Institute of Veterinary Pathology, Department of Veterinary Medicine

Freie Universität Berlin

Global Protein Analyses of Canine Mammary Gland Tumors

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Veterinarian from Berlin

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Globale Proteinexpressionsanalysen Kaniner Mammatumoren

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Für Emma & Rainer

Contents

List of Abbreviations

2D-GE	**Two**-**D**imensional **G**el **E**lectrophoresis
2D-DIGE	**Two**-**D**imensional **Di**fference **G**el **E**lectrophoresis
ADA	**A**denosine **De**a**a**minase
AID/ AICDA	**A**ctivation **I**nduced **C**ytidine **De**a**a**minase
ALDH1	**Al**dehyde **D**e**h**ydrogenase **1**
BRCA 1 / 2	**Br**east **Ca**ncer Associated Genes **1** / **2**
CALU	**Calu**menin
CMT	**C**anine **M**ammary **T**umor
CSC	**C**ancer **S**tem **C**ell
CTC	**C**irculating **T**umor **C**ell
DNA	**D**eoxyribo**n**ucleic **A**cid
ER	**E**strogen **R**eceptor
et al.	**et al**ii (latin for “and others”)
GSN	**G**el**s**oli**n**
IHC	**I**mmuno**h**isto**c**hemistry
Ki67	Antigen identified by monoclonal antibody Ki67
MS	**M**ass **S**pectrometry
p53	(Tumor) **P**rotein **53**
PCNA	**P**roliferating **C**ell **N**uclear **A**ntigen
PCR	**P**olymerase **C**hain **R**eaction
PGAM1	**P**hospho**g**lycer**a**te **M**utase **1**
PR	**P**rogesterone **R**eceptor
RAD51	**Rad**iation Induced Protein **51**
(m) RNA	(**m**essenger) **R**ibo**n**ucleic **A**cid
TNFα	**T**umor **N**ecrosis **F**actor **alpha**
TPM 1 / 3	**T**ro**p**o**m**yosin **1** / **3**
WHO	**W**orld **H**ealth **O**rganization

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1 Introduction

Despite ongoing research efforts, many questions remain regarding the biology of canine mammary tumors. It is still unclear how canine mammary carcinomas develop from normal mammary gland and which molecular features enable a carcinoma to metastasize.

1.1 Background

1.1.1 Epidemiology

Mammary gland tumors are one of the most frequent tumors in female dogs – moreover, metastasis of the primary tumor to distant organs is the most common cause of tumor-related death in these patients (Dorn et al., 1968). Approximately 100 to 200 tumors per 100,000 individuals are diagnosed every year (Dobson et al., 2002, Egenvall et al., 2005) and the median age of onset is 10 years (Boldizsar et al., 1992, Moe, 2001). In contrast, the mammary tumor incidence for male individuals is less than 1.0 per 100,000 patients per year (Withrow and MacEwen, 2007).

1.1.2 Predisposing Factors

One of the few confirmed predisposing factors for the development of canine mammary gland tumors is the time of castration. The life time risk increases from 0.05 % when spayed before first estrus up to 26 % when spayed after second estrus (Schneider et al., 1969). Later castration after the third estrus has no influence on the incidence of malignant tumors (Misdorp, 1991). Other yet less relevant predisposing factors are chemical contraceptives (Stovring et al., 1997) and obesity (Sonnenschein et al., 1991). A breed predisposition is unproven, but dachshund and poodle seem to have an increased incidence, as well as small breeds in general (Priester and McKay, 1980). An early pregnancy has no influence on the tumor incidence (Schneider et al., 1969).

1.1.3 Prognosis

The prognosis for dogs with mammary tumors depends heavily on the histologic diagnosis of the tumor. About 60 % of all canine mammary tumors (CMT) are diagnosed as benign, thus are not expected to metastasize. Out of the remaining 40 % malignant tumors, another 60 % remain local neoplasms without metastatic spread. Therefore, only 16 % of all CMT metastasize (Bostock, 1986, Benjamin et al., 1999), and are associated with an estimated survival time of 3 to 9 months, regardless of the therapy applied (Morris and Dobson, 2001).

Early differentiation between metastasizing and non-metastasizing carcinomas is still a diagnostic challenge because relevant metastasis markers are unknown.

This lack of sensitive and specific metastasis markers is mainly based on a lack of knowledge on the molecular details of mammary tumor carcinogenesis. For example, it is still uncertain whether a differentiated mammary epithelial cell or a stem-cell within the mammary gland or any differentiation status in between constitutes the origin of tumor development. Furthermore, it is unclear whether metastasis is driven by continuous accumulation of mutations during a *malignant progression* or if the dignity of a tumor is *determined early*.

1.2 Development of Malignancy

1.2.1 Tumor Development

Currently, tumor development is generally divided into three phases, called initiation, promotion and progression. All of them are equally necessary for tumor evolution (Barrett, 1993).

During *initiation*, irreversible mutations occur and directly or indirectly influence cell cycle regulation (Friedewald and Rous, 1944, Berenblum, 1957, Troll and Wiesner, 1985). In particular, proto-oncogenes and tumor suppressor genes are affected (Barrett, 1993). For instance, in 5 to 10 % of all human breast cancer patients the tumor suppressor genes BRCA 1 and 2 (breast cancer associated genes 1 and 2), major factors of the cellular DNA repair mechanism, feature mutations (Krebs in Deutschland 2007/2008). In canines, an association between CMT development and BRCA / radiation induced protein 51 (RAD 51) complex expression is assumed, too, although actual mutations are still unknown. Recent studies revealed increased mRNA levels of RAD 51 and BRCA 2 in mammary gland carcinomas and lymph node metastases in comparison to normal mammary gland, whereas expression levels were even higher in metastases than in the primary tumor. Adenomas showed reduced expression levels when compared to normal mammary gland (Klopfleisch and Gruber, 2009). Another well-known cause for mutations in breast cancer is the deletion of amine groups by activation induced cytidine deaminase (AID or AICDA). AID is activated by high estrogen levels and may thereby cause mutations in proto-oncogenes and tumor suppressor genes (Pauklin et al., 2009). A similar mechanism of mutagenesis has, however, not yet been established for CMT.

During *promotion* the initiated cells become activated by physiological selective stimuli, like growth factors, hormones or regeneration processes after tissue damage (Berenblum, 1957, Troll and Wiesner, 1985). Interestingly, a longer lasting estrogen influence in intact dogs seems to be the most predisposing factor for mammary gland tumors in canines (Schneider et al., 1969, Morris and Dobson, 2001). A possible explanation could be a chronic hormonal influence in non-neoplastic mammary epithelium, which increases the risk of an accidentally initiated cell becoming activated. However, in women the situation is different, as an early pregnancy and lactation period with high estrogen levels reduces breast cancer risk (Krebs in Deutschland 2007/2008).

The last step of tumor development, *progression*, is regarded as a clonal selection and proliferation of tumor cells. Only tumor cells which are able to survive in the adverse, hypoxic and nutritionally deficient environment of a proliferating tumor mass will finally contribute to the tumor mass. In a subsequent step, some tumor cells are able to lose their attachment to

the primary tumor, spread out systemically and may develop metastasis (Baumgärtner and Gruber, 2010, McGavin and Zachary, 2012). In malignant mammary gland tumors this full progression to metastatic disease is a rather uncommon event, since about 60 % of all carcinomas do not metastasize and stay locally, although their histological patterns are indistinguishable from that of metastasizing carcinomas (Benjamin et al., 1999).

In general, most processes of initiation and progression are unrevealed. It is still unclear whether metastatic behavior is the result of a linear malignant progression from normal mammary gland to metastatic tumors or if the capability of metastasizing is an inherent and early determined feature. Moreover, the cell of origin for CMT is still unclear.

1.2.2 Cell of Origin

In the *traditional model* of carcinogenesis, it is assumed that a somatic parenchymal cell undergoes symmetric proliferation after the initiation phase. As a consequence, the tumor mass consists of identical tumor cells, which are all able to create further tumor cells. During progression, an asymmetrical dedifferentiation and a heterogenic tumor mass may develop (La Porta, 2012).

In contrast, the so called *cancer stem cell theory* proposes that cancer stem cells (CSC) are the cells of tumor origin. Stem cells are pluripotent and exist in almost every organ for the purpose of auxiliary cells. They have the ability of self-renewal, as well as the capacity to differentiate. Cancer stem cells feature the same characteristics. Asymmetric cell division leads to the development of a heterogeneous tumor mass of CSC and differentiated tumor cells. The latter represent the majority of the tumor mass but do not have self-renewal potential and are thus not tumorigenic. Further dedifferentiation may however occur during progression, similar to the traditional model of tumor development (Ginestier et al., 2007, Charafe-Jauffret et al., 2009).

Recent studies have confirmed the cancer stem cell theory. Transplantation of human breast cancer cells into immunocompromised mice revealed that only a (CD44+/CD24-) subpopulation of tumor cells has the capacity to reproduce the heterogeneity of the primary tumor (Al-Hajj et al., 2003). Another well-established stem cell marker is aldehyde dehydrogenase 1 (ALDH1) which seems to play an important role in early differentiation of stem cells (Chute et al., 2006). It is a detoxifying enzyme which oxidizes retinol to retinoic acid (Yoshida et al., 1998, Chute et al., 2006). In human breast carcinomas increased ALDH1 activity levels identified the subpopulations with tumorigenic capacities (Ginestier et al., 2007). Similar observations have been made in multiple myeloma and acute myeloid leukemia (Matsui et al., 2004, Pearce et al., 2005).

In conclusion, evidence suggests that a stem-like cell forms the cell of origin of tumor development in most tumor types. However, it is still arguable whether the final outcome of tumor progression is determined right from the start (*early determination*) or depends on the time of tumor evolution (*malignant progression*). Thus, more details about the pathogenesis of tumors, and in particular of mammary gland tumors, would be desirable as they could reveal new diagnostic and therapeutic targets.

1.2.3 Malignant Progression

The investigation of the metastatic cascade is in the focus of cancer research worldwide. During the last 50 years several theories have been established and partly abandoned.

In 1965, J. Leighton postulated that only genotypically diverse tumor cell subpopulations in a primary tumor have metastatic potential (Leighton, 1965). Based on findings in cultured B16 melanoma cells, Fidler and Kripke complemented “Leighton’s hypothesis” with the assumption that these metastatic subpopulations arise only late during tumorigenesis by accumulation of somatic mutations (Fidler, 1973, Fidler and Kripke, 1977). In the early 1980s, metastasis assays confirmed that cultured tumor cells have increased metastatic potential in comparison to the original cell line cells (Stackpole, 1981) but most probably only due to “artificial” *in vitro* selection. In conclusion, a spontaneous metastasis model was established which proposed that all cells within a tumor have an equal capability to metastasize (Giavazzi et al., 1980, Mantovani et al., 1981, Milas et al., 1983, Vaage, 1988).

In 1984, R. P. Hill and V. Ling established the dynamic heterogeneity model which proposed that metastatic subpopulations constantly appear and disappear and generally change their genotype and phenotype within a tumor. The frequency with which they arise thereby defines the metastatic potential of the primary tumor (Hill et al., 1984, Ling et al., 1985). Three years later, R. S. Kerbel postulated the clonal dominance theory which proposed that a once developed subclone with metastatic potential will necessarily overgrow and dominate the original tumor mass (Kerbel et al., 1987, Kerbel et al., 1988).

Another model, which is similar to Fidler’s theory about tumorigenesis, is the so called “Vogelgramm”. It is named after its first descriptor Bert Vogelstein who proposed that, for colorectal cancer, approximately five different genetic or epigenetic changes must occur before a metastasizing tumor arises. Again, this theory is based on the idea that tumor cells accumulate somatic mutations during their progression and acquire thereby features of increasing malignancy, like metastatic capacity (Vogelstein et al., 1988, Fearon and Vogelstein, 1990). This multistep-carcinogenesis assumes that every benign tumor is able to metastasize under appropriate circumstances. In a recent study, Sorenmo and colleagues

hypothesized that CMT are also the result of a malignant progression with an accumulation of somatic mutations (Sorenmo et al., 2009). The assumption of a malignant progression was based on the facts that dogs with malignant tumors were significantly older than those with benign tumors, and malignant tumors were significantly larger than benign ones, and malignant tumors thus seem to be a later stage of development.

Finally, the genometastasis hypothesis by Garcia-Olmo and colleagues proposed that distant metastases occur by plasma-circulating DNA fragments of oncogenes rather than by circulating tumor cells (Garcia-Olmo et al., 1999, Garcia-Olmo and Garcia-Olmo, 2001). Nevertheless, their hypothesis has not been supported by further studies of independent research groups.

Details of the molecular processes during this multistep-carcinogenesis are still unknown. It is, for instance, unclear whether expression levels of certain specific proteins correlate with the stages of malignancy or if a once activated or deactivated protein retains the same expression level during progression from normal gland to metastasizing carcinomas.

1.2.4 Early Determination

A completely different model of the carcinogenesis and malignant progression of breast cancer was established by Laura van't Veer and colleagues. In 2002, they postulated a new theory about tumor progression which proposed metastatic capacity as an early and inherent feature of mammary gland tumors. They examined human breast cancer specimens by DNA microarray and identified an expression profile of 70 genes which enabled them to distinguish between a "good prognosis"-gene signature and a "poor prognosis"-gene signature at an early stage of tumor development (van't Veer et al., 2002). In addition to this theory, Massagué and colleagues postulated a tissue-specific gene expression in tumors with a "poor prognosis"-signature predicting the site of metastasis (Kang et al., 2003, Minn et al., 2005). In the parallel evolution model by Schmidt-Kittler, different gene signatures in the primary tumor and in disseminated tumor cells in the bone marrow of the same patient led to the assumption that metastasis formation and primary tumor development occur independently (Schmidt-Kittler et al., 2003). As it cannot be proven that disseminated cells are related to the primary tumor mass and are able to form a distant metastasis, the model is still controversial (Weigelt et al., 2005).

In conclusion, based on the findings of "good prognosis"- and "poor prognosis"-gene signatures, differences in the protein expression patterns of metastasizing and non-metastasizing canine mammary carcinomas should be detectable and may allow for the development of valuable new diagnostic and therapeutic approaches to CMT.

1.3 Prediction of Malignancy

Based on the current knowledge on canine mammary tumors, it is still difficult to distinguish between metastasizing and non-metastasizing mammary gland carcinomas before metastatic spread actually occurs. Several immunohistochemical markers have been tested but collectively failed to allow for metastasis prediction superior to histological examination of the tumor mass (Klopfleisch et al., 2011b).

1.3.1 Histological Criteria

Histopathologic examination of surgically excised tumor specimens is still the basis for mammary gland tumor diagnostics. Classification, according to the WHO (World Health Organization), is based on the tissue differentiation and the tumor dignity (Misdorp et al., 1999). Consequently, mammary gland tumors are distinguishable into:

Table 1: Classification of Canine Mammary Tumors According to the World Health Organization (Misdorp et al., 1999)

		Dignity	
		Benign Tumors	**Malignant Tumors**
Tissue Differentiation	**Epithelial / Myoepithelial**	Adenoma	(Adeno)Carcinoma
	Mesenchymal	Fibroma	Sarcoma
	Epithelial / Myoepithelial + Mesenchymal	Benign mixed Tumor	Malignant mixed Tumor (Carcinosarcoma)

Several subtypes including tubulopapillary and solid carcinomas or simple and complex adenomas are known in veterinary medicine as well (Misdorp et al., 1999). However, these are of minor importance compared to human medicine, as information on the predictive value of these subtypes or influence on the most suitable therapeutic option for each subtype are lacking for CMT (Morris and Dobson, 2001). Similarly, grading systems, like the Nottingham Grading for human breast cancer (Bloom and Richardson, 1957, Elston and Ellis, 1991, Elston, 2005), are not generally accepted in veterinary medicine up to today.

Despite this clear scheme, a reliable prognostic classification of each mammary gland tumor is still a challenge. This is particularly true for differentiating metastasizing and non-metastasizing carcinomas before a metastatic spread has actually occurred. Therefore, histopathologic examination focuses on cutting margins and blood and lymph vessels of the tumor mass and, if available, the regional lymph nodes.

However, since a reliable prognosis is possible only when disseminated tumor cells are detectable in the regional lymph nodes (Kurzman and Gilbertson, 1986, Hellmen et al., 1993, Perez Alenza et al., 1997, Chang et al., 2005, Szczubial and Lopuszynski, 2011), further diagnostic targets are needed to allow for a reliable prediction of metastatic potential before metastases are actually detectable. In recent years, several diagnostic strategies have been tested, mostly adopted from human medicine. In particular, immunohistochemical markers were transferred into veterinary medicine repeatedly.

1.3.2 Immunohistochemical Criteria

Several immunohistochemical markers have been tested for their value to predict metastasis of canine mammary tumors. For example, the proliferating cell nuclear antigen (PCNA) has been used to correlate the fraction of proliferating cells with the biologic behavior of the tumor. PCNA is located in the nucleus and acts as a cofactor of DNA polymerase delta which is mainly detectable during G1, S and M phase of the cell cycle (Moldovan et al., 2007). The so called “PCNA index” is the ratio of positively labeled cells to the sum of positive and negative cells (Klopfleisch et al., 2011b). Several studies on CMT confirmed a correlation between PCNA index and tumor dignity, since benign tumors and well differentiated carcinomas showed reduced values in comparison to malignant tumors and less differentiated carcinomas, respectively. Notwithstanding, a standardization of staining and quantification procedure is still missing. Moreover, the expression intensity of PCNA shows in parts a large variability within the same tumor (Preziosi et al., 1995, Lohr et al., 1997, Pena et al., 1998).

Ki67 (antigen identified by monoclonal antibody Ki67) is another well-known proliferation marker. Due to its expression peak in the M phase of the cell cycle and its short half-life period, detection in noncycling cells on the one hand and malignant cells with an elongated G0 phase on the other hand is still difficult (Gerdes et al., 1983). Ki67 labeling results are quantified by the “Ki67 index”, a ratio of positive to positive and negative cells, as mentioned above (Klopfleisch et al., 2011b). Similar to PCNA, increased Ki67 indices seem to be associated with increased tumor dignity. But again, staining and quantification standards are missing (Lohr et al., 1997, Pena et al., 1998, Sarli et al., 2002).

As more malignant and undifferentiated tumors have the tendency to be steroid receptor negative (Morris and Dobson, 2001), a negative correlation between estrogen (ER) and progesterone (PR) receptor status and tumor dignity is presumed. However, for the prognostic value for CMT, different studies have detected contrary results. Although most authors suppose that decreased estrogen receptor expression goes along with increased

malignancy (de Las Mulas et al., 2005, Millanta et al., 2005, Chang et al., 2009), an early, statistically significant differentiation between metastasizing and non-metastasizing carcinomas is not achievable by ER-immunohistochemistry.

In conclusion, no reliable immunohistochemical markers of metastatic potential of CMT are currently available in veterinary medicine.

1.3.3 Circulating Tumor Cells

During the metastatic cascade, single tumor cells have to lose their attachment to the primary tumor, penetrate blood or lymph vessels and travel to distant organs as circulating tumor cells (CTC; Allard et al., 2004, Attard and de Bono, 2011). CTC are a relatively rare event with less than one CTC in 10^6 peripheral blood leukocytes (Alunni-Fabbroni and Sandri, 2010). Recently, da Costa and colleagues developed the first assay for the detection of canine circulating tumor cells. Out of hundreds of possible candidate genes, known from human breast cancer or known as genes with increased expression in CMT, 12 candidates were selected as potential CTC markers. Thereby, CRYAB featured a sensitivity of 35 % and a specificity of 100 %. Since its detection was highly correlated with tumor cell invasion into blood and lymph vessels, CRYAB appears to be a promising marker for the prediction of metastatic spread of canine mammary tumors (da Costa et al., 2012).

1.3.4 Difficulties in Diagnostics

In summary, it can be stated that although mammary gland tumors are the most common tumors in female dogs and a frequent research objective worldwide, an early differentiation between metastasizing and non-metastasizing carcinomas is still impossible, before a metastatic spread has occurred. Histopathologic examination of resected regional lymph nodes still gives the best prediction regarding the patients estimated survival time, in particular when tumor cells are present. That implies that new molecular biological approaches are needed. A global protein analysis of mammary gland tumors with a comparison of metastasizing and non-metastasizing carcinomas might be a suitable approach to find differences between these two tumor groups. Differentially expressed proteins might serve as potential diagnostic targets. In addition, the reflection of the protein expression patterns of normal mammary gland, adenomas, non-metastasizing and metastasizing carcinomas might help to answer open questions about carcinogenesis and malignant progression, for instance whether protein expression levels increase or decrease over all stages of malignancy, possibly reflecting a linear malignant progression.

1.4 Global Explorative Analyses as a New Diagnostic Approach

Global explorative analyses of transcriptome and proteome are increasingly used for non-hypothesis driven studies in most areas of biomedical research. As traditional hypothesis driven approaches analyze the expression levels of single or few specific mRNA or protein targets, analyses of the full transcriptome or proteome may facilitate an observation of almost the complete set of mRNA or protein specimens (Wilkins, Pasquali et al. 1996; Velculescu, Zhang et al. 1997).

Continuing technological advances on the one hand and the perception of cells and tissues as highly complex systems gave rise to the increasing use of these explorative approaches in veterinary medicine (Klopfleisch and Gruber, 2012a). For instance, microarrays or gene chips have been used to analyze the metastasis-associated transcriptome of canine mammary carcinomas as well as the influence of tyrosine kinase inhibitors on the metabolism of canine mast cells (Klopfleisch et al., 2011a, Klopfleisch et al., 2012b).

Notwithstanding, many pathological changes arise from genetic alterations, confirmation of transcriptional findings on the proteome level are necessary anyway. In addition, posttranscriptional modifications are not detectable by transcriptome analyses at all (Greenbaum et al., 2003). Therefore, proteomics technologies have become more sophisticated in the last years, similarly to the so called transcriptomics.

Two-dimensional gel electrophoresis (2D-GE) is a well-established method for protein separation, which is even improved by the use of fluorescent dyes (2D-DIGE: two-dimensional difference gel electrophoresis; Klose, 1975, O'Farrell, 1975, Unlu et al., 1997). Due to the use of an internal standard which is applied on each gel, several tissue lysates can be analyzed at the same time, reducing gel numbers and avoiding intergel variations (Unlu et al., 1997). Thus, diverse protein expression patterns, for example, healthy versus pathologic, can be compared in search of significant changes in the expression levels. For subsequent identification of these differentially expressed proteins mass spectrometry (MS) is an appropriate approach (Hillenkamp et al., 1991, Henzel et al., 1993, Mann et al., 1993). Using these techniques, potential new diagnostic and therapeutic targets might be identifiable.

In this context, the comparison of the protein expression patterns of normal mammary gland, adenomas, non-metastasizing and metastasizing carcinomas might reveal substantial differences between these tissues on a molecular level and expose crucial details about carcinogenesis and malignant progression.

1.5 Hypotheses

Because reliable diagnostic markers for an early differentiation of non-metastasizing from metastasizing carcinomas are lacking, a prediction of the metastatic potential of canine mammary tumors is still impossible before metastasis actually occurs. Furthermore, it is still unclear whether metastatic behavior is an inherent feature or a result of a time dependent linear malignant progression.

Therefore, the present study visualized and compared the protein expression patterns of canine normal mammary gland, adenomas, non-metastasizing and metastasizing carcinomas by 2D-DIGE and identified subsequently differentially expressed proteins by mass spectrometry. Differentiation of non-metastasizing and metastasizing carcinomas was based on the lymph node status at the time of surgical excision of the primary tumor mass.

The following hypotheses were tested here:

1. The metastatic potential of canine mammary tumors is reflected in their protein expression patterns and these differentially expressed proteins are potential metastasis markers.

2. Carcinogenesis of canine mammary tumors is associated with malignant progression from normal mammary gland towards metastasizing carcinomas. On the molecular level, this malignant progression is associated with a continuous and linear change of quantitative protein expression levels over the subsequent stages of malignancy.

2 Research Publications in Journals with Peer-Review

2.1 Proteome of Metastatic Canine Mammary Carcinomas: Similarities to and Differences from Human Breast Cancer

"Proteome of Metastatic Canine Mammary Carcinomas: Similarities to and Differences from Human Breast Cancer"

Journal of Proteome Research 2010 Dec 3; 9 (12): 6380-91. Epub 2010 Nov 2

PMID: 20932060, doi: 10.1021/pr100671c

Doi: 10.1021/pr100671c

2.2 Is There a Malignant Progression Associated with a Linear Change in Protein Expression Levels from Normal Canine Mammary Gland to Metastatic Mammary Tumors?

"Is There a Malignant Progression Associated with a Linear Change in Protein Expression Levels from Normal Canine Mammary Gland to Metastatic Mammary Tumors?"

Journal of Proteome Research 2011 Oct 7; 10 (10): 4405-15. Epub 2011 Sep 27

PMID: 21888431, doi: 10.1021/pr200112q

DOI: 10.1021/pr200112q

3 Declaration of own Portions of Work in the Research Publications

3.1 Proteome of Metastatic Canine Mammary Carcinomas: Similarities to and Differences from Human Breast Cancer

Authors: Klose P, Klopfleisch R, Weise C, Bondzio A, Multhaup G, Einspanier R, Gruber AD

Year: 2010

Journal: *Journal of Proteome Research* 9 (12): 6380-91

DOI: 10.1021/pr100671c

Contributions by P. Klose: Design, preparation, completion and evaluation of experiments as well as subsequent preparation of the manuscript.

Contributions of other authors: Western Blot assay was performed by A. Bondzio. Protein identification by mass spectrometry was performed in cooperation with C. Weise. All co-authors served as councelling team regarding design and interpretation of experiments.

3.2 Is There a Malignant Progression Associated with a Linear Change in Protein Expression Levels from Normal Canine Mammary Gland to Metastatic Mammary Tumors?

Authors: Klose P, Weise C, Bondzio A, Multhaup G, Einspanier R, Gruber AD, Klopfleisch R

Year: 2011

Journal: *Journal of Proteome Research* 10 (10): 4405-15

DOI: 10.1021/pr200112q

Contributions by P. Klose: Design, preparation, completion and evaluation of experiments as well as subsequent preparation of the manuscript.

Contributions of other authors: Protein identification by mass spectrometry was performed in cooperation with C. Weise. All co-authors served as councelling team regarding design and interpretation of experiments.

4 Concluding Discussion

The aims of this study were to contribute to the basic understanding of the molecular details of carcinogenesis and metastatic cascade of canine mammary tumors (CMT) and the detection of differences in the protein expression between adenomas, non-metastasizing and metastasizing canine mammary carcinomas. To this end, a global protein analysis was performed to compare the protein expression patterns of normal mammary gland, adenomas, non-metastasizing and metastasizing carcinomas of canines.

4.1 Protein Expression Patterns

The differentiation of benign adenomas and malignant carcinomas based on their histological appearance is in most cases simple. However, an early discrimination between non-metastasizing and metastasizing carcinomas with a reliable prediction of the patient's outcome is still an unresolved challenge.

The present study therefore visualized and compared the protein expression profiles of normal mammary gland, adenomas, non-metastasizing and metastasizing carcinomas, to identify differences in their proteome. Two-dimensional difference gel electrophoresis (2D-DIGE) and mass spectrometry (MS) identified 48 proteins with significant changes in expression, comparing the different tumor stages. Most of the differentially expressed proteins revealed stepwise changes in protein expression, instead of continuing increase or decrease over all stages of increasing malignancy. This led to the conclusion that there is no "more is merrier"-principle observable in canine mammary tumors. The three different non-linear protein expression patterns which were identifiable, were designated as: „adenoma pattern", „carcinoma pattern" and „metastasis pattern".

4.1.1 Adenoma Pattern

Adenomas are defined as benign accumulations of well differentiated monomorphic tumor cells. They feature an increased cellular growth, but in contrast to carcinomas they do not show invasive growth or metastatic spread (Misdorp et al., 1999).

The *adenoma pattern* of protein expression in this study was characterized by a significant up- or down-regulation of several proteins between normal mammary gland and adenomas and consecutively constant protein expression levels in non-metastasizing and metastasizing carcinomas. Thirteen different proteins represented this pattern, of which nine were identified by mass spectrometry. Five of these proteins were up-regulated whereas four were down-regulated.

Several of the identified proteins support the above mentioned characteristics, like cellular growth and survival under oxygen deficiency by additional supply of energy. For example, phosphoglycerate mutase 1 (PGAM1) is an enzyme which is involved in glycolysis and energy supply and therefore essential for cellular growth (Ren et al., 2011). Recent studies detected increased expression levels of PGAM1 in human breast cancer and hepatocellular carcinomas (Durany et al., 2000, Ren et al., 2011). These findings are in accordance with our results, since adenomas, non-metastasizing and metastasizing carcinomas revealed an overexpression of PGAM1 in comparison to normal mammary epithelium. Calumenin (CALU) is an example for a down-regulated protein in adenomas, non-metastasizing and metastasizing carcinomas. It is a calcium-binding protein, which is localized in the endoplasmic reticulum and involved in functions like protein folding and sorting (Sahoo and Kim do, 2011). A similar decrease in protein expression has been reported in human metastatic hepatocellular carcinomas and head and neck cancer cell lines (Wu et al., 2002, Ding et al., 2004).

As an increased cellular growth and subsequent survival under hypoxic circumstances is not limited to benign adenomas, a similar protein expression in all consecutive stages of tumor progression seems biologically reasonable. Therefore, the identified proteins might serve as additional diagnostic markers, besides histological examination, to discriminate between non-neoplastic and neoplastic tissue.

4.1.2 Carcinoma Pattern

Carcinomas display a pleomorphic histological pattern with less differentiated, anisocaryotic tumor cells, regardless of their metastatic potential. Mitotic figures are frequent, as well as a lack of an intact tumor capsule. In addition to the increased cellular growth found in adenomas, carcinomas have the ability to migrate and invade the surrounding tissue (Misdorp et al., 1999). For these characteristics carcinoma cells require specific protein signatures which are reflected in the *carcinoma pattern* identified here.

A significant up- or down-regulation of several proteins between adenomas and non-metastasizing carcinomas was the main feature of the *carcinoma pattern* in this study. Normal mammary gland and adenomas on the one hand and non-metastasizing and metastasizing carcinomas on the other hand had similar protein expression levels. Nine different proteins followed the *carcinoma pattern*, of these six were identifiable by MS. Four proteins were up-regulated and two were down-regulated.

Many of these proteins have already been described in the context of human breast cancer (Winston et al., 2001, Danes et al., 2008). However, a correlation to canine mammary carcinomas has not been reported prior to this study. 14-3-3-zeta for example seems to be associated with anti-apoptotic and thereby pro-proliferative effects by down-regulation of p53 (Danes et al., 2008). These correlations have been detected in human mammary epithelium, but our findings suggest a similar connection in canines, as 14-3-3-zeta was up-regulated in both kinds of mammary carcinomas, non-metastasizing and metastasizing. Gelsolin (GSN), as an actin-binding protein, is involved in the actin cytoskeleton rearrangement (Gay et al., 2008, Litwin et al., 2009). Its down-regulation seems to support an invasive phenotype, as described before for human breast cancer (Winston et al., 2001). Again, the findings in this study on canine mammary tumors go along with the findings made in human carcinomas since both have decreased expression levels of GSN.

4.1.3 Metastasis Pattern

The main feature of the *metastasis pattern* observed here was the significant up- or down-regulation of protein expression between non-metastasizing and metastasizing carcinomas, whereas normal mammary gland, adenomas and non-metastasizing carcinomas had similar expression levels. Twenty different proteins displayed this pattern. Out of the eighteen identifiable proteins nine were up-regulated and nine were down-regulated.

Although the histological appearance of non-metastasizing and metastasizing carcinomas is often hard to distinguish on the primary tumor, their proteome seems to be most diverse as it is reflected by the large variety of differentially expressed proteins found in this study. These findings might reflect the different functions involved in the complex events of metastatic cascade, for instance the detachment of single tumor cells from the primary tumor mass, their invasion into blood or lymph vessels and most of all their survival and settlement at the metastatic site (Raubenheimer and Noffke, 2006). Many of the proteins of the *metastasis pattern* have been described to be relevant for metastatic spread in other species or other cancer types (Gines et al., 2002, Thal et al., 2008, Wu et al., 2010).

For instance, tropomyosin 1 and 3 (TPM1 and TPM3) are cytoskeletal actin-binding proteins (Raval et al., 2003, He et al., 2004). TPM1 has been reported to induce anoikis (detachment-induced apoptosis) in human breast cancer cells and functions thereby as a tumor suppressor (Raval et al., 2003). A similar function can be assumed for canine mammary epithelium, as metastasizing carcinomas revealed decreased protein expression levels of TPM1 in comparison to normal mammary gland, adenomas and non-metastasizing carcinomas. TPM3 in contrast, provides the epithelial-mesenchymal transition and the

dissemination of tumor cells (Li et al., 2006, Choi et al., 2010) which explains the increased protein levels in metastasizing carcinomas. Other examples are the serpins (serin protease inhibitors) maspin (mammary serine protease inhibitor; SERPINB5) and bomapin (serpin peptidase inhibitor; SERPINB10). Maspin is also a well-known tumor suppressor. Its down-regulation is associated with malignant behavior, especially tumor cell invasion and metastasis (Zou et al., 1994, Sager et al., 1997, Stark et al., 2010). These findings made in invasive human breast carcinomas are in accordance with our observation of a decrease in the protein expression in metastasizing carcinomas. The up-regulation of bomapin in metastasizing carcinomas is comprehensible, as a cytoprotective effect against tumor necrosis factor alpha (TNFα)-induced cell death is indicated for this protein (Schleef and Chuang, 2000).

However, not only proteins with impact on the metastatic spread were differentially expressed between non-metastasizing and metastasizing carcinomas. In addition to the above mentioned findings, several proteins with proliferative activity or cell motility-association have been identified, as, for example, the proliferating cell nuclear antigen (PCNA) and adenosine deaminase (ADA).

4.2 News about Malignant Progression

The continuum from a benign adenoma towards a malignant carcinoma is called *malignant progression*. It is often mentioned in the context of mammary gland tumors or colorectal tumors (Baumgärtner and Gruber, 2010, McGavin and Zachary, 2012). However, the existence of malignant progression is mainly based on the fact that intermediate stages are visible between the two extremes of benign adenomas and metastasizing carcinomas, but a continued monitoring of the complete sequence of events *in vivo* has not been described. A relatively new idea about tumor development assumes an *early determination* of different tumor entities and disputes malignant progression in this way (van't Veer et al., 2002).

The protein expression patterns identified in the present study on canine mammary tumors might support both hypotheses; the continuous *malignant progression* with an increasing quantity of different proteins or the *early determination* of different entities – adenomas, non-metastasizing and metastasizing carcinomas – which are reflected by different protein signatures.

The fact that proteins which discriminate between normal mammary gland and adenomas have a similar expression level at all consecutive tumor stages supports the theory of *malignant progression*. Therefore, the quantity of a single protein does not increase or decrease over all stages of increasing malignancy, but the quantity of different proteins. Hence, the occurrence of pro-proliferative proteins may represent the first step of malignant progression from normal mammary gland towards adenomas. Whereas cell motility-associated proteins may reveal the next step towards carcinomas and the appearance of metastasis-associated proteins constitutes the terminal of malignant progression.

On the other hand, the fact that not only new features appear in each pattern, like cellular growth in the *adenoma pattern*, invasion in the *carcinoma pattern* and metastatic spread in the *metastasis pattern*, but also additional proteins for similar characteristics, like growth and invasion, are visible, substantiates the theory of *early determination*. These findings suggest that carcinomas and in particular metastasizing carcinomas have, in addition to the features found in adenomas, separate mechanisms for the same properties.

A clear approval or disapproval of the existence of a malignant progression was therefore not possible. It can however be stated that protein expression in canine mammary tumors does not follow the “more is merrier”-principle. To the contrary, a stepwise, saturating change in the protein expression with persisting expression levels in the consecutive tumor stages was observed in this global protein analysis.

4.3 Conclusion

In conclusion, the global protein analysis with comparison of protein expression patterns of normal mammary gland, adenomas, non-metastasizing and metastasizing carcinomas revealed the following results about carcinogenesis and metastatic spread:

The majority of identified proteins followed one of the three major expression patterns. These, designated as „adenoma pattern“, „carcinoma pattern“ and „metastasis pattern“, were characterized by a stepwise increase or decrease in protein expression between the examined tumor stages and a subsequent persistence on the same level in the consecutive stages. This implies that canine mammary tumors do not underlie the “more is merrier”-principle, as no constant and linear changes of quantitative protein expression levels over the different stages of increasing malignancy were detectable.

Many of the proteins identified in these patterns have been described to be relevant for carcinogenesis and metastasis in other species or other cancer types before. Interestingly, proteins reflecting the *adenoma pattern* were mainly associated with cellular proliferation, proteins from the *carcinoma pattern* with an invasive phenotype and *metastasis pattern*-proteins with metastatic spread. Furthermore, additional proteins with similar functions were found in the subsequent tumor stages, for example pro-proliferative proteins in the *metastasis pattern*. This last observation supports the hypothesis of an *early determination* of different entities which was established in recent years (van't Veer et al., 2002).

Hypothesis one was supported since metastasis-associated protein expression patterns were identifiable in canine mammary tumors. Moreover, these differentially expressed proteins may serve as potential new diagnostic targets to differentiate at an early stage between non-metastasizing and metastasizing carcinomas. In contrast, the second hypothesis was not supported since no continuous and linear change of quantitative protein expression levels was detectable over the different stages of increasing malignancy.

4.4 Outlook

The findings in the present study facilitated a clear discrimination between non-metastasizing and metastasizing canine mammary gland carcinomas on a molecular level, notwithstanding their histological similarity. Of course, further studies are needed to prove their authenticity, reliability and applicability as new diagnostic markers for routine usage.

Several issues have to be investigated. First, it is necessary to evaluate whether the detected genes with changes in their protein expression are central players in the carcinogenesis and metastasis of CMT, so called “driver” genes, or if these genes with differential protein expression levels are “passengers” and part of a reactive phenotype. Therefore cell culture studies are needed to knock down or overexpress single proteins and observe molecular consequences. Furthermore, their usefulness as routine markers by Western blot or immunohistochemistry needs to be tested. Especially the slight differences in the fold changes might cause difficulties. No exclusive expression or loss of expression of a single protein in one tumor type was detectable in the whole study. The question if this is caused by the method of 2D-DIGE, or if such a biological event is too rare to be detected, should be investigated as well. Finally, applications on larger and more homogenous populations would be helpful.

Proceeding from the assumption that mammary gland tumors feature the so called *malignant progression*, there is still no convincing evidence that benign tumors are able to dedifferentiate into malignant and metastasizing tumors. In order to prove this complete sequence of events *in vivo*, it would be necessary to remove and analyze only half of a primary tumor and monitor the residual tumor mass. Of course, this scenario is unacceptable for humans and canines.

5 Summary

Global Protein Analyses of Canine Mammary Gland Tumors

Patricia Schlieben

Although scientists worldwide do research on tumor development and metastatic cascade of canine tumors, many molecular details of this process are still unknown. For instance, it is still an intricate problem to distinguish between a non-metastasizing and a metastasizing canine mammary carcinoma before metastases are actually detectable. Furthermore, it is unclear whether metastatic behavior is an early inherent feature or a late result of a linear malignant progression.

Therefore, the present study visualized and compared the protein expression patterns of canine normal mammary gland, adenomas, non-metastasizing and metastasizing carcinomas (each n=6) by two-dimensional difference gel electrophoresis and identified subsequently differentially expressed proteins by mass spectrometry. Differentiation of non-metastasizing and metastasizing carcinomas was based on the histological examined lymph node status at the time of surgical excision of the primary tumor mass.

The following two hypotheses were tested here:

1. The metastatic potential of canine mammary tumors is reflected in the protein expression patterns and these differentially expressed proteins reveal potential metastasis markers.

2. Carcinogenesis of canine mammary tumors is associated with malignant progression from normal mammary gland towards metastasizing carcinomas. On the molecular level, this malignant progression is associated with a continuous and linear change of quantitative protein expression levels over the subsequent stages of malignancy.

In total, 48 different proteins featured significant changes in the comparisons: normal mammary gland versus adenomas, adenomas versus non-metastasizing carcinomas and non-metastasizing versus metastasizing carcinomas. Most of them followed three major expression patterns, which were designated as “adenoma pattern”, “carcinoma pattern” and “metastasis pattern”. The main characteristic of these patterns was a stepwise but not linear increase or decrease in protein expression with a subsequent persistence on the same expression level in the consecutive tumor stages. Interestingly, the comparison of non-metastasizing and metastasizing carcinomas revealed the majority of differentially expressed proteins, notwithstanding their histological similarity. Since many of these proteins have been described as relevant for carcinogenesis and metastasis in other species or other cancer types before, they may serve as potential metastasis markers for canine mammary tumors in the future.

In conclusion, the first hypothesis was supported since metastasis-associated proteins were identifiable in the present global protein analysis. On the contrary, the second hypothesis was not supported since no continuous and linear change of quantitative protein expression levels was detectable over the different stages of increasing malignancy.

6 Zusammenfassung

Globale Proteinexpressionsanalysen Kaniner Mammatumoren

Patricia Schlieben

Obwohl Mammatumoren seit längerer Zeit im Mittelpunkt der Forschung stehen, sind noch immer viele Aspekte ihrer Entstehung und molekularbiologische Details ihrer Metastasierung unbekannt. Beispielsweise stellt sich eine frühe Differenzierung zwischen nicht-metastasierenden und metastasierenden Karzinomen noch immer als sehr schwierig dar, bevor Metastasen detektierbar sind. Darüber hinaus ist bis heute nicht geklärt, ob Tumorzellen von Beginn an metastatisches Potential besitzen, oder es erst im Zuge der so genannten linearen malignen Progression entsteht.

Um diesen Fragen auf den Grund zu gehen, wurden in der vorliegenden Studie die Proteinexpressionsmuster von gesunder Milchdrüse, Adenomen, nicht-metastasierenden und metastasierenden Karzinomen der Milchdrüse des Hundes (je n=6) mittels zweidimensionaler differenzieller Gelelektrophorese verglichen und differenziell exprimierte Proteine im Anschluss mittels Massenspektrometrie identifiziert. Die Unterscheidung von nicht-metastasierenden und metastasierenden Karzinomen erfolgte anhand des histologischen Lymphknotenstatus zum Zeitpunkt der Entfernung des Primärtumors.

Die Prüfung der folgenden zwei Hypothesen stand im Mittelpunkt dieser Arbeit:

1. Metastatisches Potential von kaninen Mammatumoren spiegelt sich in deren Proteinexpressionsmustern wieder und die dabei unterschiedlich exprimierten Proteine stellen potentielle Metastasierungsmarker dar.

2. Die Karzinogenese kaniner Mamatumoren ist mit einer malignen Progression vom Normalgewebe hin zum metastasierenden Karzinom assoziiert, welche sich auf molekularbiologischer Ebene durch einen kontinuierlichen und linearen An- oder Abstieg in der Proteinexpression auszeichnet.

Insgesamt zeigten 48 verschiedene Proteine signifikante Expressionsunterschiede in den folgenden Vergleichen: Normalgewebe versus Adenome, Adenome versus nicht-metastasierende Karzinome und nicht-metastasierende Karzinome versus metastasierende Karzinome. Die meisten dieser Proteine folgten einem der drei Expressionsmuster, welche wie folgt benannt wurden: „Adenommuster“, „Karzinommuster“ und „Metastasierungsmuster“. Charakteristisch für die einzelnen Muster waren jeweils der stufenweise An- oder Abstieg in der Proteinkonzentration zwischen zwei Tumorstadien und die anschließende Persistenz auf dem erreichten Niveau in allen nachfolgenden Stadien. Interessanterweise ergab der Vergleich von nicht-metastasierenden und metastasierenden Karzinomen die meisten

differentiell exprimierten Proteine, obwohl sich diese beiden Tumorstadien histologisch kaum unterscheiden. Da viele der identifizierten Proteine bereits als relevant für die Karzinogenese und Metastasierung beschrieben, allerdings noch nicht im Zusammenhang mit kaninen Mammatumoren erwähnt wurden, stellen diese potentielle neue Metastasierungsmarker für die Tiermedizin dar.

Abschließend kann die erste Hypothese unterstützt werden, da Metastasierungs-assoziierte Proteine in der vorliegenden globalen Proteinexpressionsanalyse identifiziert wurden. Die zweite Hypothese hingegen konnte nicht gestützt werden, da keines der identifizierten Proteine einen kontinuierlichen und linearen An- oder Abstieg in seiner Expression über alle Malignitätsstufen hinweg aufwies.

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8 Publications

"*Fluorescence Difference Gel Electrophoresis* (DIGE) zur Identifikation Metastasierungs-relevanter Proteine bei kaninen Mammatumoren"

P. Klose, A.D. Gruber, A. Bondzio, R. Klopfleisch

53. Annual Conference of the German Veterinary Medical Society, Section Veterinary Pathology, Fulda, Germany (13.-14.03.2010)

"*Fluorescence Difference Gel Electrophoresis* (DIGE) zur Identifikation Metastasierungs-relevanter Proteine bei kaninen Mammatumoren"

P. Klose, A. Bondzio, C. Weise, A.D. Gruber, R. Klopfleisch

5. Post graduate research symposium at the Freie Universität Berlin, Berlin, Germany (02.07.2010)

"Identification of Differentially Expressed Proteins in the Primary Tumors of Metastasizing Canine Mammary Tumors Using 2D-DIGE and Mass Spectrometry"

P. Klose, A. Bondzio, C. Weise, A.D. Gruber, R. Klopfleisch

28. Annual Meeting of the European Society of Veterinary Pathology and European Conference of Veterinary Pathologists, Belgrade, Serbia (08.-11.09.2010)

"Proteome of Metastatic Canine Mammary Carcinomas: Similarities to and Differences from Human Breast Cancer"

Klose P, Weise C, Bondzio A, Multhaup G, Einspanier R, Gruber AD, Klopfleisch R

Journal of Proteome Research 2010 Dec 3; 9 (12): 6380-91. doi: 10.1021/pr100671c

"Proteomanalysen der malignen Progression von kaninen Mammatumoren"

P. Klose, C. Weise, A. Bondzio, A.D. Gruber, R. Klopfleisch

54. Annual Conference of the German Veterinary Medical Society, Section Veterinary Pathology, Fulda, Germany (12.-13.03.2011)

"Proteomanalysen der malignen Progression von kaninen Mammatumoren"

P. Klose, A. Bondzio, C. Weise, A.D. Gruber, R. Klopfleisch

6. Post graduate research symposium at the Freie Universität Berlin, Berlin, Germany (01.07.2011)

"Is There a Malignant Progression Associated with a Linear Change in Protein Expression Levels from Normal Canine Mammary Gland to Metastatic Mammary Tumors?"

Klose P, Weise C, Bondzio A, Multhaup G, Einspanier R, Gruber AD, Klopfleisch R

Journal of Proteome Research 2011 Oct 7; 10 (10): 4405-15. doi: 10.1021/pr200112

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Selbstständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbstständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Berlin, den 10.10.2012 Patricia Schlieben