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# Immunohistochemical demonstration of metallothionein in benign and malignant canine mammary tumours

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**Summary.** Immunocytochemical demonstration of metallothionein (MT) has been reported as a useful prognostic tool in human breast cancer. The aim of this study was to determine the immunohistochemical location of MT in canine mammary tumours and its possible correlation with the morphologic characteristics of these tumours. Surgical specimens from spontaneous malignant (n=20) and benign mammary neoplasms (n=20) were processed for routine histological examination and immunohistochemical study. An indirect immunoperoxidase technique, using monoclonal antibody E9 against horse MT was employed. Intensity of the stain, the percentage of immunoreactive tumour cells and immunohistochemical overexpression of MT was estimated for each case. Metallothionein overexpression, defined as those cases with more than 10% immunopositive cells, was detected in both benign and malignant mammary tumours. However, strong immunostaining intensity was seen in benign tumours, whereas in malignant tumours immunopositive cells stained weakly. Positive MT immunostaining occurred in neoplastic epithelial cells, and some chondrocytes present in mixed mammary tumours. However, staining intensity was variable in immunopositive cells. Differences in staining intensity between the primary malignant mammary tumour, tumour emboli and metastatic cells within a lymph node were also noted. Myoepithelial cells and connective tissue did not stain for MT. We concluded that metallothionein immunostaining cannot be used as a diagnostic or prognostic tool in canine mammary neoplasms. However, results of this study support the hypothesis that MT has a role in tumour proliferation and tumour progression.

**Key words:** Metallothionein, Mammary gland, Canine, Immunohistochemistry, Breast

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## Introduction

Mammary tumours have been recognized as the second most common tumours in dogs (Hampe and Misdorp, 1974; Moulton, 1990; Destexhe et al., 1993). Classification of mammary tumours is mostly based on standard histopathology (Fowler et al., 1974) and although the prognosis is dependent on microscopic examination of submitted biopsies, classification of mammary neoplasms remains subjective and often confusing. Difficulties in establishing a uniform nomenclature are partially due to the diverse criteria which can be applied to classify these tumours. Canine mammary tumours have different proportions of epithelial, myoepithelial, and connective tissue components. In addition to the diverse histogenesis, the pattern of arrangement of neoplastic cells can also vary from papillary, tubular, cystic to medusoid, and the proportion of fibrous connective tissue stroma may be minimal or prominent such as in scirrhous carcinomas. An important criterion to classify a neoplasm should be the presence or absence of malignant behaviour, as evidenced by infiltration of adjacent tissues and/or lymphatic and blood vessel invasions. It is estimated that approximately 60% of canine mammary tumours are benign (Hampe and Misdorp, 1974; Moulton, 1990), but some neoplasms originally thought to be benign microscopically, ultimately become malignant and metastize throughout the body (Misdorp et al., 1971). Conversely, other mammary tumours appear malignant morphologically yet are benign in behaviour (Hampe and Misdor, 1974; Brealy, 1989).

Research on metallothionein (MT), a metal-binding protein, has been traditionally focused on its role in metal homeostasis and toxicity (Hamer, 1986). In recent years, the role of MT in carcinogenesis (Cherian, 1994) and its potential applications differentiating aggressively malignant from benign neoplasms, has received increased attention. MT has been found to be elevated in ovarian neoplastic cells (Murphy et al., 1991) and its over expression in some cancer cell lines has been found to confer resistance to chemotherapy (Beck et al., 1979;

Andrews et al., 1987; Kelleey et al., 1988). Increased expression of MT has been described in some types of human cancer including thyroid carcinomas (Nartey et al., 1987), malignant melanomas (Zelger et al., 1993) and mammary cancer (Fresno et al., 1993; Schmid et al., 1993; Bier et al., 1994; Haerslev et al., 1994; Douglas-Jones et al., 1995; Goulding et al., 1995). In human breast cancer studies, tumours with a better prognosis had lower levels of MT expression than those tumours with a less favourable prognosis (Fresno et al., 1993; Schmid et al., 1993; Bier et al., 1994; Douglas-Jones et al., 1995; Goulding et al., 1995).

The aim of this study was to determine the immunohistochemical location of MT in canine mammary tumours and its possible correlation with morphological characteristic of these tumours.

## Materials and methods

A search was performed on the pathology records held at the Atlantic Veterinary College. The most recent surgical specimens from spontaneous malignant (n=20), and benign mammary tumours (n=20) were selected. Diagnosis was made using the World Health Organization classification (Hampe and Misdorp, 1974). Cases with multiple tumours were not included in this study.

### *Histopathology*

The paraffin blocks of mammary tissue were retrieved and sections 6 $\mu$m thick were cut and processed for routine haematoxylin and eosin (HE) staining and immunocytochemistry. Staining for argyrophilic proteins of nucleolar organizer regions (AgNORs) was performed on 5 $\mu$m-thick sections as described by Ofner et al. (1995).

### *Immunohistochemistry*

#### Metallothionein

Tissue sections 6 $\mu$m thick were applied to 0.1% w/v poly L-lysisine coated slides. All reagents were prepared in Dulbecco's phosphate buffered saline (D-PBS) and used at room temperature. Tissues were deparaffinized, hydrated and treated sequentially with 1.5% hydrogen peroxide for 30 minutes to block endogenous peroxidase activity. Non-specific tissue binding sites were blocked with 1% normal goat serum for 30 minutes. Monoclonal antibody E9 against horse MT (a kind gift from A. Cryer, J. Kay and J.M. Stark, University of Wales College of Medicine, Cardiff, UK) was selected as the primary antibody. This antibody is reactive against a conserved epitope shared by the I and II isoforms of human, rat and horse metallothionein (Jasani and Elmes, 1991). The primary antibody at 1:12000 was applied for 60 minutes. Next, slides were treated with horseradish peroxidase-goat anti-mouse IgG at 1:200 for 60 minutes and substrate for 4 minutes.

Substrates used consisted of 18 mL D-PBS, 7 $\mu$L of 30% hydrogen peroxide and 10 mg 3,3'-diaminobenzidine. Between each step, the slides were washed in D-PBS/0.1% Tween-20 for 10 minutes. Slides were counterstained with aqueous 4.7% haematoxylin for 5 seconds, dehydrated in a graded ethanol series and cleared with xylene. Slides were mounted with Flo-texx and examined with a Zeiss light microscope.

Specificity of the staining reaction was assessed in several different control experiments: prior absorption of the primary antibody with rabbit MT, substitution of the primary antibody with D-PBS or normal mouse serum (1:100), and omission of both primary and secondary antibodies.

Slides were assessed for staining intensity, percentage of immunoreactive cells, location of immunoreactivity within the cell, and type of cell staining. Intensity of the stain was classified as weak (+), moderate (++) and strong (+++) immunoreactivity. The score described by Fresno et al. (1993) was used to estimate the percentage of immunoreactive tumour cells as follows: 0, negative staining; +, less than 10% tumour cells stained; ++, 10-50% of cells stained; +++, more than 50% of cells stained.

MT overexpression was defined as those cases with more than 10% of positive tumour cells (Fresno et al., 1993; Haerslev et al., 1995).

#### Actin

Myoepithelial cells were distinguished from luminal epithelial cells utilizing the peroxidase anti-peroxidase method on paraffin-embedded sections. Anti-human alpha-smooth muscle actin (Dako Corp. Carpinteria, CA) was used as the primary antibody, and rabbit anti-mouse serum (Sigma Chemical Co., St. Louis, MO) was used as the secondary antibody.

## Results

The breed, age, size of the tumour, histological diagnosis and immunohistochemical reactivity of benign and malignant mammary tumours are presented in Tables 1 and 2 respectively. The age range for benign mammary tumours was 5-12 years, with an average of 8.2 years. The age range for malignant mammary tumours was 6-14 years, with an average of 9.6 years. Both benign and malignant mammary tumours often occurred in sporting breeds, Poodles and Dachshunds.

### *Histological findings*

Benign tumours were diagnosed as papillary or complex adenomas, and benign mixed tumours. Lobular hyperplasia occurred in conjunction with both malignant and benign mammary tumours. Malignant tumours were classified as solid carcinomas, papillary and tubular adenocarcinoma; complex and anaplastic carcinomas, or

**Table 1.** Breed, age, size, morphology diagnosis and proportion of metallothionein-positive cells in 20 benign mammary tumours in dogs.

| CASE No. | BREED | AGE (years) | SIZE (cm) | DIAGNOSIS | CARTILAGE % | EPITHELIUM % | MYOPEPITHELIUM % | MT STAINING INTENSITY | PROPORTION MT POSITIVE CELLS |
|---|---|---|---|---|---|---|---|---|---|
| 1 | Akbash | 8 | 1 | Papillary adenoma | - | 95 | - | ++ | ++ |
| 2 | Yorkshire terrier | 12 | 0.5 | Mixed | 5 | 20 | 75 | +++ | + |
| 3 | Maltese | 10 | 8 | Mixed | 60 | 20 | 20 | ++ | ++ |
| 4 | Siberian Husky | 6 | 1 | Complex adenoma | - | 50 | 50 | ++ | + |
| 5 | Standard Poodle | 6 | 2.5 | Complex adenoma | - | 40 | 60 | +++ | ++ |
| 6 | Pomeranian | 7 | 2 | Complex adenoma | - | 80 | 20 | ++ | + |
| 7 | Terrier cross | 6 | 2 | Papillary adenoma | - | 90 | - | + | +++ |
| 8 | Golden Retriever | 8 | 5 | Mixed | 15 | 70 | 15 | ++ | ++ |
| 9 | English Setter | 5 | 1 | Mixed | 50 | 20 | 30 | ++ | + |
| 10 | Pomeranian | 12 | 2 | Mixed | 40 | 30 | 20 | ++ | ++ |
| 11 | Springer Spaniel (FS) | 8 | 3 | Mixed | 45 | 10 | 45 | +++ | + |
| 12 | Dachshund | 8 | 1.5 | Mixed | 30 | 20 | 50 | +++ | + |
| 13 | Lhasa Apso cross | 9 | 1.5 | Mixed | 10 | 55 | 35 | + | + |
| 14 | Mixed (FS) | 7 | 1.5 | Complex adenoma | - | 50 | 50 | + | ++ |
| 15 | Scottish Terrier (FS) | 7 | 2.5 | Mixed | 80 | 10 | 10 | + | + |
| 16 | Cocker Spaniel | 9 | 6 | Mixed | 45 | 15 | 40 | ++ | + |
| 17 | Poodle (FS) | 8 | 1.5 | Mixed | 30 | 15 | 55 | +++ | + |
| 18 | Mixed | 8 | 6 | Mixed | 45 | 10 | 55 | ++ | + |
| 19 | Mixed | 11 | 2.5 | Mixed | 20 | 30 | 50 | +++ | ++ |
| 20 | Poodle | 9 | 0.5 | Papillary adenoma | - | 60 | 40 | ++ | ++ |

**Table 2.** Breed, age, size, morphology diagnosis and proportion of metallothionein-positive cells in 20 malignant canine mammary tumours.

| CASE No. | BREED | AGE (years) | SIZE (cm) | TUMOUR TYPE | MT STAINING INTENSITY | PROPORTION MT POSITIVE CELLS |
|---|---|---|---|---|---|---|
| 1 | Sheltie (FS) | 11 | 2 | Anaplastic carcinoma | + | + |
| 2 | Pomeranian | 12.5 | 2 | Complex adenocarcinoma | ++ | ++ |
| 3 | Dachshund (FS) | 11 | 1 | Solid adenocarcinoma | + | + |
| 4 | Mixed | 11 | 5 | Malignant mixed mammary tumor | + | +++ |
| 5 | West Highland West Terrier | 13 | 1.5 | Papillary adenocarcinoma | + | + |
| 6 | Labrador cross (FS) | 9 | 2 | Anaplastic carcinoma | - | - |
| 7 | Alaska Husky | 9 | 3 | Tubular adenocarcinoma | + | + |
| 8 | Belgian Sheepdog (FS) | 8 | 2 | Papillary adenocarcinoma | ++ | ++ |
| 9 | Britain Spaniel (FS) | 13 | 1.5 | Anaplastic adenocarcinoma | ++ | + |
| 10 | English Spaniel | 7 | 0.5 | Tubular adenocarcinoma | ++ | + |
| 11 | Terrier cross (FS) | 12 | 0.5 | Tubular adenocarcinoma | ++ | + |
| 12 | Springer Spaniel | 7 | 2.2 | Tubular adenocarcinoma | ++ | ++ |
| 13 | Maltese (FS) | 8 | 1.3 | Solid adenocarcinoma | +++ | +++ |
| 14 | Springer Spaniel | 10 | 1 | Tubular adenocarcinoma | ++ | + |
| 15 | Golden Retriever (FS) | 8 | 2 | Solid adenocarcinoma | ++ | ++ |
| 16 | Bouvier | 7 | 1.5 | Complex adenocarcinoma | + | + |
| 17 | Terrier x Poodle (FS) | 14 | 2.5 | Solid adenocarcinoma | + | +++ |
| 18 | Corgi cross | 9 | 2 | Tubular adenocarcinoma | ++ | + |
| 19 | Doberman cross | 6 | 1 | Tubular adenocarcinoma | - | - |
| 20 | Golden Retriever | 6 | 1.5 | Solid adenocarcinoma | + | - |

malignant mixed tumours.

Evaluation of slides stained for AgNORs revealed that although malignant tumours generally had increased number of AgNORs per neoplastic cell compared to benign tumours, there were a few benign tumours with equally high numbers of AgNORs.

Papillary adenoma was diagnosed in 3 of 20 cases. This tumour was characterized by presence of well-demarcated lobules formed from dilated or cystic intralobular ductules or interlobular ducts. Lobules were composed of papillated sheets of neoplastic epithelial cells forming polypoid to frond-like structures, supported by a fibrovascular stalk. The neoplastic cells were small cuboidal with poorly defined cell borders, scant acidophilic cytoplasm and a small single, oval to cigar-shaped vesicular nucleus containing one or more indistinct nucleoli. The cell nuclei exhibited 2-3 fold anisokaryosis and 0-2 mitotic figures for x40 field were seen.

Complex adenoma was diagnosed in 4 cases. Complex adenoma had proliferations of myoepithelial cells and secretory epithelial cells arranged into tubuloacinar structures. The epithelial component was characterized by uniformly-sized small cells with a small

distinct nucleus with vesicular or coarsely granular chromatin pattern. Myoepithelial cells varied from spindle to plump with variable amounts of pale eosinophilic extracellular matrix associated with them. Cases characterized by papillary projections of epithelial cells in which there was proliferation of myoepithelial cells in the stalk tissue were also diagnosed as complex adenomas.

Benign mixed mammary tumour was diagnosed in 13 of the 20 cases. A diagnosis of mixed mammary tumour was made when there was a multilobulated, well-demarcated area of neoplastic proliferation of mesenchymal, myeopithelial and glandular epithelial cells. The epithelial cells were forming solid cords, papillary projections, follicular and cystic structures. The glandular epithelial component was characterized by small cuboidal to columnar epithelial cells, with slight loss of polarity but low mitotic index (0-1 per x40 field). The mesenchymal component accounted for 5-80% of the total neoplastic mass, and varied from fibrous to fibromucinous, loose myxomatous, and chondroid to ossified tissue.

Solid carcinoma was diagnosed in 5 of the 20 cases. A diagnosis of solid carcinoma was made when luminal epithelial neoplastic cells were arranged in solid sheets, cords and nests without tubular or other lumen formation. The neoplasm was often multilobulated and exhibited a central area of necrosis. Neoplastic cells were pleomorphic and had indistinct cell borders and scant eosinophilic cytoplasm. The cell nuclei were medium to large, round to oval with evenly distributed chromatin, and one or more prominent nucleoli. Mitotic figures were common (approximately 2 per x40 field). Necrosis of individual cells was often seen. Neoplastic cells infiltrated adjacent tissues and were often seen within lymphatics.

Papillary adenocarcinoma was diagnosed in 2 of the 20 cases of malignant mammary tumours. Low columnar or cuboidal luminal epithelial cells were arranged in pedunculated papillary projections within the lumen of dilated ductular and lobular structures. There was piling up of cells covering the papillae and loss of polarity. Duct-like structures were cystic in 1 of the 2 cases (diagnosed as papillary cystadenocarcinoma). The nuclei were hyperchromatic, large, round to ovoid with 1-2 variably-sized nucleoli. Mitotic rate was moderate (up to 3 mitotic figures per x40 field). Infiltration of surrounding tissue and invasion of lymphatics was seen in both cases. Lymph node metastasis was detected in one case.

Tubular adenocarcinoma was found in 7 of the 20 cases of malignant mammary tumours. This neoplasm was characterized by multilobulated and poorly encapsulated proliferation of luminal epithelial cells forming anastomosing glandular structures (tubules and acini). Neoplastic cells were often piling up into the lumen and exhibited marked loss of polarity, they varied considerably in size and shape, had indistinct cytoplasmic borders and variable amounts of lightly basophilic cytoplasm. The neoplastic cells have medium to large (up to three fold anisokaryosis), round to oval euchromatic nuclei with prominent, often multiple nucleoli. Mitotic figures were common (2-7 per x40 field). Focal areas of necrosis and haemorrhage were often seen scattered throughout the tumour. Lymphatic invasion and clusters of neoplastic cells within the lumen of some veins were often seen. Clusters of lymphocytes and plasma cells were located at the periphery of neoplastic aggregates.

Complex carcinoma was diagnosed in 2 of the 20 cases of malignant mammary tumours. This tumour was composed of proliferation of neoplastic luminal epithelial cells arranged in a tubular to papillary pattern and surrounded by numerous myoepithelial cells. Neoplastic epithelial cells had morphologic characteristics similar to those described for tubular and papillary adenocarcinomas. Mitotic activity was high. Myoepithelial cells were polyhedral or spindle-shaped with vacuolated cytoplasm and arranged in a stellated reticulated pattern.

Mixed malignant mammary tumour was diagnosed in 1 of the 20 cases of spontaneous malignant mammary tumours. This neoplasm was composed of luminal epithelial cells, myoepithelial cells and connective tissue. The stroma varied from fibrous to fibromucinous to chondromucinous. Diagnosis of malignant mammary tumour was based on presence of carcinomatous changes.

Anaplastic carcinoma was diagnosed in 3 of the 20 cases of malignant mammary tumours. This tumour was characterized by a poorly circumscribed and non-encapsulated mass consisting of sheets and cords of neoplastic epithelial cells embedded in a prominent connective tissue stroma. Neoplastic cells were occasionally forming small ill-defined tubular structures or thin cords. Neoplastic cells were pleomorphic, cuboidal to polygonal, with poorly delineated, pale basophilic cytoplasm. The nuclei were hyperchromatic, often large (upon to 4-fold anisokaryosis), round to oval, with multiple prominent nucleoli. Mitotic rate was high (4-10 mitotic figures per x40 field). Collagenous stroma was abundant in 2 of the 3 cases (diagnosed as scirrhous carcinoma) and there was closely intermingling of the neoplastic epithelial cells with the fibrous stroma. Scattered foci of lymphocytes were seen at the periphery of the tumour. Invasion of lymphatics was a common feature.

### *Immunohistochemistry*

In normal mammary tissue, most alveolar and ductal epithelial cells were weakly positive for MT but sporadic cells stained intensely. Reactivity was mainly intracytoplasmic, and located at the apex portion of the cytoplasm. Although normal mammary lobules stained for MT, the staining was usually heterogeneous within the lobules and the cells with the most intense staining appeared hyperplastic, as evidenced by a large nucleus

and cytoplasm relative to non-staining normal epithelial cells. As the number of lobular layers of epithelium increased both the proportion of immunopositive cells and intensity of the stain increased. The wall of arteries served as internal positive control for actin immunostaining. Myoepithelial cells in normal mammary gland and within neoplastic tissue stained strongly with actin (Fig. 1), but did not stain with MT.

Intensity of the MT immunostain in normal tissue often correlated with that of the adjacent neoplastic tissue. In benign mammary neoplasms MT immunostaining intensity was strong and it was seen in the cytoplasm and, occasionally, the nucleus of luminal epithelial cells. As demonstrated in Table 1, the percentage of MT immuno-positive cells in benign tumours often hinged on the proportion of luminal epithelial cells present in each tumour. The degree of MT immunostaining decreased in terms of number of immunopositive cells and staining intensity in less differentiated anaplastic neoplastic cells. In malignant tumours immunostaining was faint but within some carcinomas it was possible to find small clusters of intensely staining glandular epithelial cells.

Results of MT immunostaining intensity and overexpression of all tumours examined are presented in Tables 1 and 2. Cumulative results of metallothionein overexpression in specific benign and malignant mammary tumours are presented in Table 3.

**Table 3.** Classification of mammary tumours, distribution and Metallothionein (MT) staining intensity.

| TYPE OF TUMOUR | MT OVEREXPRESSION |
|---|---|
| *Benign tumours* | |
| Papillary adenoma | 3/3 |
| Complex adenoma | 2/4 |
| Benign mixed tumour | 4/13 |
| *Malignant tumours* | |
| Solid adenocarcinoma | 3/5 |
| Papillary adenocarcinoma | 1/2 |
| Tubular adenocarcinoma | 1/7 |
| Complex adenocarcinoma | 1/2 |
| Malignant mixed tumour | 1/1 |
| Anaplastic carcinoma | 0/3 |

### Papillary adenoma

MT overexpression (more than 10% of MT positive tumour cells) was detected in the three cases examined. Staining intensity in epithelial cells was moderate, but cells lining papillary projections stained strongly for MT (Fig. 2a) and negative for actin (Fig. 2b).

### Complex adenoma

MT overexpression was observed in 2/4 cases. Epithelial cells arranged in tubules and acini stained mild to moderate for MT, but the papillated parts stained strongly. Myoepithelial cells and mesenchymal cells were negative.

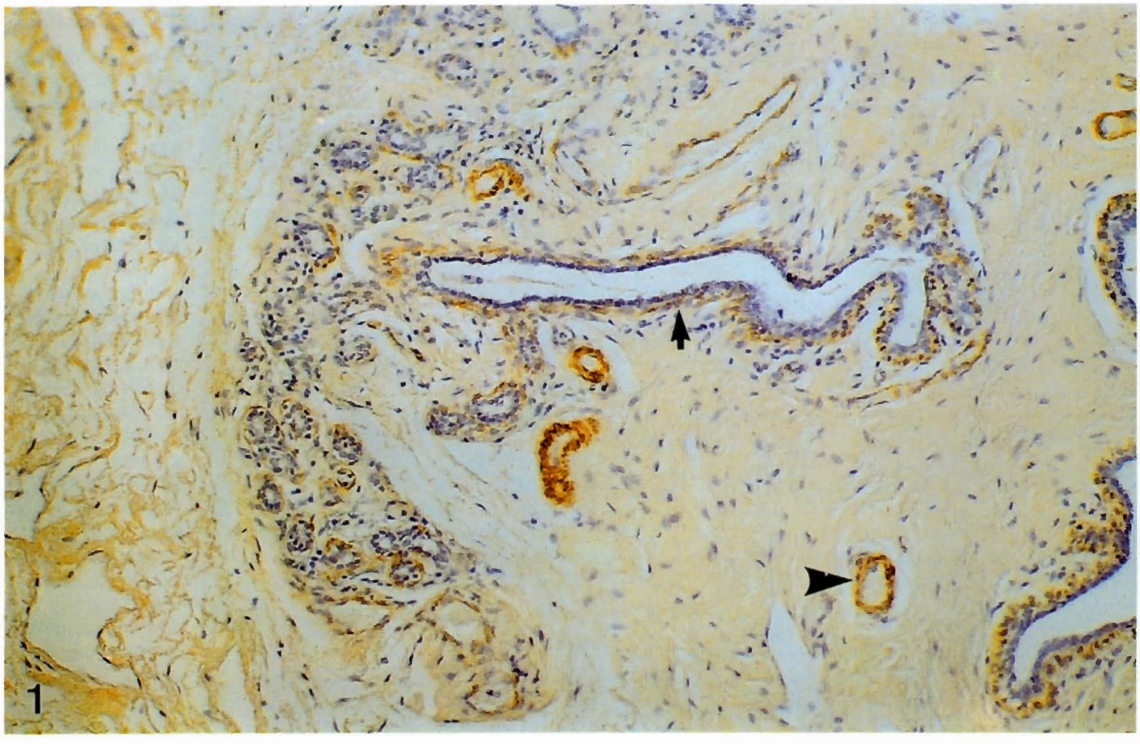


**Fig. 1.** Canine mammary gland. Positive immunostaining in myoepithelial cells (arrow) and smooth muscle cells (arrow head). Anti-human alpha-smooth muscle actin antibody with haematoxylin counterstain. x 180

Benign mixed tumour

The proportion of immunopositive cells was related to the proportion of luminal epithelium present in the tumour, and varied therefore from 1 to 50%. MT overexpression was detected in 4 out of 13 cases.

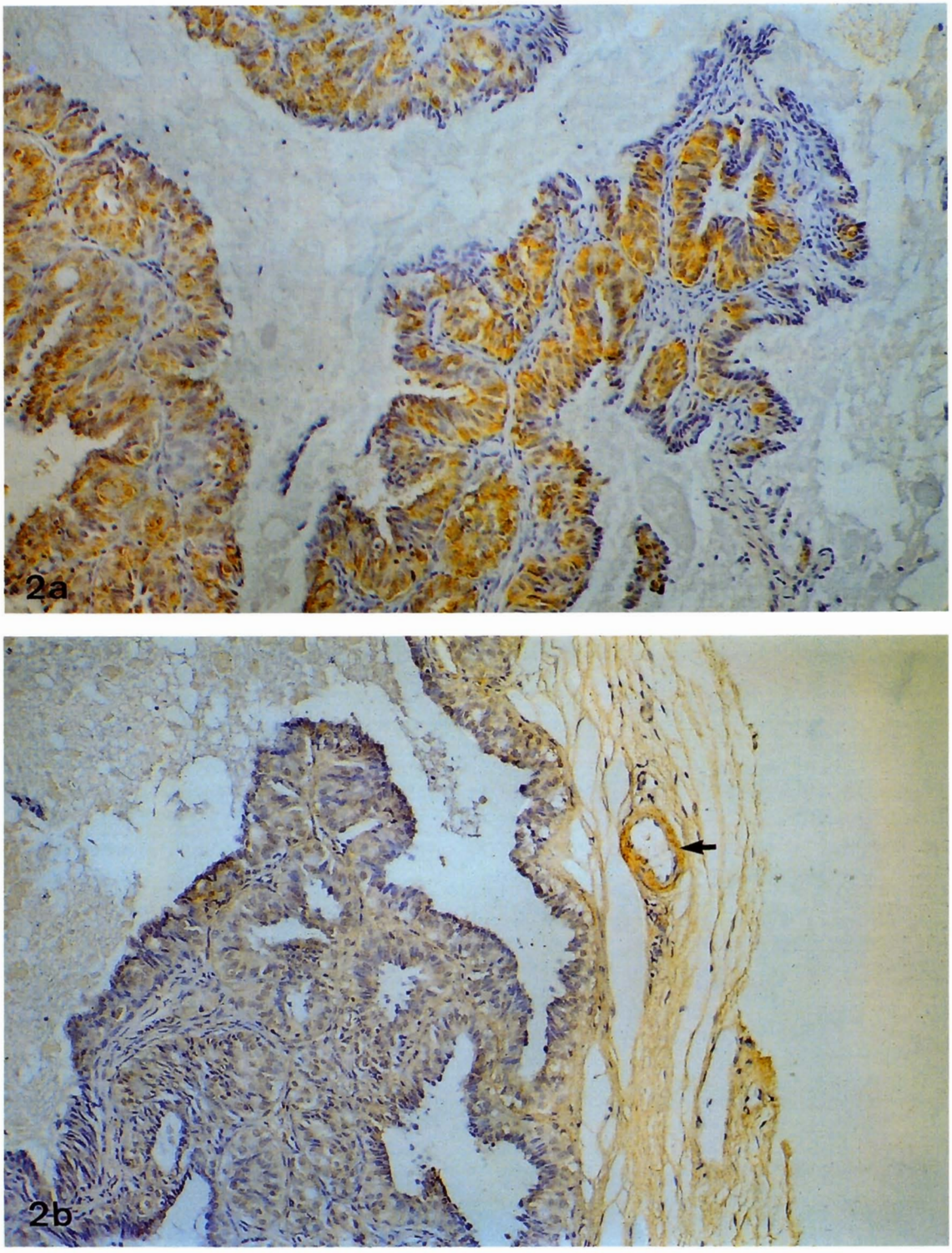


**Fig. 2. a.** Papillary adenoma. Canine mammary gland. Strong positive MT-immuno-staining in epithelial cells within papillary projections. E9 against horse metallothionein and haematoxylin counterstain. x 180. **b.** Papillary adenoma. Canine mammary gland. Cells lining papillary projections are negative for actin, compared with smooth muscle of adjacent blood vessel (arrow). Anti-human alpha-smooth muscle actin antibody with haematoxylin counterstain. x 180

Intensity of the immunostaining in epithelial cells was moderate to strong and both the cytoplasm and nuclei stained. Spindle shaped myoepithelial cells stained negative. Star-shaped vacuolated myoepithelial cells had faint cytoplasmic and nuclear stain. Myxomatous, fibrous and cartilaginous tissues were negative except for a few chondrocytes which stained positive (Fig. 3). Some papillated structures were lined by immunopositive epithelium but the core was composed of myoepithelium or cartilage, which did not stain for MT.

Solid adenocarcinoma

Overall staining varied from 0 to 50% of the tumour and MT overexpression was observed in 3 out of 5 cases examined. Staining intensity in immunopositive epithelial cells varied from weak to strong. Necrotic non-staining cores were often bordered by several layers of non-staining epithelial cells.

Papillary adenocarcinoma

MT overexpression was observed in 1 out of 2 cases. Epithelial cells' MT immunostaining intensity was generally weak and the staining was mainly cytoplasmic. Staining intensity was higher (moderate) at the base of the fronds rather than the tips. Myoepithelial cells were negative. Metastatic luminal epithelial cells within a regional lymph node stained moderately positive for MT.

Tubular adenocarcinoma

The proportion of immunopositive cells in the tumour varied from 0-50% and MT overexpression was found in only 1 of the 7 cases examined. The staining intensity was weak to moderate and heterogeneous within the neoplastic mass, with scattered immunopositive epithelial cells (Fig. 4). A few necrotic epithelial cells stained weakly positive.

Complex adenocarcinoma

MT overexpression was detected in 1 out of 2 cases examined. A few epithelial cells at the centre of neoplastic lobules stained moderate. Weak immunostaining was seen in the case with less than 10% immunopositive cells. Myoepithelial cells were negative.

Malignant mixed mammary tumour

MT overexpression was detected in the case examined. Immunostaining intensity was weak. Myoepithelial cells were negative.

Anaplastic carcinoma

Negative staining was seen in one case and less than 10% of the cells stained in the other 2 cases examined. The immunostaining intensity was weak to moderate. Tumour emboli within lymphatics also stained weakly (Fig. 5). Fibrous connective tissue was negative for MT.

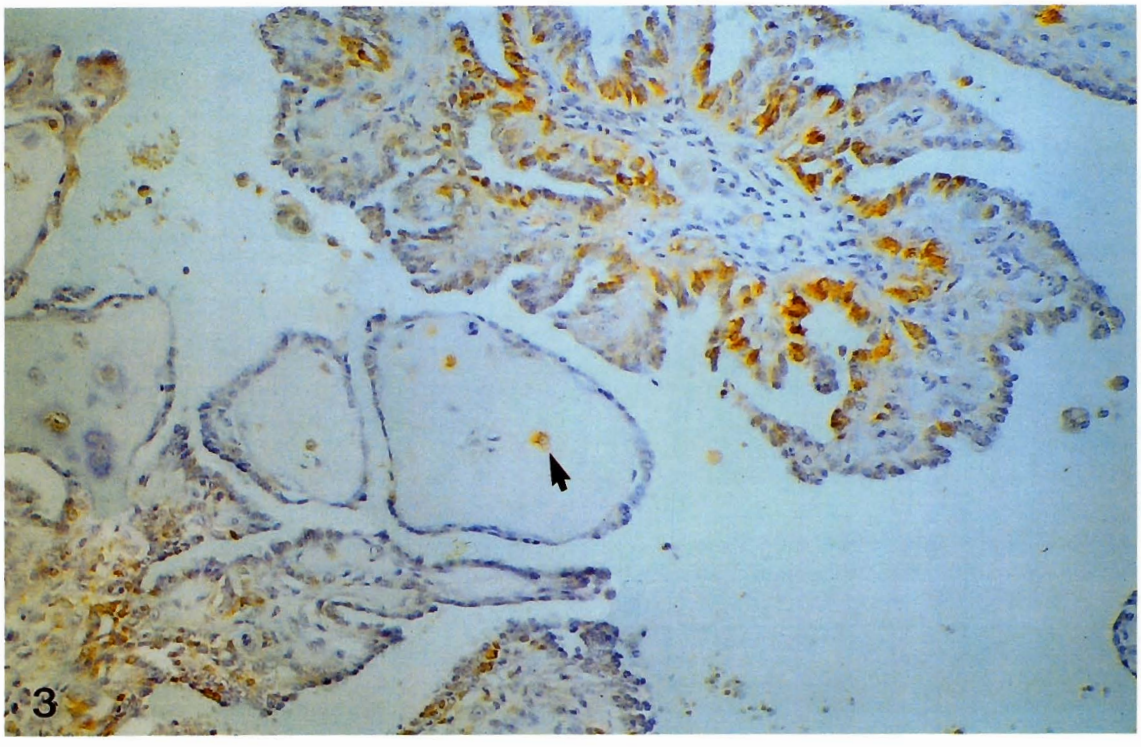


**Fig. 3.** Mixed mammary tumour. Canine mammary gland. Weak to moderate immunostaining in a few chondrocytes (arrow) and some epithelial cells lining papillated structures. Myxomatous and cartilaginous tissues are negative. E9 against horse metallothionein and haematoxylin counterstain. x 180

## Discussion

In the present study the breed susceptibility and age at the time of detection were in agreement with previous reports of canine mammary neoplasia. Priester (1979) described a preponderance of sporting breeds among

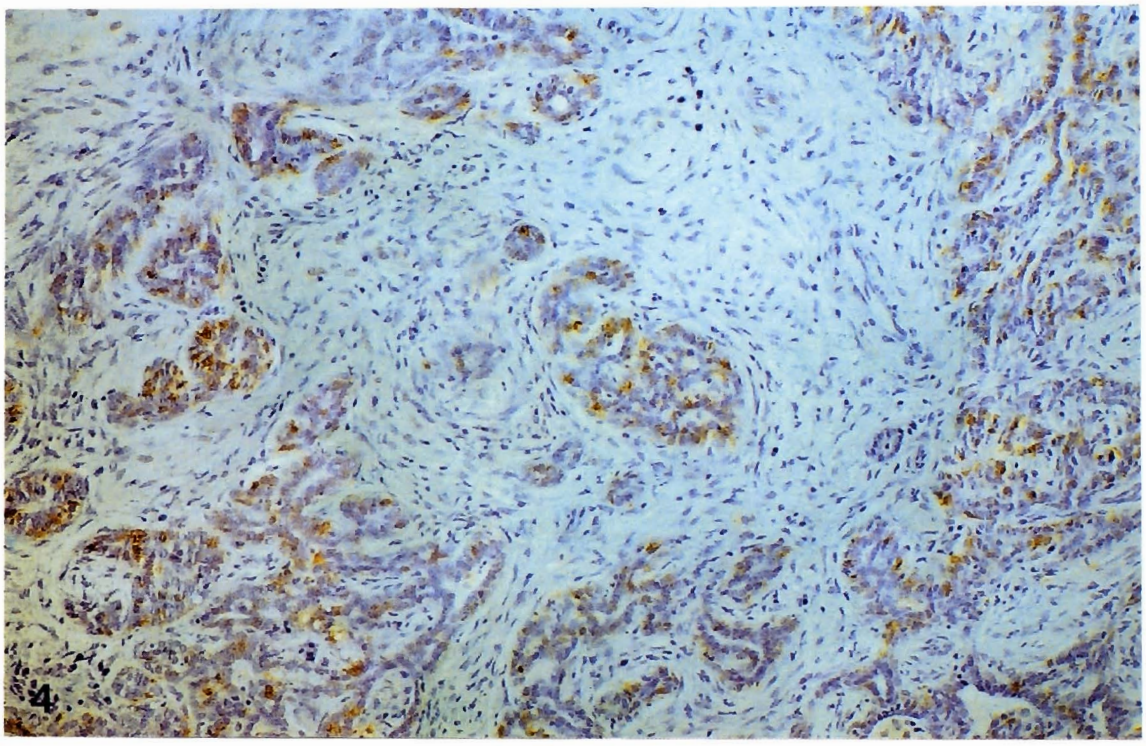


**Fig. 4.** Tubular adenocarcinoma. Canine mammary gland. Scattered neoplastic cells stain weak to moderate. E9 against horse metallothionein and haematoxylin counterstain. x 180

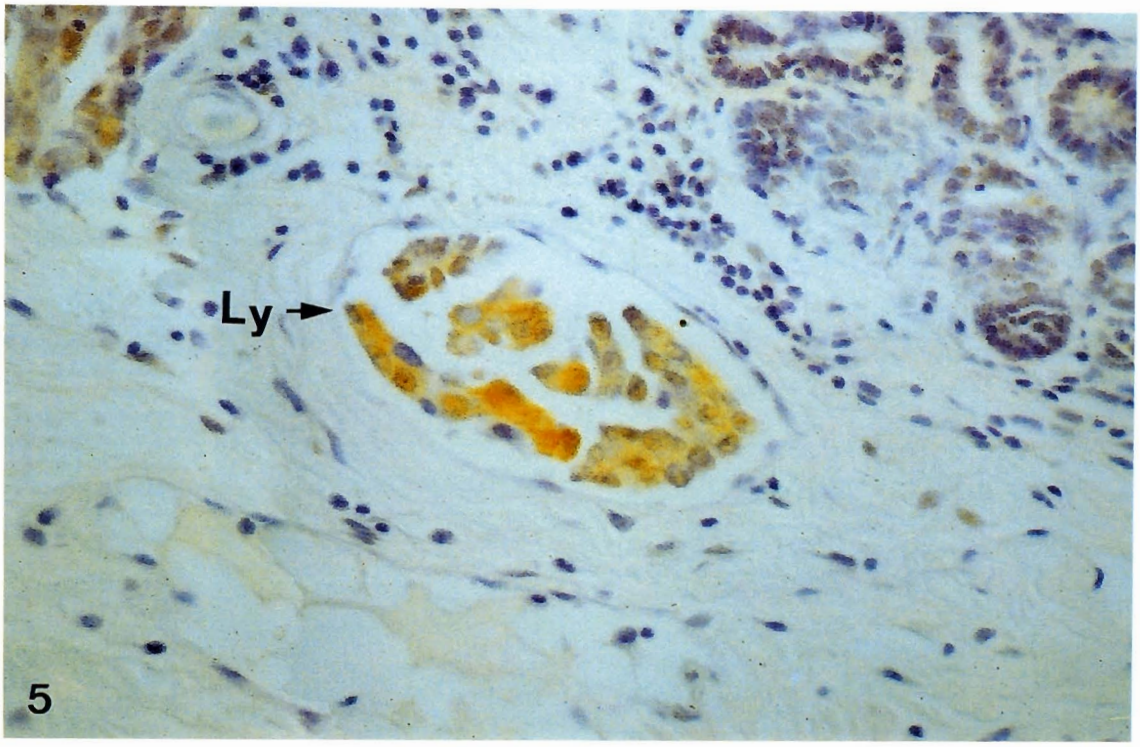


**Fig. 5.** Anaplastic carcinoma. Canine mammary gland. Weak to moderate MT-immuno-staining in tumour emboli within lymphatics (Ly). E9 against horse metallothionein and haematoxylin counterstain. x 240

those at high risk of mammary neoplasms. This higher incidence may be associated with genetic factors as many of the sporting breeds are interrelated (Priester, 1979). A genetic component in the occurrence of mammary tumours has been reported in dogs (Dorn and Schneider, 1976), and in some types of human breast cancer (Anderson, 1974). Mammary tumours are considered a disease of the middle-aged and old bitch (Moulton et al., 1986; Destexhe et al., 1993; Hellmén et al., 1993). The age range for mammary neoplasms is 10.4 to 13.9 years, but benign tumours occur 1 to 2 years earlier (Priester, 1979; Moulton et al., 1986). The differences in the age of onset of benign and malignant mammary tumours in dogs have been interpreted as a potential support to the hypothesis of a sequence of mammary tissue transformation from dysplasia to hyperplasia to benign neoplasm, culminating with malignant neoplasms (Priester, 1979). Some authors have found morphological evidence that certain benign tumours may arise from lobular hyperplasia (Hampe and Misdorp, 1974) and malignant mammary tumours may develop on benign lesions (Brealey, 1989).

The diagnosis and prognosis of canine mammary tumours based on routine histopathology are challenging due in part to their diverse histogenesis (Griffey et al., 1993). Canine mammary tumours with overt signs of malignancy, such as pronounced infiltrative growth, ulcerated skin and lymph node metastasis do not present a diagnostic challenge for clinicians. However, many canine mammary tumours are not at an advanced stage of development when first detected. To improve the accuracy of the prognosis, other diagnostic tools have been added to conventional histopathology (Hellmén et al., 1988).

Immunohistochemical methods have been used to demonstrate the presence of basal epithelium- and luminal epithelium-specific cytokeratins within ductular and alveolar epithelium in human breast tissue (Dairkee et al., 1985) and canine mammary gland (Griffey et al., 1993). Expression of smooth muscle actin has been described in myoepithelial cells in the canine (Vos et al., 1993), human and feline mammary glands (Martín de las Muelas et al., 1994). Positive MT immunostaining has been described in myoepithelial cells in both benign epithelium and human breast carcinoma, and only occasionally in ductal epithelial cells (Schmid et al., 1993; Fresno et al., 1993; Bier et al., 1994; Haerslev et al., 1994; Douglas-Jones et al., 1995). In our study, luminal epithelial cells stained positive for MT, but myoepithelial cells were negative for MT and positive for alpha-smooth muscle actin. The reason for this discrepancy is not known.

There is much controversy about the histogenesis of canine mammary tumours and the origin of mesenchymal components (Fowler et al., 1974; Hampe and Misdorp, 1974; Hellmén and Lindgren, 1989). Mesenchymal tumours of the canine mammary gland may originate from the myoepithelial cells (Fowler et al., 1974). Specifically, it has been suggested that the chondromucinous change found in mixed mammary tumours develops from myoepithelial ground substance (Moulton, 1990), and that cartilage and bone arise from metaplasia of myoepithelial cells (Moulton et al., 1986; Destexhe et al., 1993). In the present study, we found morphologic evidence of a progressive transformation from myoepithelial cells to cartilage in mixed mammary tumours.

Recent research suggests that MT is associated with cell proliferation and differentiation which are both occurring at an accelerated rate in growing tumours (Cherian, 1994). The presence of MT in the developing liver and the endodermal yolk sac of mice suggests that the developmental profile of MT is similar to other oncofetal gene products such as alpha-fetoprotein and that it has potential use as a marker for aggressive tumour behaviour (Fresno et al., 1993). However, it is not known whether tumours with MT overexpression originate from cells expressing MT already in their normal state or MT expression should be regarded as a secondary phenomenon caused by one or more factors responsible for MT Induction (Bier et al., 1994). Synthesis of MT can be induced in tissues by metal ions such as zinc, copper, and cadmium as well as by endogenous factors such as glucocorticoids, interferon, interleukin-1, lipopolysaccharide, ultraviolet light, progesterone, and vitamin D3 (Hamer, 1986; Nartey et al., 1987).

MT has the ability to bind to large quantities of metal ions, which emphasize its function as an intracellular reservoir for essential ions. MT plays an important role in detoxification of toxic metals such as zinc and copper (Goering and Klaasen, 1984; Hamer, 1986). Histochemically detectable copper, zinc and iron have been found in malignant melanoma (Bedrick et al., 1991). Furthermore, studies qualifying trace metal concentrations in benign and malignant tumours, including breast carcinomas, have demonstrated increased levels of copper or zinc in malignant tumours when compared with corresponding benign tumors (Santoliquido et al., 1976; Margalioth et al., 1983). The presence of high levels of copper in tumour tissue is interesting since this metal has been implicated in the generation of hydroxyl free radicals that can damage DNA. Copper complexes are reduced by the superoxide radicals or other reducing agents to the cuprous state, and then they react with hydrogen peroxide to form hydroxyl radicals. These radicals may cause double-stranded DNA breakage, which is not repairable by cellular mechanisms (Samuni et al., 1981). Cells under oxidative stress are likely to overexpress MT as a protective response to the DNA-damaging influence of hydroxyl radicals (Thornalley and Vasak, 1985). Decrease in the cytotoxic activity of certain anticancer drugs and increased resistance in MT-rich cells occurs after exposure ot ionizing radiation. However, the mechanism by which MT contributes to this protection is still unclear. Studies have shown that cancer cell lines originating from leukemic cells (Beck et al., 1979), ovarian carcinoma (Andrews et al., 1987;

Kelleey et al., 1988), prostatic carcinoma (Webber et al., 1988) with increased MT expression are more likely to be resistant to chemotherapy. Ovarian carcinoma cells with acquired resistance to anticancer agents often show an increase in MT mRNA and MT content (Kelleey et al., 1988).

Our finding of variations in the intensity and distribution of the stained cells within the same specimen is shared by others. Douglas-Jones et al. (1995) suggested that the immunostaining heterogeneity is a reflection of a wide variety of expression of MT among malignant cells in the same duct, indicating the possibility of a clonal selection process to occur during progression of the tumour. In the present study the staining intensity was higher in benign compared to malignant mammary tumours, however, a few malignant tumours stained strongly. We used a similar score and definition of MT overexpression to that described in human breast cancer (Fresno et al., 1993; Haerslev et al., 1995), and detected immunohistochemical MT overexpression in both benign and malignant canine mammary tumours. Conversely, in human breast tumours MT immunostaining has been consistently found to correlate with a poor prognosis in invasive duct carcinomas whereas lack of MT expression has been found in normal lobular epithelium and little or no expression in lobular carcinomas (Fresno et al., 1993; Schmid et al., 1993; Bier et al., 1994; Douglas-Jones et al., 1995; Goulding et al., 1995). Although breast tumours of favourable prognostic type have significantly lower levels of MT expression than tumours of poor prognosis subtypes, invasive lobular carcinomas are constantly MT-negative irrespective of their clinical course (Schmid et al., 1993). The cause for the inconsistency between MT immunopositivity results in canine and human mammary tumours is not clear. Most immunohistochemical studies of MT have focused in breast cancer (Schmid et al., 1993; Haerslev, 1995; Fresno et al., 1993; Douglas-Jones et al., 1995; Goulding et al., 1995), thus it is possible that similar to the situation in canine mammary tumours MT may also be present in benign breast tumours. Bier et al. (1994) found that in addition to ductal carcinoma *in situ* and invasive ductal carcinoma, MT immunohistochemical expression also occurs in benign breast lesions such as adenosis, scleradenosis, papillomas and epitheliosis, which they interpreted as a possible indication that MT overexpression may occur during cell proliferation and tumour progression. Similarly, since high levels of MT were found in both benign and malignant epithelial tumours, it was suggested that MT increase may occur depending on the type of tumour, cellular origin, morphological heterogeneity or stage of growth (Cherian, 1994). This concept is also supported by other authors, who proposed a physiological role for MT in cellular proliferation in human colonic cancer cells (Nagel and Vallee, 1995). Therefore, positive MT immunostaining in epithelial cells in both benign and malignant canine mammary tumours, in some chondrocytes in mixed mammary tumours, and the differences in staining intensity in tumour emboli and metastatic cells within lymph nodes may be a manifestation of cell proliferation, rather than an indication of neoplastic behaviour.

We conclude that MT immunostaining cannot be used as a diagnostic tool to distinguish benign and malignant mammary neoplasms in dogs. The role of MT overexpression in the prognosis of canine mammary tumours is questionable, but the pathogenesis of MT overexpression in benign and malignant mammary tumours and its role in tumour proliferation and tumour progression requires clarification.

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