Detection of cell origin by immunohistochemistry in canine mammary tumours

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SUMMARY

The main objective of this study was to improve the tumour type diagnosis using immunohistochemical markers for epithelial (cytokeratins AE1/AE3) and myoepithelial cells (cytokeratins AE1/AE3, calponin, α-smooth muscle actin (α-SMA)) and for mesenchymal components (vimentin) in the case of canine mammary tumours. Tumours were obtained from 43 female dogs in different ages and breeds and were classified according to WHO-AFIP after histopathological examination. The tumours were diagnosed as in situ carcinoma (3 cases), complex carcinoma (12 cases), tubulopapillary carcinoma (12 cases), solid carcinoma (1 case), spindle cell carcinoma (1 case) and carcinosarcoma (14 cases). Cytokeratins AE1/AE3 were expressed in all cases except in the spindle cell carcinoma and vimentin was expressed by mesenchymal cells in all cases except in the in situ carcinoma whereas α-SMA and calponin were specifically detected in complex carcinoma and in carcinosarcoma (88.5% and 69.2%, respectively). However, calponin immunostaining was generally moderate to strong whereas the α-SMA detection remained often weak. These results emphasize the interest of immunohistochemical markers for identifying the tumour cell origin in canine mammary tumours.

Keywords: Dog, mammary tumour, pathology, immunohistochemistry, cell origin, cytokeratins AE1-AE3, α-smooth muscle actin, calponin, vimentin.

RéSUMÉ

L’objectif de cette étude a été d’améliorer le diagnostic du type de tumeur dans le cas de tumeurs mammaires chez la chienne en utilisant des marqueurs immunohistochimiques des cellules épithéliales (cytérakétines AE1/AE3), myoépithéliales (cytérakétines AE1/AE3, calponine et α-actine du muscle lisse (α-SMA)) et mésenchymateuses (vimentine). Les tumeurs provenaient de 43 chiennes de différents âges et de différentes races et elles ont été classées d’après des critères histopathologiques (WHO-AFIP) en carcinome in situ (1 cas), carcinome complexe (12 cas), carcinome tubulopapillaire (12 cas), carcinome solide (1 cas), carcinome à cellules fusiformes (1 cas) et carcinosarcome (14 cas). Les cytérakétines AE1/AE3 ont été exprimées dans tous les cas sauf dans le cas du carcinome à cellules fusiformes et la vimentine a aussi été détectée dans les cellules mésenchymateuses dans toutes les tumeurs à l’exception du carcinome in situ alors que l’α-SMA et la calponine n’ont été mises en évidence que dans les carcinomes complexes et dans les carcinosarcomes (dans respectivement 88.5% et 69.2% des cas). Cependant, l’expression de la calponine s’est avérée en général modérée à forte alors que celle de l’α-SMA est restée le plus souvent faible. Ces résultats soulignent l’intérêt des marqueurs immunohistochimiques dans la détermination de l’origine des cellules tumorales lors de tumeurs mammaires chez la chienne.

Mots clés : Chien, tumeur mammaire, pathologie, immunohistochimie, origine cellulaire, cytérakétines AE1-AE3, α-actine du muscle lisse, calponine, vimentine.

Introduction

Mammary tumours are the most common tumour type in the female dog [16]. Common risk factor in the development of canine mammary tumours is increased lifetime exposure to endogenous or exogenous oestrogens [4, 16]. Other risk factors include ovariectomy after 2.5 years of age, irregular oestrus cycles and obesity [15]. Because canine mammary tumours have great histomorphological heterogeneity, the accurate diagnosis of these tumours is quite difficult. Several immunohistochemical studies have shown diagnostic importance of specific luminal and myoepithelial cell markers in canine mammary tumours. Immunohistochemical techniques have critical role on distinguishing these cells and may allow accurate diagnosis of canine mammary tumours [8, 9, 11, 12, 19].

Normal mammary glands are composed of 2 cell types that luminal and myoepithelial and these cell types express different types of proteins. The luminal epithelial cell types express different cytokeratins (CKs); myoepithelial cells express more specific markers, such as alpha smooth muscle actin (α-SMA) and calponin [1, 17]. Cytokeratin AE1/AE3 is a mixture of two monoclones of cytokeratins and both are expressed in the epithelial cells [10]. The cytokeratin AE1/AE3 detection has been widely used for detection of the epithelial origin of malignant cells in tumours [17]. Calponin is a 3 kDa protein and is a specific marker for determination of smooth muscle and myoepithelial cells of breast especially in humans. Myoepithelial cells of mammary glands and the myofibroblasts in the stroma of breast tumours express α-SMA [13]. Vimentin is a member of class-III intermediate filaments and it is especially found in mesenchymal cells. Because of this, it can use as a mesenchymal and myoepithelial cell marker in the tumours [17].
The aim of this study was to improve the diagnosis value using immunohistochemical detection of cytokeratin AE1/AE3, α-SMA, calponin and vimentin as markers in canine mammary tumours.

Material and Methods

TISSUE SAMPLES AND HISTOPATHOLOGY

Surgically removed 43 canine mammary tumours or biopsy specimens were collected in Department of Pathology, Faculty of Veterinary Medicine, University of Adnan Menderes, Aydın, Turkey among 2003 and 2010. Biopsy specimens were fixed in 10% neutral formalin solution, embedded in paraffin, sectioned at 5 µm and stained routinely with Haematoxylin-Eosin and examined microscopically.

IMMUNOHISTOCHEMISTRY

For the immunohistochemical staining, tumour sections were routinely processed according to standard protocols. Avidin-biotin peroxidase complex method was used on tumour sections (avidin-biotin peroxidase complex, Invitrogen Histostain Plus Detection Kit, USA). All of the used markers were shown in Table I. Tissue sections were deparaffinised, rehydrated and then antigen retrieval was applied by microwave heat for 10 minutes at medium voltage in a 10 mM citrate buffer, pH 6.0. After cooling at room temperature, the sections were incubated in 3% hydrogen peroxide (H₂O₂) for 30 minutes and then washed by phosphate buffer saline (PBS), pH 7.2, 3 times. Nonspecific staining was eliminated by 10 minutes incubation with normal goat serum at the room temperature. Excess normal serum was removed and slides were then incubated with primary antibody (cytokeratin AE1/AE3, vimentin, α-SMA, calponin) at 4°C overnight. After washing the slides, the sections were incubated with biotinylated secondary antibody for 15 minutes and replaced in the streptavidin, horseradish peroxidase (HRP) conjugate for 15 minutes at the room temperature. The colour was developed with 3, 3'-diaminobenzidine tetrahydrochloride (DAB, DAKO)-H₂O₂ in PBS for 5 minutes. Slides were counterstained with Harris haematoxylin, dehydrated and mounted with Entellan (Merck). In all slides, non-tumoural areas were used as internal positive control and specificity of primary markers was confirmed.

On each slide, different fields were observed and immunopositive reactions were demonstrated by the presence of brown cytoplasmic staining. The results were evaluated semi-quantitatively. The semi-quantitative evaluation (SQS) was performed as follows: +, weak expression; ++, moderate expression; ++++, strong expression; −, negative. The tumours were classified according to WHO-AFIP (World Health Organization–Armed Forces Institute of Pathology) classification.

Results

Among the 43 female dogs investigated in this study, 40 were 4-14 years old (10.30 ± 2.33 years) and age was

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Recommended dilutions</th>
<th>Stained tissues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-αSMA mouse monoclonal antibody (Abcam / ab-18147)</td>
<td>0.5 – 1.0 mg/L</td>
<td>Smooth muscle, myoepithelial cells</td>
</tr>
<tr>
<td>Anti-calponin rabbit monoclonal antibody (Abcam / ab-46794)</td>
<td>1:100 – 1:250</td>
<td>Smooth muscle, myoepithelial cells</td>
</tr>
<tr>
<td>Anti-vimentin mouse monoclonal antibody (Abcam / ab-8069)</td>
<td>1:50 – 1:100</td>
<td>Mesenchymal tissues</td>
</tr>
<tr>
<td>Anti-human cytokeratin mouse monoclonal antibody (Dako / Clones AE1-AE3)</td>
<td>1:50</td>
<td>Epithelial cells</td>
</tr>
</tbody>
</table>

αSMA: α-Smooth Muscle Actin

Table I: Immunohistochemical method: primary antibodies, dilutions used and stained tissues.

<table>
<thead>
<tr>
<th>Staining intensity</th>
<th>αSMA</th>
<th>Calponin</th>
<th>Vimentin</th>
<th>Cytokeratins (AE1-AE3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No staining (-)</td>
<td>20 (46.51%)</td>
<td>25 (58.13%)</td>
<td>1 (2.32%)</td>
<td>1 (2.32%)</td>
</tr>
<tr>
<td>Weak (+)</td>
<td>8 (18.60%)</td>
<td>1 (2.32%)</td>
<td>19 (44.18%)</td>
<td>4 (9.30%)</td>
</tr>
<tr>
<td>Moderate (+++)</td>
<td>14 (32.55%)</td>
<td>5 (11.62%)</td>
<td>18 (41.86%)</td>
<td>16 (37.20%)</td>
</tr>
<tr>
<td>Strong (+++)</td>
<td>1 (2.32%)</td>
<td>12 (27.90%)</td>
<td>5 (11.62%)</td>
<td>22 (51.16%)</td>
</tr>
<tr>
<td>Total for positive staining</td>
<td>23 (53.48%)</td>
<td>18 (41.86%)</td>
<td>42 (97.67%)</td>
<td>42 (97.67%)</td>
</tr>
</tbody>
</table>

Table II: Intensity of immunohistochemical staining in canine mammary gland tumours (n = 43).
undetermined in 3 of them; there were 5 crossbred dogs, 5 cockers, 3 golden retrievers, 2 German shepherds, 2 Pinschers, 1 Pointer, 1 Boxer, 1 Doberman, 1 Spits, 1 medium Poodle, seventeen belonged to terrier breeds including one Bolognese terrier, and breed was not known for four cases.

All of the cases used in this study were examined histopathologically before immunohistochemical examination. After the immunohistochemical examination, these cases could be reclassified. According to histopathological and immunohistochemical findings, all of the cases were diagnosed as malignant tumours: in situ carcinoma (3 cases), complex carcinoma (12 cases), tubulopapillary carcinoma (12 cases), solid carcinoma (1 case), spindle cell carcinoma (1 case) and carcinosarcoma (14 cases). Histopathologically, marked pleomorphic features (figure 1) such as atypia, hyper- or hypochromasia in the epithelial and myoepithelial components (intertubular spindle cells), infiltrative growth, moderate or high mitotic activity, necrosis and mononuclear cell infiltrations in the stroma and intertubular areas were observed in all cases.

Figure 1: Carcinosarcoma, marked pleomorphic features of tumour cells and luminal papillary projections (arrows) in the carcinomatous area of the tumour, Haematoxylin-eosin, X 20.

The expression intensity of the different markers immunohistochemically investigated in the 43 canine malignant mammary tumours was summarized in Table II.
Cytokeratins AE1/AE3 were expressed in the all tumour cases except in the spindle cell carcinoma case. They were observed in the cytoplasm of normal epithelial cells, luminal epithelial (figure 2A) and myoepithelial cells of the tumours. The staining intensity was more often moderate to strong (in 90.5% of the positive tumours).

The α-SMA was expressed in the great majority (23/26, i.e. 88.5%) of complex carcinoma and carcinosarcoma cases, but its expression remained weak to moderate. The α-SMA staining was detected in the cytoplasm of intertubular spindle cells (neoplastic myoepithelial cells) (figure 2B) except near the blood vessel walls. Calponin was moderately to strongly expressed in all cases of complex carcinoma (n = 12) and more occasionally (6/14) in carcinosarcoma cases. Calponin immunostaining was also identified in the cytoplasm of intertubular spindle (figure 2C) and in polygonal shaped myoepithelial cells.

Vimentin was weakly to moderately expressed in all cases of canine mammary tumours except in the in situ carcinoma case. The protein was detected in the cytoplasm of mesenchymal cells of the tumour including fibrocytes, lipocytes as well as myoepithelial cells (figure 2D). In addition, chondroid matrix cells and osseous tissues were intensely stained for vimentin.

**Discussion**

Canine mammary tumours may originate from different cell types including luminal epithelial, myoepithelial and stroma cells. For accurate diagnosis and prognosis of canine mammary tumours, differentiation of these cell types is very important. Therefore, some specific markers such as cytokeratins, vimentin and α-SMA were used by immunohistochemical techniques in the canine mammary tumours [11, 17, 19]. In this study, vimentin, α-SMA, cytokeratin AE-1/AE-3 and calponin were used as immunohistochemical markers in order to identify the lumino-epithelial and myoepithelial origin of tumour cells in canine mammary tumours.

Normal and tumoural myoepithelia have a complex immunophenotype (epithelial and smooth muscle characteristics) and because of this, high-molecular-weight cytokeratins and α-SMA have been used as markers of myoepithelial origin in tumours [7, 11, 14, 21]. The cytokeratin AE1/AE3 monoclonal antibody is a combination of 2 monoclones (AE1 and AE3) and these antigens are expressed during epithelial cell differentiation in tumours. Cytokeratins are specific epithelial markers and the detection of their expression in tumours has been widely used for specification of the epithelial origin of malignant cells [2, 3]. In the present study, cytokeratins AE-1/AE-3 were detected in luminal epithelial and myoepithelial cells in all cases except in the spindle cell carcinoma case. This finding revealed that the spindle shaped cells have not a myoepithelial origin.

Calponin is a 34-kDa smooth muscle specific protein considered as a quite sensitive marker of myoepithelial cells in human breast carcinomas [5, 6] and as a more specific marker of myoepithelial cells than α-SMA in human breast tumours [20]. In this study, calponin was identified in non-luminal polygonal and spindle shaped cells in all complex carcinoma cases and in 6 carcinosarcoma cases. However, α-SMA was seen in intertubular spindle cells (neoplastic myoepithelial cells) except in the blood vessel walls in 12 cases of complex carcinoma and in 5 cases of carcinosarcoma. Based on these results, in agreement with human literatures, calponin appeared as a more reliable immunohistochemical marker than α-SMA for demonstrating the myoepithelial origin of tumoural cells in canine mammary tumours.

Vimentin is a 57 kDa intermediated filament protein and it is stated as an important diagnostic marker in the histogenesis of tumours cells and mesenchymal components [18]. In this study, vimentin was observed in mesenchymal cells, chondroid matrix cells, osseous tissues and myoepithelial cells.

As a conclusion, the present study shows the importance of calponin as an immunohistochemical marker of myoepithelial tumoural cells in canine mammary tumours. Based on these results, the consideration of histopathological findings alone may lead to misdiagnosis of canine mammary tumours and, for this reason histopathological findings should be sustained by immunohistochemical findings before a definite diagnosis.

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**References**


