Immunohistochemical detection of tumour cell proliferation and intratumoural microvessel density in canine malignant mammary tumours

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Abstract

The objective of this study was to investigate the correlation between different histological types and grades of canine malignant mammary tumours, tumour cell proliferation and their angiogenic activity using immunohistochemical markers. Mammary tissue samples from 47 bitches with mammary cancer were evaluated. The expression of cellular proliferation marker Ki-67 and endothelial marker Von Willebrand's factor (vWF) were immunohistochemically demonstrated. The tumours with the highest Ki-67 and vWF expressions were found to share similar histomorphological features. Simple solid carcinoma had the highest levels of Ki-67, vWF, and higher histological grade while complex carcinomas, osteosarcomas, and carcinosarcomas had the lowest ones. The differences between the expressions of Ki-67 and vWF in different tumour types were considered to be of great importance in determination of biological behaviour and prognosis of these tumours. This study is one of the few studies that evaluate these differences among the subtypes of malignant canine mammary tumours.

Keywords: dog, mammary tumour, proliferation marker, intratumoural microvessel density, prognosis.

Introduction

Mammary tumours are the most common neoplasms in bitches and constitute an important problem in veterinary medicine (1). Histologically, approximately 41%-53% of mammary tumours in bitches are diagnosed as malignant (17). The incidence of mammary carcinomas in dogs is three times higher than in humans (15, 26). Histopathological and clinical aspects of canine mammary tumours have been widely reviewed; nevertheless, the presence of considerable variations in the biological behaviour, even among histologically malignant tumours, often does not allow an accurate prognosis using microscopic examination alone (9). Several efforts have been made towards the adoption of criteria to standardise the diagnosis, such as understanding tumour behaviour and progression, and the evaluation of prognostic factors including morphology, oncogene expression, and gene alterations. The knowledge and adoption of these criteria are fundamental for the selections and success of therapies which could prevent tumour recurrence and increase survival rates (1).

Veterinary oncologists seek information concerning the histological variant of the tumour and other prognostic indicators. Proliferative activity of tumour cells, known as proliferation index (PI) (30) is considered as a morphological indicator of tumour aggressiveness, which is an important prognostic factor to define the criteria of histomorphological diversity in determination of outcome and therapeutic strategy (23). The immunohistochemical identification of antigens specific to dividing cells using monoclonal antibodies directed against Ki-67 offers a useful approach in the evaluation of the growth fraction (8, 14).

The growth, invasion, and metastatic capability of malignant tumours highly depend on the presence of sufficient blood supply. This procedure is termed angiogenesis, which indicates a complex physiological process involving development of new blood vessels from pre-existing vascular network (4, 12). Without the ability to recruit new vascular network, most tumours...
would never grow beyond 2-4 mm³ in diameter and would remain localised to the primary site (7).

Intratumoural microvessel density (IMD) is assessed to determine angiogenesis, which indicates a progressive development, invasion, and metastatic capacity of the tumour, thus setting the groundwork for therapeutic approach with the development of anti-angiogenic drugs (5, 10). These drugs have been widely used in human medicine, but they have only been introduced in the treatment protocol of animal cancer in the USA and Europe since early 2000s (15).

Weidner (31) contends that the IMD is a more reliable prognostic factor in human cancer than histological type, grade, and stage of tumours. However, the prognostic significance of IMD has been documented in a limited number of canine and feline neoplasms, such as mammary tumours (15, 20).

In order to predict the biological behaviours of these tumours, the present study investigates the tumour cell proliferation and angiogenic activity of canine malignant mammary tumours of different subtypes and grades by using immunohistochemical markers. In the present study, von Willebrand factor (vWF) has been applied as an endothelial marker.

Material and Methods

Animals and samples. Total mastectomy materials from 47 bitches admitted to the Obstetrics and Gynaecology Department of Istanbul University Veterinary Faculty were investigated. The Age of the bitches at the time of tumour removal ranged from 4 to 15 years (median 9.5 years). Among the animals that underwent mastectomy, 20 gave birth, 27 never gave birth, and 15 were spayed.

The tissue samples were fixed in 10% buffered formalin, processed by routine methods, embedded in paraffin wax, sectioned at 4 µm, and stained with haematoxylin and eosin (H&E stain). Histopathological findings were recorded by two pathologists and used to classify the tumours according to the criteria of a recently validated system (16). Tumour malignancy was histologically graded by Nottingham method modified by Elston and Ellis (3). Histological grade of carcinomas was determined on the basis of three morphological features: tubule formation, nuclear pleomorphism, and mitotic counts. Each feature was scored on a scale from 1 to 3. The scores were then added together to obtain an overall tumour grade as follows: 3-5 points – well differentiated (grade 1); 6-7 points – moderately differentiated (grade 2); 8-9 points – poorly differentiated (grade 3) in 40× lens. Tumours were classified histologically according to their most aggressive component. According to Misdorp (17), there is no grading system for mammary sarcomas.

Immunohistochemistry. Immunohistochemistry was performed using a streptavidin-biotin-peroxidase complex method. Sections were collected on Polysine™ slides. For antigen retrieval, Proteinaz K was used in sections labelled with vWF and heated citrate buffer (pH 6.0, 10 nM) was used for Ki-67 immunolabelling. Endogenous peroxidase activity was blocked with 30% hydrogen peroxide in methanol for 10 min at room temperature. The sections were then rinsed with phosphate buffered saline (PBS, pH 7.4). Non-specific staining was prevented by serum blocking solution. Subsequently, the sections were incubated with primary Ab Ki-67 (Clone MIB-1; Dako®, Denmark, Code No. M 7240) prepared in 1:100 dilution at 4°C overnight. Antibody against vWF (factor VIII-related antigen) (DakoCyttomation®, Denmark, Code No. A 0082) was diluted 1:1000, and the incubation was conducted at room temperature for 2 h. The 2nd Generation LAB-SA Detection System (Histostain®-Plus Kits Zymed®) was applied according to the manufacturer’s instructions. Immunolabelling was visualised by 3-amino-9-ethylcarbazole (AEC) for Ki-67 and 3,3-diaminobenzidine tetrahydrochloride (DAB) for vWF as chromogen. The preparations were counterstained with Mayer’s haematoxylin. Positive (canine haemangiomia for vWF and small intestine for Ki-67) and negative (mammary tumours with primary antisem omitting and replaced with PBS solution) control slides were prepared simultaneously.

Cellular proliferation analysis. Tumour proliferative fraction was assessed by counting the Ki-67-positive cells. The sections were examined under light microscope (Olympus BX50) initially at low power magnification (40×). Ten areas with high numbers of positive cells were identified. Within each area, a high-power magnification (400×) was selected and a total of 2000 cells were counted. The average number of positive cells in 10 fields represented the tumour PI.

Microvessel density analysis. Immunohistochemistry was used to identify vascular endothelium by staining for vWF antigen. The method originally described by Vermeulen et al. (29) was modified. In the first step of Vermeulen’s approach the area of the highest neovessel density (the so called “hot spot”) was identified with light microscopy (Olympus BX50) by scanning the whole tumoural section at low power magnification (100×). Then, the second step the individual microvessels were counted in an adequate area at high power magnification (400×). Positive staining for vWF was characterised by the presence of brown cytoplasmic staining. All stained endothelial cells or cell clusters were counted as one microvessel. The presence of a lumen was not required and vascularity in areas of necrosis was not evaluated. The average microvessel density of 10 fields from each tumour within the hot spot was calculated.

Statistical analysis. Kruskal Wallis test and Mann Whitney U test were performed to compare subtypes of malignant mammary tumours in terms of Ki-67 expression, vWF expression, and degree of...
malignancy. Chi square test was performed in order to compare reproductive history (birth rate and spaying status) of different subtypes of malignant mammary tumours.

Results

Clinical and pathological findings. The differences between different histological types of mammary tumours with respect to reproductive history (birth rate and spaying status) of the dogs were not significant (P > 0.05). Relapse of tumours and overall survival times were not included in the analysis because of the lack of communication with dog owners, and thus this information was absent from the medical records of the examined animals.

Histopathological findings. Histological diagnosis included 12 complex carcinomas, 11 simple tubulopapillary carcinomas, 7 carcinosarcomas, 6 osteosarcomas, 8 simple solid carcinomas, and 3 spindle cell carcinomas. The histological grades of each tumour, their proliferation index (PI; Ki-67), intratumoural microvessel density (IMD; vWF) values, and age and reproductive status of the dogs are presented in Table 1.

Immunohistochemical findings. Ki-67 positive reaction was expressed as nuclear granular staining often with prominent nucleolar positivity. The cytoplasmic membrane and cytoplasm of endothelial cells of control and neoplastic tissues displayed strong and specific brown immunopositivity when labelled with vWF antibodies.

Statistical significance (P < 0.001, P < 0.001) was detected between different subtypes of tumours with respect to Ki-67 (PI) and vWF (IMD) expression levels (Table 2).

Immunostaining revealed only a small ratio of positive cells in grade I carcinomas (Fig. 1A) and the immunoreactive tumour cells increased in parallel with histological malignancy grade values (Fig. 1B). The significance of histological grading on PI and IMD was high (P < 0.001, P < 0.01 respectively), as shown in Table 2.

Proliferation index. The tumours with the highest PI were simple solid carcinomas, while carcinosarcomas had the lowest PI.

Intratumoural microvessel density. The density of vWF expression in the tumours increased concurrently with the histological grade (Figs 2A, B). Simple solid carcinomas revealed high vWF expression, while osteosarcomas had the lowest one.

In terms of PI and IMD, the difference between simple solid carcinoma and mesenchymal/epithelial + myoepithelial tumours (osteosarcoma, complex carcinoma, and carcinosarcoma) were significant (P < 0.001-0.05).

Table 1. Distribution of histological grading of canine mammary tumours, proliferation index (PI, Ki-67), and intratumoural microvessel density (IMD, vWF) mean values, age, and reproductive status of the dogs

<table>
<thead>
<tr>
<th>Histological diagnosis</th>
<th>n (%)</th>
<th>Grade of malignancy</th>
<th>PI (Ki-67)</th>
<th>IMD (vWF)</th>
<th>Age</th>
<th>Birth</th>
<th>Spayed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malignant mammary tumour</td>
<td>47 100</td>
<td>I II III</td>
<td>82.78</td>
<td>9.5 (4-15)</td>
<td>27</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Simple solid carcinoma</td>
<td>8 17</td>
<td>I II III</td>
<td>188.79</td>
<td>12.1 (10-15)</td>
<td>4</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Spindle cell carcinoma</td>
<td>3 6.4</td>
<td>I II III</td>
<td>132.53</td>
<td>9.6 (8-11)</td>
<td>4</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Simple tubulopapillary carcinoma</td>
<td>11 23.4</td>
<td>I II III</td>
<td>64.10</td>
<td>9.8 (7-12)</td>
<td>7</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Complex carcinoma</td>
<td>12 25.5</td>
<td>I II III</td>
<td>55.60</td>
<td>12.92</td>
<td>7</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Osteosarcoma</td>
<td>6 12.8</td>
<td>Not graded</td>
<td>66.32</td>
<td>9.5 (6-12)</td>
<td>5</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Carcinosarcoma</td>
<td>7 14.9</td>
<td>Not graded</td>
<td>30.37</td>
<td>9.3 (6-11)</td>
<td>4</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Mean values for proliferation index (PI; Ki-67), intratumoural microvessel density (IMD, vWF), and age of the dogs in different histological subtypes of the tumours

<table>
<thead>
<tr>
<th>PI (Ki-67)</th>
<th>IMD (vWF)</th>
<th>Age</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simple solid carcinoma</td>
<td>37.56</td>
<td>43.50</td>
</tr>
<tr>
<td>Spindle cell carcinoma</td>
<td>36.00</td>
<td>31.33</td>
</tr>
<tr>
<td>Tubulopapillary carcinoma</td>
<td>28.36</td>
<td>31.33</td>
</tr>
<tr>
<td>Complex carcinoma</td>
<td>15.67</td>
<td>14.25</td>
</tr>
<tr>
<td>Osteosarcoma</td>
<td>18.83</td>
<td>9.75</td>
</tr>
<tr>
<td>Carcinosarcoma</td>
<td>15.21</td>
<td>18.79</td>
</tr>
</tbody>
</table>

* Differences between the means of histological subtypes of the tumours with different superscript letters in the same row are significant (P < 0.05)
Fig. 1A. Simple tubulopapillary carcinoma, well-differentiated. Few cells showing nuclear immunostaining for Ki-67. Streptavidin-biotin-peroxidase; bar – 20 µm. B. Spindle cell carcinoma, poorly differentiated. Diffuse nuclear staining of tumour cells for Ki-67. Streptavidin-biotin-peroxidase; bar – 20 µm.

Fig. 2A. Simple tubulopapillary carcinoma. IHC for vWF revealed a small number of scattered microvessels. Streptavidin-biotin-peroxidase; bar – 50 µm. B. Simple solid carcinoma. IHC for vWF revealed numerous irregularly shaped microvessels without lumen. Streptavidin-biotin-peroxidase; bar – 20 µm.

Discussion

Since canine malignant mammary tumours show wide variations in terms of their biological activity, it is extremely difficult for a veterinary clinician to estimate the prognosis of an individual case. Prognostic factors can be defined as specific clinical, pathological, and biological characteristics of individuals and their tumours that permit prediction of clinical outcome and survival of patients without subjecting them to additional and adjuvant therapies after initial surgery (1). The study of prognostic factors is of utmost importance, as it enables prediction of the behaviour and clinical outcome of mammary tumours using individualised therapeutic protocols with appropriate intensity and effectiveness (11, 23).

The age of the patient is one of the most important risk factors since it has been well-documented that the incidence of canine mammary tumours is directly correlated with advancing age (11, 17, 28). In the present study, the average age was 9.5 years (ranging from 4 to 15 years) in dogs with malignant tumours, which was similar to that reported in previous studies (2, 27).
The statistical difference between ages of dogs with different kinds of malignant mammary tumours was significant (P < 0.05). The dogs diagnosed with simple solid carcinomas were older than the rest, with an average age of 12.1 years. While the mean age was 7.5 in dogs with complex carcinoma, it was 12.1 in dogs with simple solid carcinoma, and the age difference was found to be highly important in the comparison between these two groups (P < 0.001). We concluded that simple solid carcinoma encountered in older ages has the worst prognosis.

In the present study, reproductive history of the dogs was found to have no effect on histological type, grade, PI, and IMD of the tumours.

Histological grading of the canine mammary carcinoma is significantly related to prognosis: a high histological grade is associated with low overall survival (25). In the present study, although the types of the tumours were not equal in number, 50% of simple solid carcinomas were grade III, whereas complex adenocarcinomas and simple tubulopapillary carcinomas were mostly grade I. Similarly, Pena et al. (19) reported that 94.1% of grade III canine mammary tumours did not show myoepithelial proliferation.

Molecular markers have been evaluated as information sources for prognosis and to predict the behaviour of various types of tumour in humans and animals. PI scores of simple solid carcinomas were higher than the other carcinomas (23). On the contrary, in another study (32), PI scores of simple tubulopapillary carcinomas were found to be higher than those of solid carcinomas, yet with complex carcinomas showing the highest PI values. In this study, PI scores of mammary carcinomas of epithelial origin, such as simple solid carcinomas and spindle cell carcinomas, were higher than those of carcinomas, complex carcinomas, and osteosarcomas which consist of both epithelial and myoepithelial tissues, as well as cells of mesenchymal origin. The difference between PI scores of these two tumour groups was found to be statistically significant, except for simple tubulopapillary carcinomas. This was associated with the less malignant nature of simple tubulopapillary carcinomas considering the low PI scores of these tumours.

Understanding the growth and metastatic capability and prognostic significance of the tumours has provided substantial contribution to development of the methods for determination of angiogenesis, and development of anti-angiogenic drug-based treatment protocol (6, 24). The relation between expression status of angiogenic factors and the biological behaviours of the tumours has not been fully understood in animals. It is obvious that in terms of treatment, no progress has been made with respect to the methods of angiogenesis assessment (15, 20).

In general, our findings were compatible with those of the previous studies (15, 20, 21). Restucci et al. (21) reported that along with solid carcinoma, mixed carcinoma also has high microvessel density. In another study Restucci et al. (22) claimed that less differentiated phenotypes had higher numbers of microvessels than the more differentiated malignancies. In this study, IMD scores of canine malignant tumours varied widely according to the subtypes of the tumour. IMD was directly correlated with histological grade in the tumours showing similar histological features. Simple solid carcinomas revealed high angiogenic activity in contrast to osteosarcomas, complex carcinomas, and carcinosarcomas.

Among the malignant mammary tumours, histological grading was found to be statistically significant in terms of PI and IMD (P < 0.001, P < 0.01 respectively), and the density of positive immunoreactivity increased progressively from grade I to grade III. The results regarding PI and IMD were consistent with previous studies (15, 21).

Histomorphological features of canine mammary tumours are rather complex, and therefore microscopic evaluation alone might be insufficient to determine their biological behaviours. In this study, the correlation between tumour grade and their biological behaviours could not be established due to the lack of follow-up communication with dog owners. However, when previous studies on biological behaviour were reviewed, it was concluded that grade Ki-67 expression and IMD can be helpful in predicting tumour behaviour. Karayannopoulou et al. (13) found significant differences in survival between cases with different tumour grades. They reported that grade II and III carcinomas were found to have 21-fold increased risk of death as compared with grade I carcinomas. The mortality rate of grade III tumours was also 7 times higher than that of grade II tumours. In another study, Pena et al. (18) reported that increased Ki-67 expression was an objective prognostic parameter in predicting the postsurgical behaviour of canine mammary carcinomas, but it was not objective prognostic parameter for predicting metastases, disease free survival, and overall survival. Graham and Myers (10) stated that IMD was higher in metastatic tumours than benign tumours, but there was no significant difference between malignant tumours with and without metastases.

In conclusion, although tumour subtypes were not equal in number, a correlation has been identified between their histological type and grade of malignancy, proliferation capacity, and angiogenic activity. The tumours with the lowest and highest proliferation capacity and angiogenic activity were detected to share similar histomorphological features. Tumours showing expansive growth pattern, such as simple solid carcinomas, showed the highest levels of proliferation capacity, angiogenic activity, and degree of differentiation, while complex carcinomas, osteosarcomas, and carcinosarcomas had lower values. Increased Ki-67 and vWF expression seems to be associated with increasing malignancy of canine.
mammary tumours. They are valuable prognostic factors, which are associated with tumour aggressiveness and the degree of differentiation. Thus, the application of anti-angiogenic drug-based treatment protocol in animals with high levels of vWF expression is recommended. Therefore, assessment of these factors in different sub-types of the mammary tumours may have significantly contributed to the determination of the outcome of tumours, as well as the establishment of an accurate therapeutic protocol for canine malignant mammary tumours.

Conflicts of Interest Statement: The authors declare that there is no conflict of interest regarding the publication of this article.

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