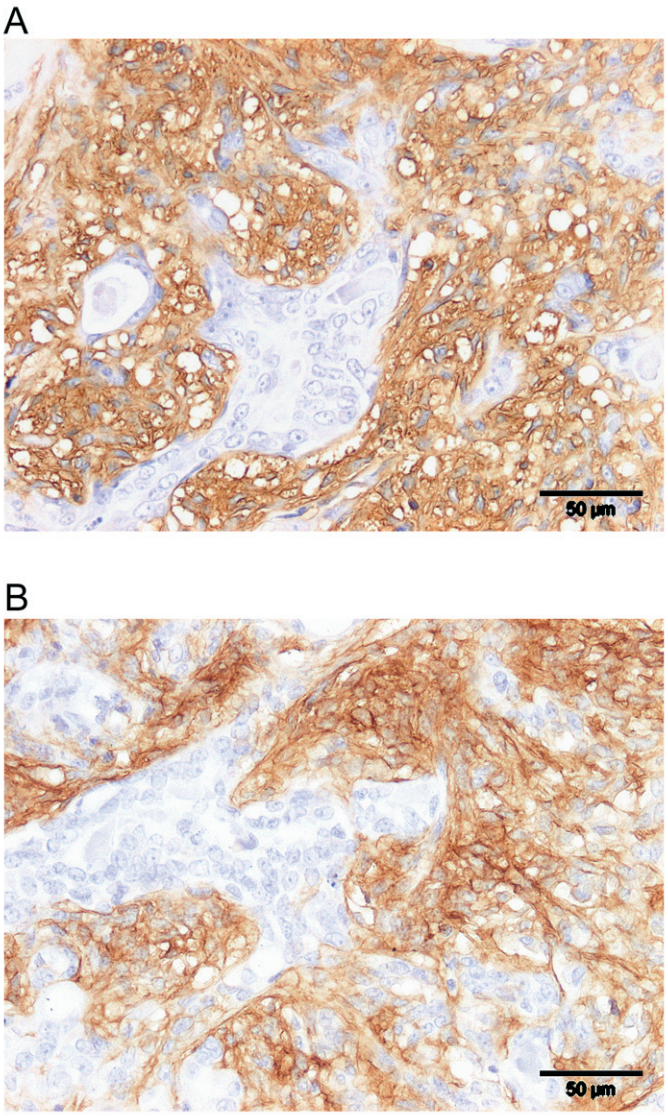

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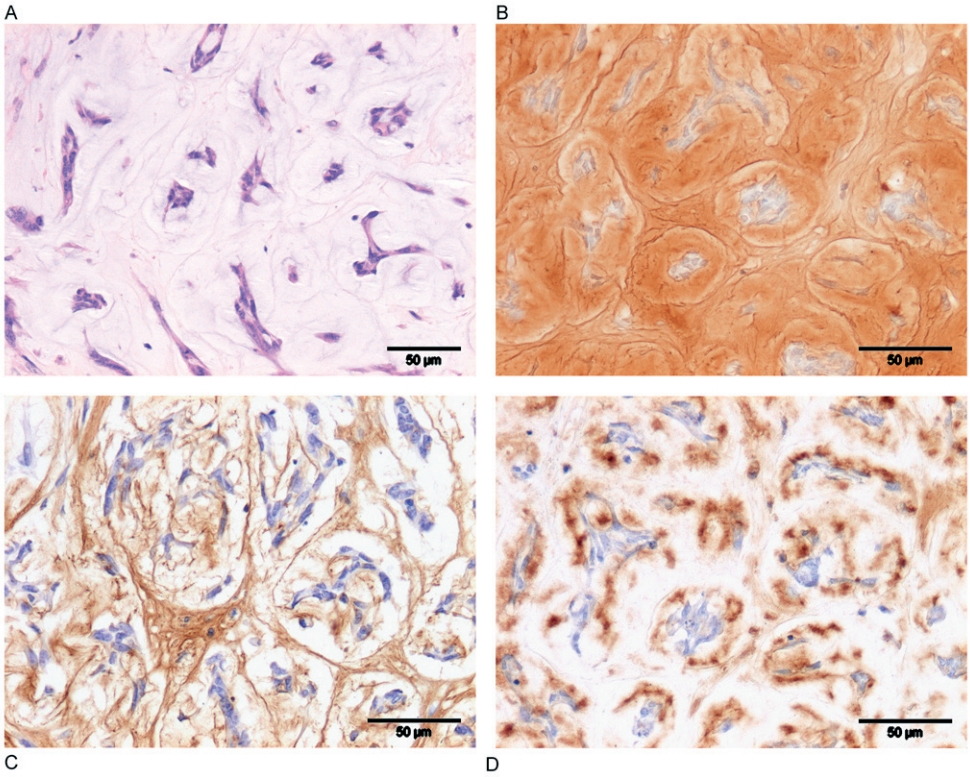

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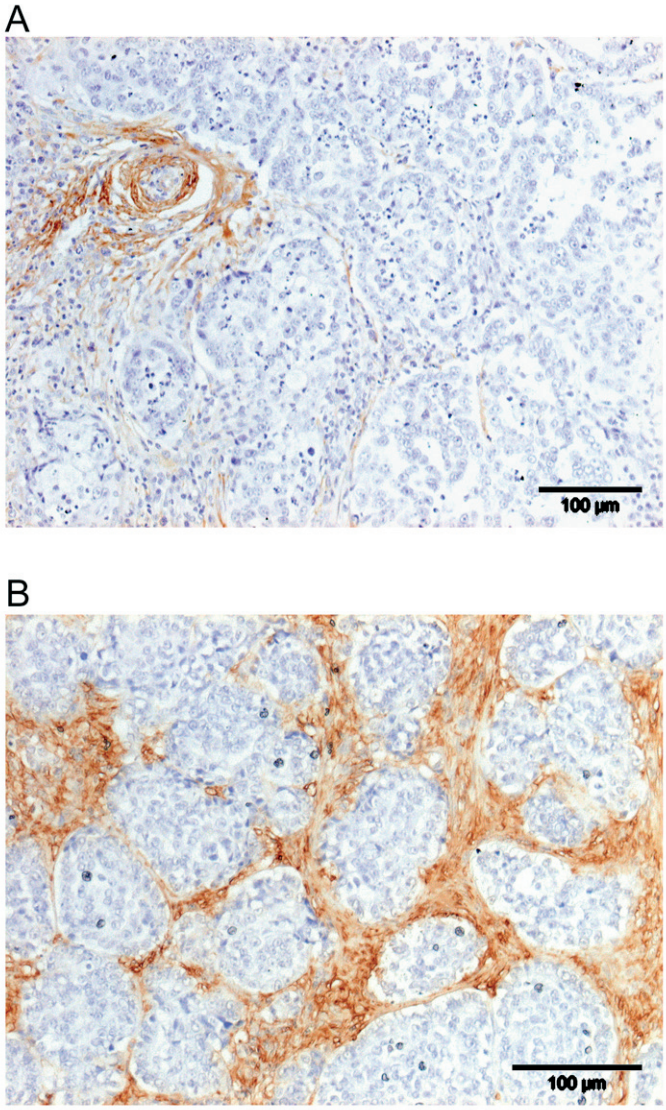

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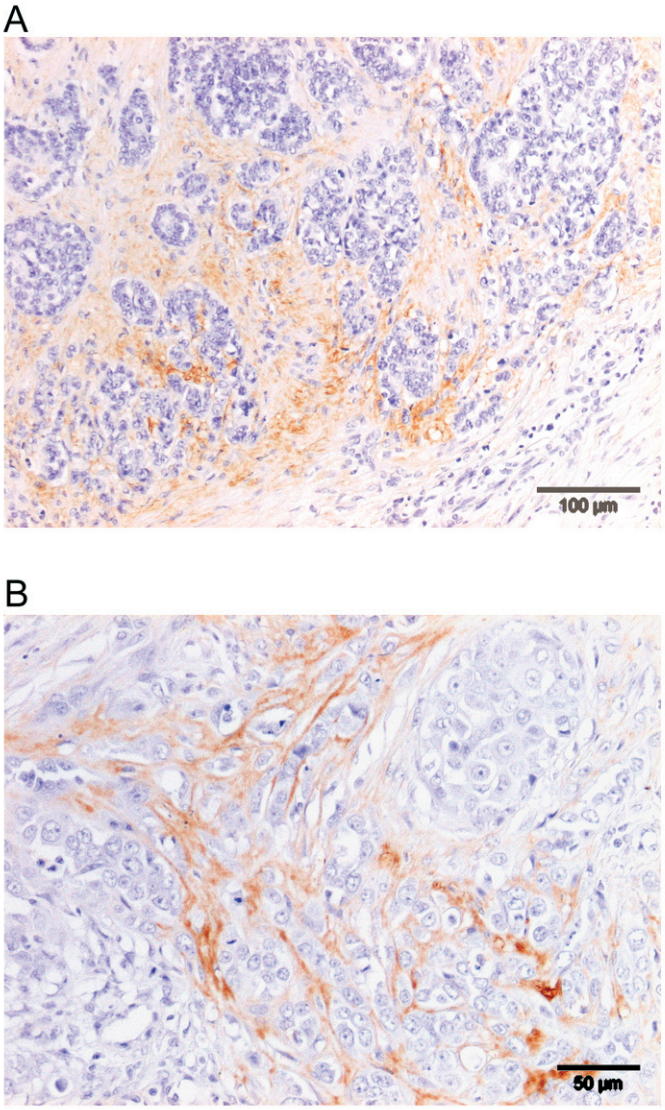

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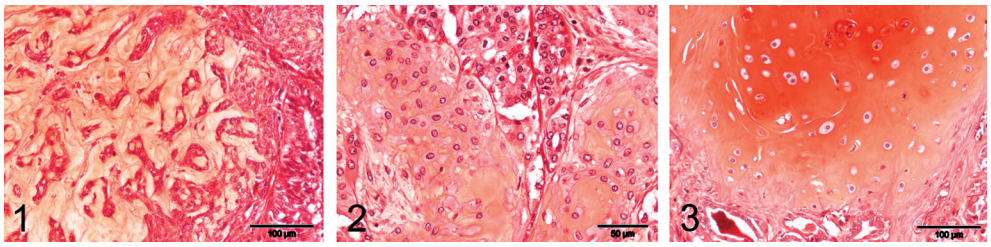

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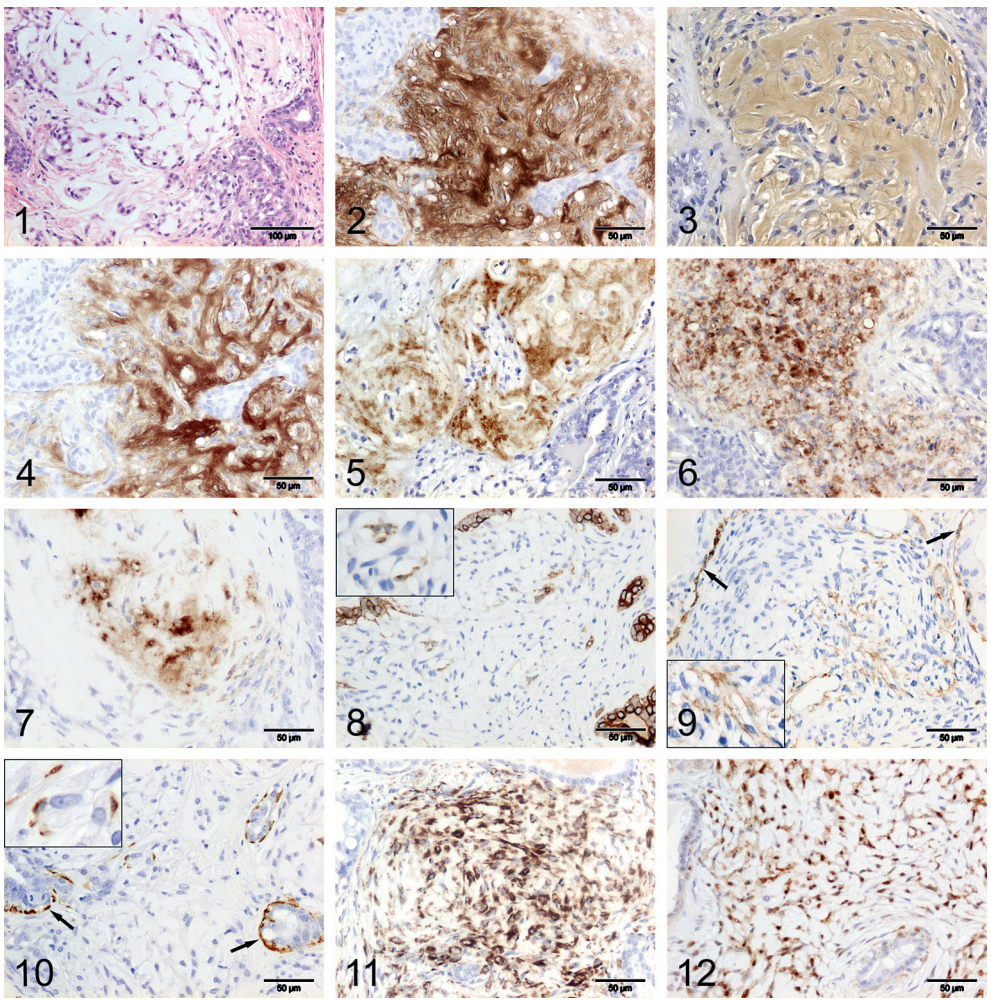

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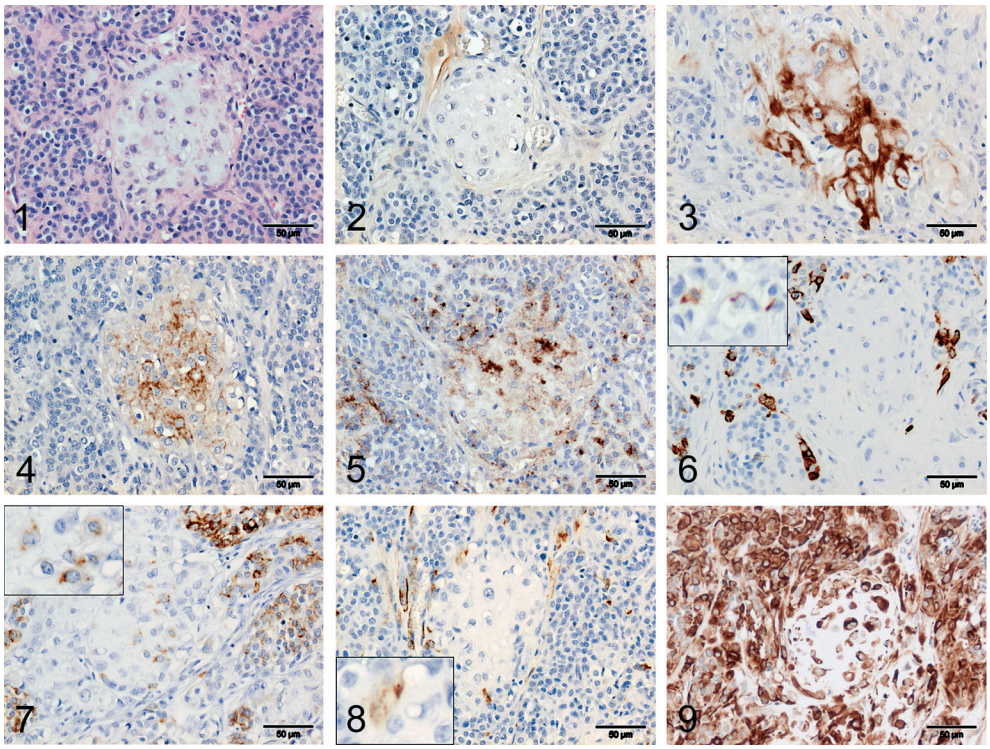

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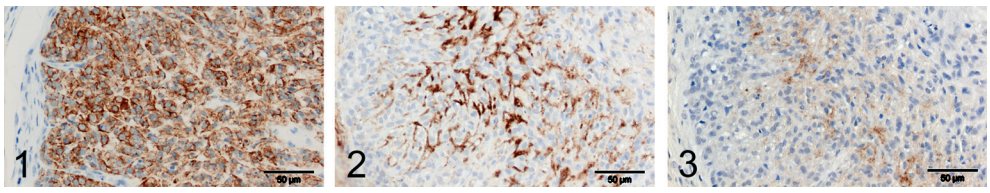

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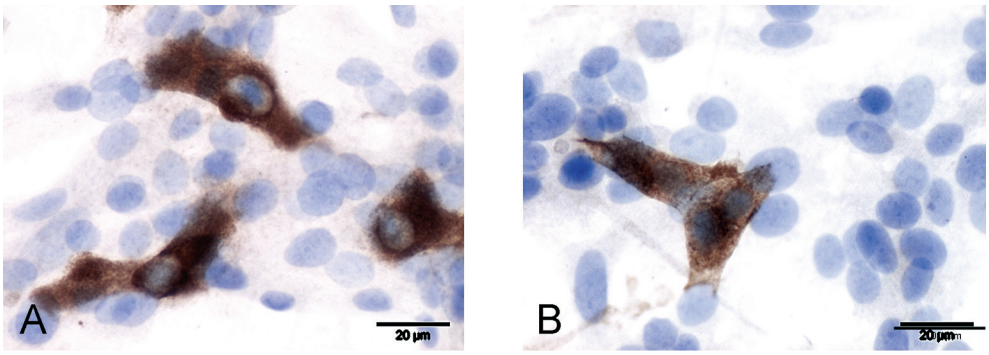

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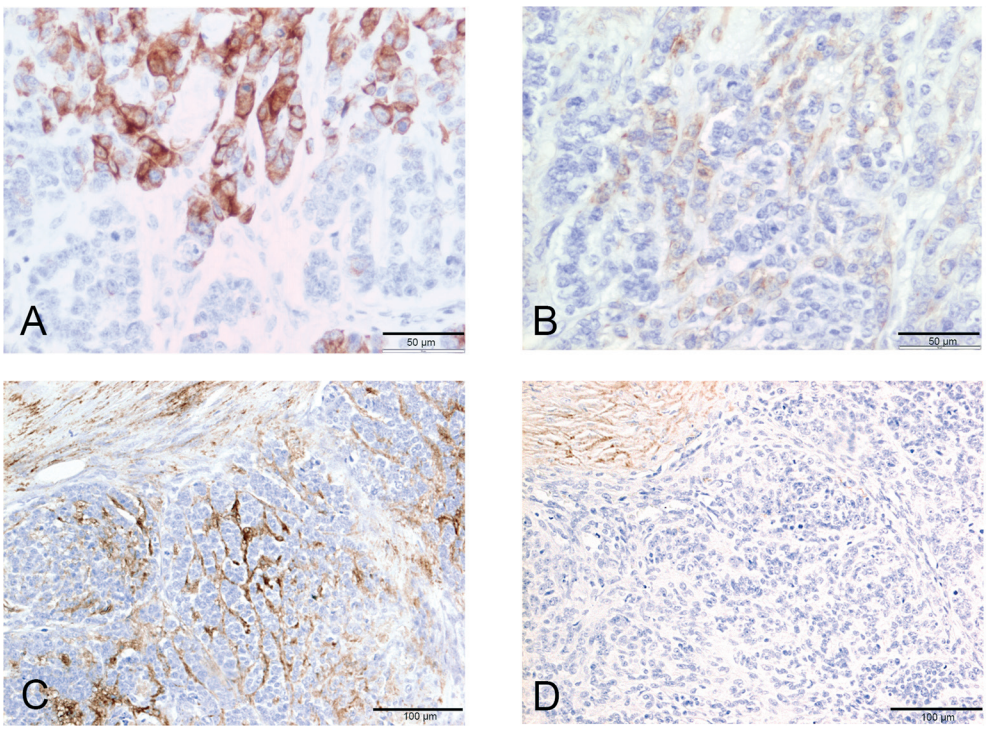

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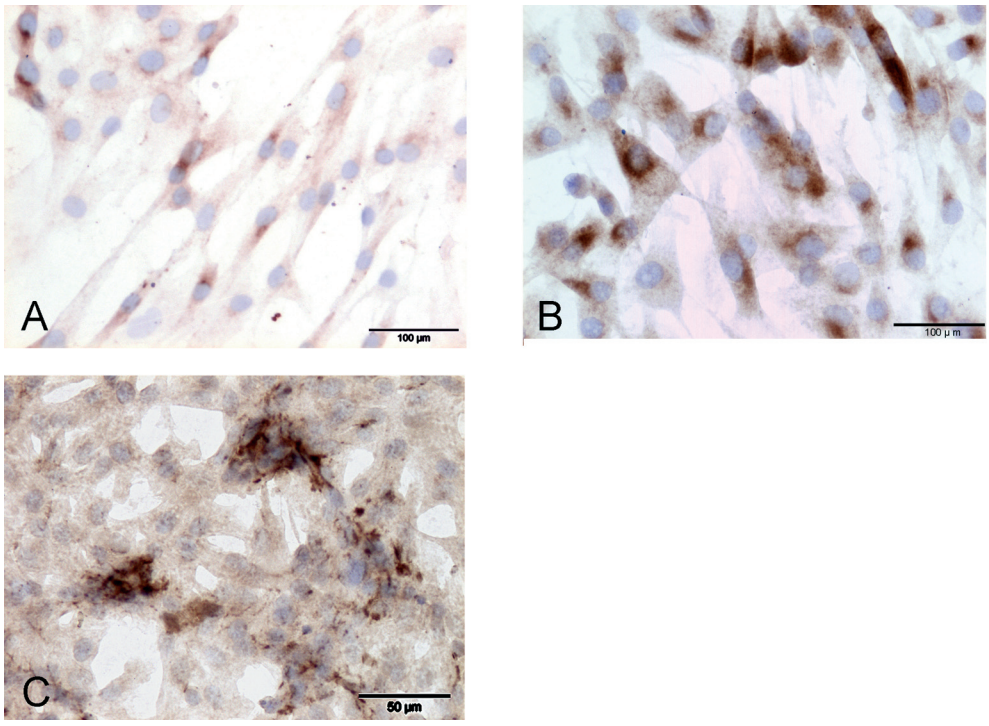

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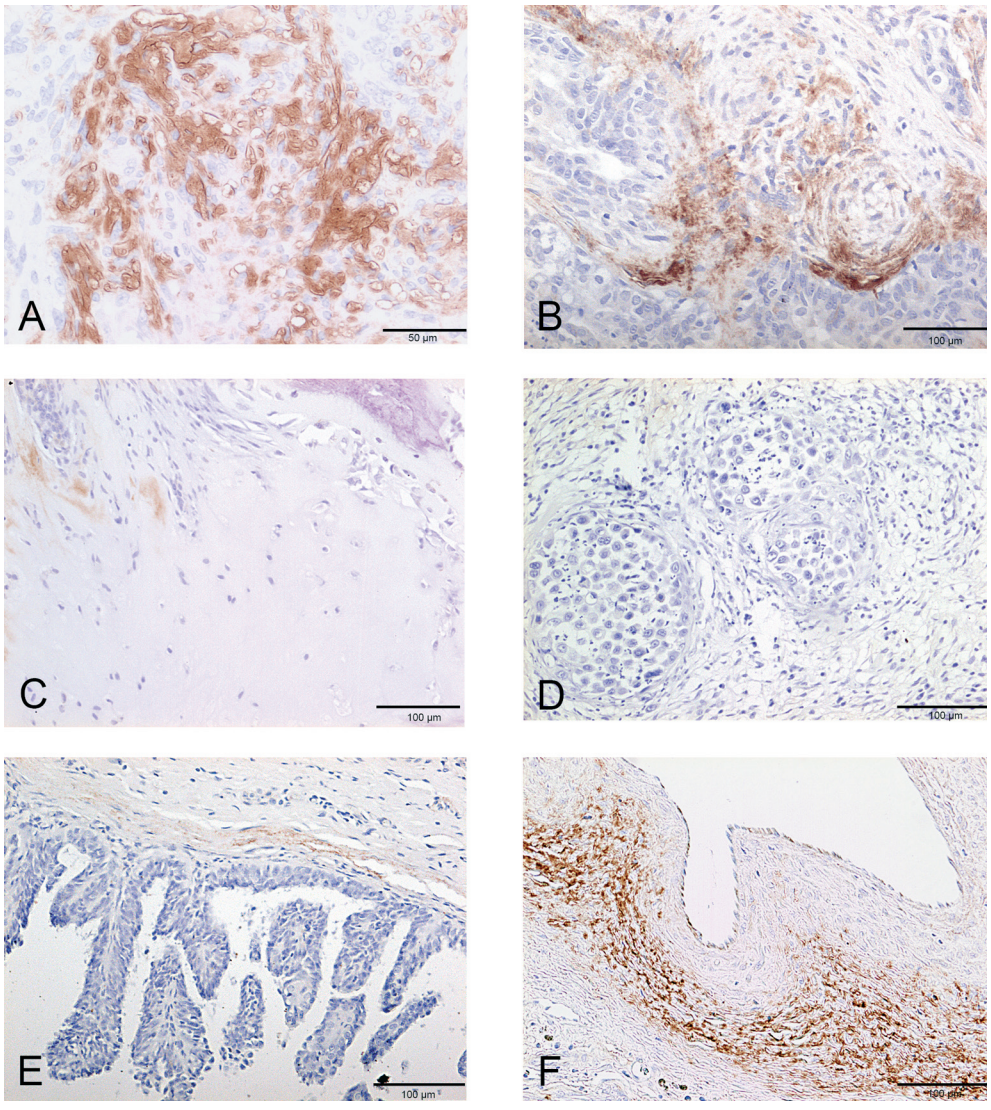

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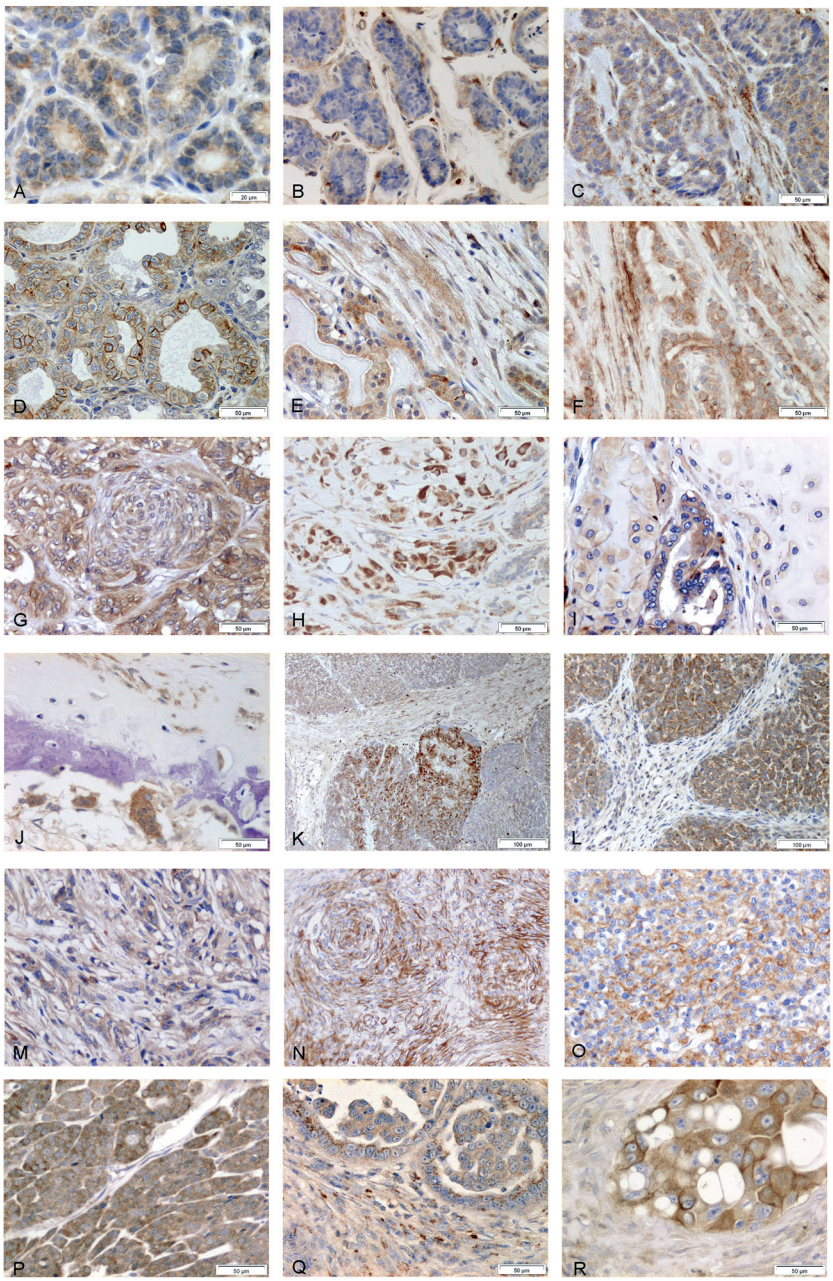

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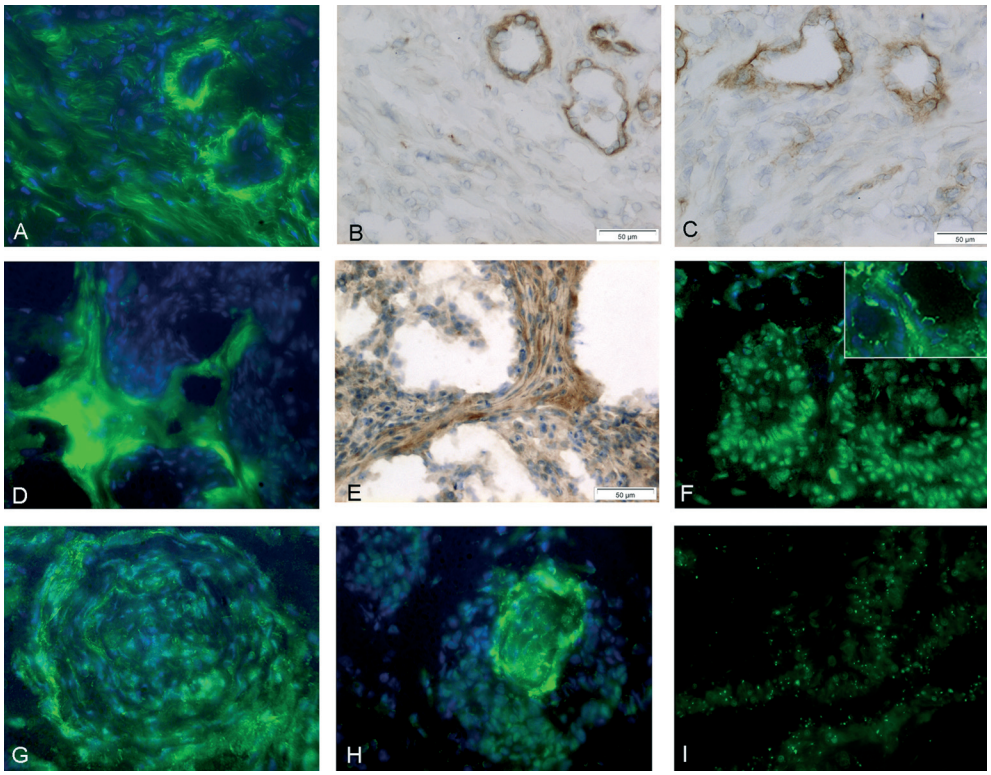

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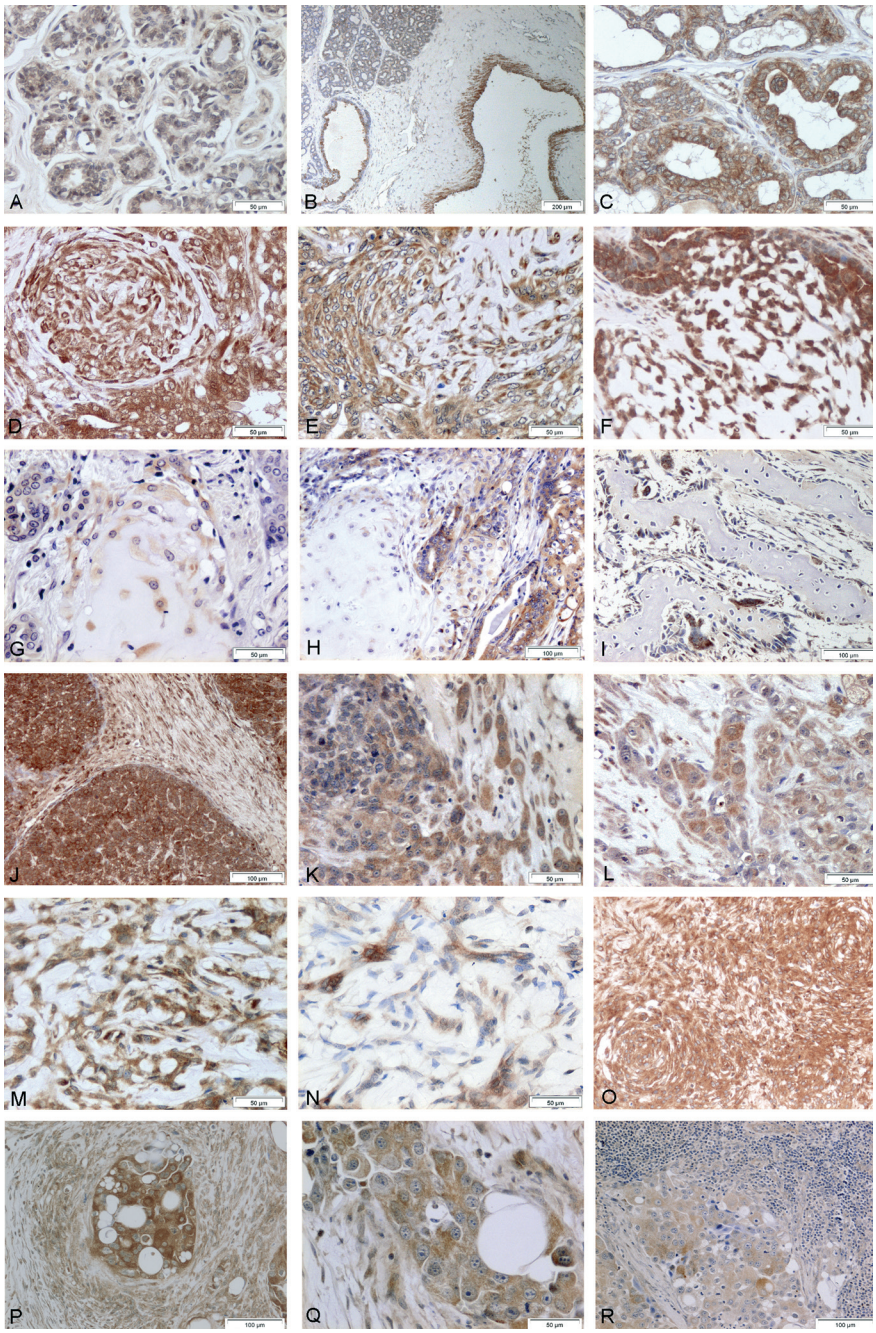

Chapter 5 - Figure 6