Occurrence of metallothionein immunostaining in feline mammary epithelial neoplasms

S.D. ERGINSOY1, M. SOZMEN*, M. CALDIN2 and T. FURLANELLO2

1 Department of Pathology, Faculty of Veterinary Medicine, University of Kafkas, 36040 Kars, Turkey
2 Veterinary Clinic and Laboratory “San Marco”, V. Sesto, 114/0C, Padova, Italy

* Corresponding author : Dr. Mahmut SOZMEN Department of Pathology, Faculty of Veterinary Medicine, University of Kafkas, 36040 Kars - TURKEY
Tel.: +90 474 2426801-ext : 1202 - Fax: +90 474 2426853 - E-mail: msozmen@hotmail.com

SUMMARY

Metallothioneins (MTs), a set of ubiquitous low-molecular-weight proteins, were detected immunohistochemically using a monoclonal antibody (E9) against a conserved epitope of I and II isoforms in a series of 19 feline mammary tumours. In a semi-quantitative analysis, unequivocal MT expression in the tumour cells was observed in 2/3 cases of benign and 8/16 malignant mammary neoplasms. The mean immunoreactive score was higher in benign mammary neoplasms compared to malignant tumours. But, MT was differently expressed according to the type of malignant tumours. The MT expression was demonstrated in 80% of feline ductular mammary carcinomas, whereas its occurrence in mammary gland adenocarcinomas was only 36%. It is concluded that immunohistochemically demonstrated MT expression is associated with feline benign mammary tumours and ductular carcinomas.

Keywords : cat - ductular carcinoma - mammary tumour - metallothionein - immunohistochemistry.

Introduction

Mammary tumours are the third most frequent group in queens [18] and they bear similarities to those found in human including metastatic pattern that primarily concerns regional lymph nodes and lungs [17]. The prognostic assessment of human patients with breast cancer has become increasingly important, and additionally variables are needed to give supplementary information that can lead to a better understanding of the biology of mammary carcinoma. For this reason, spontaneous feline mammary tumours offer a valid and appropriate animal model to study human cancer biology [17]. Recently, the role of metallothioneins (MTs) in carcinogenesis has received significant attention in both human [12-14, 21] and animals [2, 6, 16].

Metallothioneins are a family of small (61 amino acids), cysteine-rich (20 residues), low molecular weight (6-7 kDa) proteins with high binding affinity to metal ions, such as zinc, copper, and other group II heavy metal ions [10]. MTs play important roles in various physiological processes and its synthesis can be induced by many factors, including heavy metals, hormones and chemical and physical stress [2]. In addition, the differential MT expression in various human tumours indicates the involvement of MT in carcinogenesis [11, 12]. MTs are often associated with tumour growth by affecting both cell proliferation and death [15]. It has been shown that expression of the MTs detected via immunohistochemistry has different prognostic significance in various human tumours [11]. The significance of MT expression in feline mammary tumours has not yet been adequately assessed and there are a few published studies in the literature. In limited studies, MT expression in feline mammary tumours revealed the role of MTs in tumour proliferation and progression [3]. In the present study, we investigated immunohistochemically MT overexpression in 19 cases of feline mammary gland tumours using a monoclonal antibody (against I and II isoforms) and we correlated the immunohistochemical findings with the histologic tumour type of the respective animals.

Materials and methods

Formalin-fixed, paraffin-embedded blocks of 19 (3 benign and 16 malign) spontaneous feline mammary neoplasms were selected retrospectively from Veterinary Clinic and Laboratory “San Marco” (Padova, Italy). The histological type was based on both the BENJAMIN et al. [1] and the WHO classifications [18] with some modifications.
Sections from all the tissue samples were cut 4 µm and processed for immunohistochemical examination by a streptavidin-biotin-peroxidase method. Tissue sections were placed on 3-aminopropyltriethoxysilane (Sigma, St. Louis, Montana, USA) coated slides, dewaxed and hydrated. Antigen retrieval was facilitated by heating in citrate buffer (pH 6.0) for 20 min in a microwave oven with a power of 600 watt. The slides were then dipped in freshly prepared absolute methanol containing hydrogen peroxide 3% v/v for 20 min to block endogenous peroxidase activity. A mouse monoclonal antibody that reacts with human and rabbit MT (Clone : E9, Dako Corporation, CA, Carpinteria, USA, code M0639) was used at a dilution of 1:600 for 60 min in this study. The monoclonal antibody used equally recognises the I and II isoforms, independently of the metal status of the proteins. After washing with phosphate-buffered saline (PBS), the slides were incubated with biotinylated rabbit anti-mouse immunoglobulin G (Dako, Carpinteria, USA) diluted 1:300 in PBS for 60 min at room temperature. Sections then were incubated with streptavidin peroxidase complex (ABC ; Dako Corporation, CA, Carpinteria, USA) diluted 1:300 in Tris-buffered solution (TBS) for 60 min at room temperature. The slides were then treated for 5 min at room temperature with 3,3’ diaminobenzidine tetrahydrochloride (DAB ; Sigma, St. Louis, Montana, USA) in distilled water (0.5 mg DAB/ml) containing hydrogen peroxide 30% v/v. Finally, sections were counter-stained with Mayer’s hematoxylin, dehydrated and mounted. Negative control tissue sections were incubated with normal rabbit serum. The specificity of the MT monoclonal antibody was tested by pre-absorption techniques in which the antibody was pre-absorbed with an excess (100 µmol) of the initial antigen (horse MT ; Sigma, St. Louis, USA).

The percentage of the total area of the MT positive cells were assessed quantitatively under a light microscope with a 10X ocular with grids and a 40X objective. A total of 10 high-power fields were randomly chosen. The findings were categorised as follows : (0) no positively staining tumour cells ; (1) 5-25%; (2) 26-50%; (3) 51-75% ; (4) >75% of tumour cells positive. The intensity of MT staining was assessed semi-quantitatively for the cytoplasm and nucleus of the neoplastic cells separately as follows : (0) none ; (1) weak ; (2) moderate ; (3) intense immunolabeling. After multiplication of the grades for intensity and percentage score (cytoplasm and nucleus separately with each maximal value of 12), the scores were added together and per definition were called the immunoreactive score with a maximum of 24.

**Results**

Sporadic MT expression was observed in both cytoplasm and nucleus of epithelial cells lining ducts and lobules of normal mammary glands stemming from healthy epithelial tissues surrounding tumours. In neoplastic tissues, the immunohistochemical localisation of MT was both cytoplasm and nuclei of epithelial and myo-epithelial cells (Figure 1). A mosaic pattern was observed for MT immunolabeling in some tumours, with neoplastic cells showing high heterogeneity of staining, from negative to strongly positive (Figure 2). There was no correlation between staining intensity and percentage of staining cells.

Unequivocal MT expression was shown in 2 out of 3 benign mammary tumours examined (Figure 3, Table I). However, MT localisation could be detected in eight out of 16 cases of feline malignant mammary tumours (50%). Between the malignant tumours, unequivocal MT expression was demonstrated in 80% (4/5) feline ductular carcinomas while, MT expression could be detected in only 36% (4/11) cases of feline adenocarcinomas (Table I).

A statistical analysis could not be performed because of limited number of benign neoplasms. However, in terms of immunoreactivity score, the MT expression was still unequivocal for the benign neoplasms with a mean score of 7 whereas the score in malignant tumours remained low (maximum 50% tumour cell positive).

<table>
<thead>
<tr>
<th>Epithelial neoplasm classification</th>
<th>Total number of tumours</th>
<th>MT negative tumours</th>
<th>MT positive tumours</th>
<th>Immunoreactive score of MT positive tumours †</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Benign Tumours</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ductal hyperplasia</td>
<td>1</td>
<td>-</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Adenosis</td>
<td>1</td>
<td>-</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Fibroadenoma</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Malignant Tumours</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma*,#</td>
<td>11</td>
<td>7</td>
<td>4</td>
<td>3, 1, 2</td>
</tr>
<tr>
<td>Ductular carcinoma**,**,†</td>
<td>5</td>
<td>1</td>
<td>4</td>
<td>2, 2</td>
</tr>
</tbody>
</table>

† Immunoreactive score: the frequency rates for the numbers of stained nuclei and cytoplasm were assessed semi-quantitatively as follows: (1) 5-25%; (2) 26-50%; (3) 51-75%; (4) >75% of tumour cell positive
* 5 simple and 6 complex adenocarcinoma
** 4 simple and 1 complex ductular carcinoma
* Solid, tubular, and/or papillary
† Intralobular or interlobular origin

Table I. — Immunohistochemically detected MT in feline mammary tumours (n = 19).
OCCURRENCE OF METALLOTHIONEIN IMMUNOSTAINING IN FELINE MAMMARY EPITHELIAL NEOPLASMS

Discussion

In human neoplasms, MTs are commonly used in studies investigating the prognostic value of this protein. However, in animals, MT immunoreactivity has only been studied previously in experimental rodent tumour models [16] and cases of spontaneous canine and feline mammary neoplasms [3, 6].

In this study, morphologically ‘normal’ mammary epithelial cells were found to contain immunocytochemically detectable MT, whereas previous studies of MT expression in benign or malignant feline neoplasms have not pointed out this phenomenon [3]. In the present study, MT expression observed in most of benign feline mammary tumours contrasted with its occurrence in 50% of the corresponding malignant tumours, suggesting an inverse correlation of MT expression with malignancy. The observed MT staining expression in our cases were different to those described in the literature [3, 6] where MT expression was detected in the feline malignant mammary tumours with an occurrence of 31% (8/26) [3] but not in the benign mammary tumours. However, in the present study, MT expression was detected in both feline benign mammary neoplasms (2/3) and malignant tumours (50% ; 8/16). The reason for this difference is not clear but it may be the result of differences in tissue fixation period or staining procedure and/or indicate some differences in the tumour types investigated.

Previously, it was suggested that MT plays a role for preventing carcinogenesis in epithelial tissues [3]. However, the high expression of MT in feline malignant mammary tumours [3], and in human breast cancer tissues [13, 14, 21] contrasted with this hypothesis. This paradox could be explained by the metal occupancy state of the expressed MT [3]. MT is present either as an apo- (metal-free) or a holo- (metal-bound) forms [20]. The anti-carcinogenic effect of MT was associated with holo-MT chelated with zinc metal ions stemming from Zn-associated transcriptional factors [23]. This form of MT is able to protect genomic DNA against a variety of DNA-damaging alkylating agents. Indeed, MT-null cells are associated with increased rate of both spontaneous and mutagenic DNA damage [7]. Consequently, it was postulated that the expressed MT in benign canine mammary tumours would be the holo form of MT [3], while the MTs detected in feline ductal carcinomas were probably apo forms. It was previously shown that apo-MT abrogated Zn-dependent p53 DNA binding activity by removing Zn [9]. However, in mice and rat solid tumours, persistently elevated presence of apo-MT was detected without any evidence of zinc deficiency [20]. The reason why tissues differently expressed MTs is not yet clear. It was suggested that tissues in which persistent stress related mutation occurred were associated with the enhancement of MT expression (apo form) and with carcinogenic process [20].

The results revealed that MT expression differed according to the type of malignant tumours, suggesting a role of MT in carcinogenesis and tumour progression in cats. The MT expression demonstrated in 80% of feline ductal mammary carcinomas contrasted with its occurrence in only 36% of the corresponding feline mammary gland adenocarcinomas. The antibody used for immunocytochemical detection of tumour-associated MT overexpression, is unable to distinguish between either MT-I and MT-II isoforms, or metal bound (holoMT) and metal-free (apoMT) forms. It is, therefore possible that the tumour type differences noted in the...
present study are related to isoform/metal status of MT overexpression characteristic of a given type of tumour.

Another point of our study is, to the best of our knowledge, the ductular carcinoma in cats. Carcinomas arising from the ducts have only been reported in dogs [1, 5, 19]. This kind of carcinoma has largely been ignored in the major morphologic classifications [18]. FOWLER et al. [5] evaluated the biological behaviour of the canine ductular carcinomas more malignant than that of the lobular carcinomas arising from alveolar epithelium. MONLUX et al. [19] reported that canine ductal carcinomas corresponded to nearly all of the metastatic lesions seen at the Tulsa, Oklahoma, neoplasm registry. In the present report, the solid lesions in feline ductular carcinomas commonly had central necrosis in a comedo-carcinoma pattern. In humans, the presence of necrosis has been linked to local recurrence of ductal carcinoma in situ (DCIS) and development of invasive breast carcinoma [22]. In human duct breast carcinomas, MT overexpression has been related to poor histological type and grade DCIS with central necrosis [4, 8]. The immunohistochemical findings in feline ductular mammary carcinomas, which closely resembled to those reported in human breast cancer, provide further support for a possible mechanistic involvement of MT in tumour progression. Furthermore, recognition of the ductular carcinomas as a separate morphologic category would have a significant impact on determining the prognosis for feline mammary neoplasms and will be of value in assisting the pathologist and oncologist in evaluating mammary carcinoma prognosis. All of the above points are good arguments for the importance of reclassification of ductal mammary neoplasms, particularly those arising from small intra- or interlobular ductules.

References