Immunohistochemical demonstration of p53 protein and metallothioneins in canine mammary tumours

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SUMMARY

In the study, p53 protein and metallothioneins (MT) were investigated by immunohistochemistry in canine mammary tumours (n = 50). These tumours (5 benign mixed tumours (BMT) and 45 malignant tumours whose 36 malignant mixed tumours (MMT) and 9 adenocarcinomas (AC)) mainly involved ingunal (n = 18), caudal thoracic (n = 11) and caudal abdominal (n = 10) glands. Positive p53 protein immunolabelling of nucleus and/or cytoplasm of glandular epithelial cells and more rarely of ductal epithelial cells were observed in all malignant tumours. Furthermore, a moderate to marked p53 staining was evidenced in 75% of MMT and in 44% of AC. Forty-four (98%) malignant tumours also exhibited metallothionein (MT) positive immunostaining of nucleus, cytoplasm or both. The MT positivity was moderate to intense in 82% of positive tumours (30 MMT and 6 AC). The p53 protein and MT expressions with a low to a moderate intensity were detected in 2 and 3 benign mixed tumours respectively and 2 of them simultaneously expressed the 2 markers. These results suggest that the subcellular accumulation of p53 protein and MT is associated with tumour malignancy and that positive MT and p53 protein benign tumours would evolve into malignant tumours.

Keywords: Dog, mammary gland, p53 protein, metallothionein, tumour, immunohistochemistry.

RÉSUMÉ

Détection immunohistochimique de la protéine p53 et des métallothionéines dans les tumeurs mammaires chez le chien

Cette étude a eu pour objectif de rechercher l’expression de la protéine p53 et des métallothionéines (MTs) par immunohistochimie dans les tumeurs mammaires chez le chien. Parmi les 50 cas étudiés, 5 étaient des tumeurs mixtes bénignes (TMB) alors que 45 étaient malignes (36 tumeurs mixtes malignes (TMM) et 9 adénocarcinomes (AC)) et ces tumeurs ont principalement affecté les glandes inguinales (n = 18), les glandes thoraciques caudales (n = 11) et les glandes abdominales caudales (n = 10). La protéine p53 a été détectée dans toutes les tumeurs malignes, dans le noyau et/ou le cytoplasme des cellules épithéliales glandulaires principalement et plus rarement des cellules épithéliales des canaux mammaires. En outre, le marquage de p53 a été modéré à élevé dans 75 % des TMM et 44 % des AC. La présence des métallothionéines dans le noyau et/ou le cytoplasme a également été mise en évidence dans 44 tumeurs malignes (98 %) et 82 % des tumeurs positives (30 TMM et 6 AC) ont présenté un marquage modéré à intense. La protéine p53 et les MTs ont été exprimées dans, respectivement 2 et 3 tumeurs bénignes, avec une intensité faible à moyenne et ces 2 marqueurs ont été simultanément détectés dans 2 d’entre elles. Ces résultats suggèrent que l’accumulation intracellulaire de la protéine p53 et des MT est associée à la malignité de la tumeur et que les tumeurs bénignes positives de la protéine p53 et les MT pourraient devenir malignes.

Mots clés : Chien, glande mammaire, protéine p53, métallothionéine, tumeur, immunohistochimie.

Introduction

Mammary tumours in dogs are the most common tumours following skin tumours [33]. The incidence and frequency of these tumours reported in all parts of the world has shown some regional differences. Investigations have shown that about 25-50% of tumours in dogs were mammary tumours and the rest were hyperplasia, adenoma, and myoepithelioma [12, 18, 40]. In recent years, several prognostic factors have been used to determine the characteristics of these tumours in humans and animals [7]. In order to ascertain these factors, canine mammary tumours are used as a good model for human breast tumours [47].

Metallothioneins (MTs) are low molecular weight proteins with high cystein content, which are selectively bound to zinc (Zn), copper (Cu) and other group II heavy metals [3, 48, 56]. Because of their great binding affinity characteristic to metal divalent ions, they are thought to play a role in Zn and Cu homeostasis, heavy metal detoxification, metal transport, and even in protecting the cell from oxidative stress [6, 23]. Their role in cell proliferation and carcinogenesis by embryogenesis differentiation is not clearly understood. These also provide drug resistance to some chemotherapeutics and prevent the side effects of anticancer drugs [46]. The genesis and release of free radicals induced by some metal ions lead to unrecoverable damage on DNA, and MTs act as cell scavenger of metal ions [21], offering in this way a relative protection to the induced oxidative stress. But, on the other hand, it is postulated that they can inactivate the Zn dependent p53 protein, a major tumour suppressor factor, by chelating Zn$^{2+}$, resulting in neoplastic cell proliferation [11].

The p53 protein is a specific cellular oncoprotein detected in mouse sarcomas [9] and in other tumours. Nevertheless, it has been shown that this protein exists not only in tumoral, but also in normal tissues. Therefore, the p53 protein is evaluated in two groups as “wild type (normal) p53 protein” and...
“mutant p53 protein”. The half-life of “wild type p53 protein” is very short (6-30 seconds) and it is not sufficiently concentrated in tissues to be identified by immunohistochemistry. The half-life of “mutant p53 protein” is longer and it accumulates in the cell nucleus making detection easy. The P53 gene is a suppressor gene for tumour growth. Changes in this gene are detected by cytogenetic, molecular, and immunohistochemistry methods in many tumours [29, 41, 43]. Detection of the p53 mutant protein, particularly by immunohistochemistry as a marker of p53 gene mutation and its accumulation in malignant tumours of high grade [7, 29, 55] have lead to consider the p53 protein as a bad prognostic factor.

The aim of this study was to investigate the accumulation of p53 protein and MT in malignant and benign mammary tumours in dog and to correlate them with the tumour type and grade.

Materials and Methods

ANIMALS

Fifty female dogs, 6 - 15 years old for most of them, with mammary tumours were used in this study: 23 Terriers, 3 Boxers, 3 Cockers, 1 Kangal, 1 Ireland Setter, 1 Doberman, 1 Rotweiler, 1 Poodle, 1 Pointer and 15 Mongrels. A complete physical examination based on TNM (Tumour, Nodule and Metastasis) classification [37] was performed on all dogs: thoracic radiographs (3 views), a complete haematological analysis and biochemical profiles were obtained on each dog to evaluate the occurrence of pulmonary metastasis and of paraneoplastic syndromes and to estimate the general health status.

As resection method of the mammary glands, the unilateral radical mastectomy technique was performed when mass/masses occurred through the mammary chain but when mass/masses occurred in both chains, bilateral radical mastectomy was practiced according to the standard surgical method and general anaesthesia protocols [19]. The surgically removed mammary chains / masses were evaluated histopathologically and immunohistochemically analyses of histological specimens were done.

HISTOLOGICAL AND IMMUNOHISTOCHEMICAL METHODS

The masses were fixed in 10% formalin, processed routinely, and embedded in paraffin. Then, 5-6 µm sections were prepared and stained with haematoxylin-eosin (HE).

Immunohistochemical staining was performed using the standard avidin-biotin peroxidase complex (ABC, Dako, Carpinteria, USA) method. The sections were heated in a microwave oven, in 0.01 M citric acid for 5 min at 700 watts and then cooled for 20 min. Endogenous peroxidase was blocked by immersing the sections in 0.3% hydrogen peroxide in absolute methanol for 30 min. Subsequently, the slides were incubated with the respective anti p53 PAb 240 (monoclonal antibody targeted mutant p53) and anti metallothioneins primary rabbit antibodies (Dako) diluted in PBS (phosphate buffer saline) to 1:500 for 60 min at room temperature. After washing with PBS, the sections were incubated for 20 min with biotinylated goat anti-rabbit antibodies at room temperature. After washing again, the immune complexes were detected by the streptavidin-biotin horseradish peroxidase complex using amino-ethyl carbazole (AEC) as chromogen (DAKO). The Mayer’s haematoxylin was used for counterstaining. Negative control sections were treated as described above except that primary antibodies were omitted. Slides were read with an optic microscope (Leica, DM 4000 B) at X100 magnification and positive cells for MTs or for p53 protein were counted on 10 different microscopic fields. The following immunohistochemical scores were established: 0 (no immune positive cell), 1+ (less than 10%), 2+ (10-50%) and 3+ (more than 50%).

Results

Among the 50 tumours, 5 were benign (5 benign mixed tumours) (figure 1A) and 45 were malignant (36 malign mixed tumours (figure 2A) and 9 carcinomas (figures 3A and 4A). The tumoral masses affected mainly the inguinal glands (n = 18), caudal thoracic glands (n = 11) and caudal abdominal glands (n = 10), and more occasionally cranial abdominal glands (n = 6) and cranial thoracic glands (n = 5) (Table I).

<table>
<thead>
<tr>
<th>Tumour types</th>
<th>N</th>
<th>Affected mammary glands</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Cr. T.</td>
</tr>
<tr>
<td>Benign tumours</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benign mixed tumour</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>Malignant tumours</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malign mixed tumour</td>
<td>36</td>
<td>3</td>
</tr>
<tr>
<td>Carcinoma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tubular adenocarcinoma</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Papillary cystic adenocarcinoma</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Ductal adenocarcinoma</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>50</td>
<td>5</td>
</tr>
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N: number of cases; Cr. T.: cranial thoracic glands; Ca. T.: caudal thoracic glands; Cr. A.: cranial abdominal glands; Ca. A.: caudal abdominal glands; I.: inguinal glands.

Table I: Histological classification (according to the World Health Organization [18]) and localisation of the 50 canine mammary tumours.
In the present study, the relative frequency of the different types of mammary tumours and their localization on mammary chain were in accordance with previous data [25] although the incidence of benign tumours appeared low compared to the GILBERTSON’s study [15].

Recently, many prognostic factors have been used to determine the characteristics of human and animal tumours [7]. Among these reagents, p53 protein, a nuclear non-histone protein, plays an important role in the cell cycle control. When DNA is damaged, the p53 protein gene is activated and the expressed p53 protein is involved in DNA repair, regulation of cell proliferation and in programmed cell death [17, 54]. Some p53 gene mutations are frequently found in many types of lung, colon, and thoracic cancers in humans and are strongly involved in malignancy progression because they lead to the loss of the p53 tumour suppressive function [2, 8, 26]. In animals, mutations are also encountered in canine mammary tumours [29] and osteosarcomas [31, 51], and in haematopoietic tumours in cats [36]. The p53 protein accumulation has been also found in canine mammary tumours [14, 20, 50] and in squamous cell carcinomas of cats and

### Table II: Immuno-positivity of the p53 protein and of metallothioneins (MTs) according to the type of the canine mammary tumours (n = 50).

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<tbody>
<tr>
<td>Benign (n = 5)</td>
<td>2 (40%)</td>
<td>0 (0%)</td>
<td>1 (20%)</td>
<td>2 (40%)</td>
</tr>
<tr>
<td>Malignant (n = 45)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMT (n = 36)</td>
<td>0 (0%)</td>
<td>1 (3%)</td>
<td>0 (0%)</td>
<td>35 (97%)</td>
</tr>
<tr>
<td>AC (n = 9)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>9 (100%)</td>
</tr>
</tbody>
</table>

MMT: malignant mixed tumours; AC: adenocarcinoma.

### Table III: Frequencies of positive p53 protein and MT (metallothionein) immunolabelling in canine mammary tumours (n = 50).

Discussion

In the present study, the relative frequency of the different types of mammary tumours and their localization on mammary chain were in accordance with previous data [25] although the incidence of benign tumours appeared low compared to the GILBERTSON’s study [15].

Recently, many prognostic factors have been used to determine the characteristics of human and animal tumours [7]. Among these reagents, p53 protein, a nuclear non-histone protein, plays an important role in the cell cycle control.
In agreement with these previous studies, a total of 94% (47/50) canine mammary tumours, 2 benign tumours and all malignant tumours, showed variable p53 positivity in the present study. Intense positive p53 staining is mostly observed in malignant tumours (69%), suggesting that p53 accumulation would be a malignancy criterion to detect a poor prognosis and increased malignancy potential. The p53 staining occurrence in benign cases would be considered as a high malignancy risk, despite a benign histopathological appearance. HAGA et al. [16] stated that p53 protein accumulation in canine mammary tumours showed 50-60% positivity compared with human breast or mouse mammary tumours, and this high positivity was explained by the use of multiple monoclonal antibodies. With the anti-p53 BP53-12 monoclonal antibody which recognizes N epitope, or the anti-p53 PAb122 monoclonal antibody which recognizes C epitope, 50% and 60% respectively of tumours were positive, whereas the percentage of positive tumours was enhanced when the 2 antibodies were simultaneously used. Therefore, by the use of multiple monoclonal antibodies related with N, C, and even mid-region epitopes, the positive rate is reported to be higher and these studies will be useful not only for

**FIGURE 1**: Benign mixed tumour: (A): haematoxylin-eosin (HE), X100; (B): p53 protein positivity in glandular epithelium (arrows), avidin-biotin peroxidase complex method (ABC), X100; (C): MT positivity in glandular epithelium (arrows), avidin-biotin peroxidase complex method (ABC), X100.

**FIGURE 2**: Malignant mixed tumour: (A): haematoxylin-eosin (HE), X100; (B): p53 protein positivity in glandular epithelium (arrows), avidin-biotin peroxidase complex method (ABC), X100; (C): MT positivity in glandular epithelium (arrows), avidin-biotin peroxidase complex method (ABC), X100.
canine mammary tumours, but also for humans. However, KANAYA et al. [24] reported that p53 protein intensive staining in dogs is only observed in malignant myoepithelioma, where no staining was obtained in mammary tumours and in other tumour types. While the rate of p53 protein positivity was determined as 20-50% in human breast cancer [8, 34], great differences of p53 protein immuno-positivity were observed in canine mammary tumours according to the used antibodies and the tumour types [1, 14, 27, 45]. Among these, PAb421 did not give any results in dogs by immuno-histochemistry [27] and CM-1 (anti-wild type and mutant p53 protein) and PAb240 (anti-mutant p53) gave positive results both in benign and malignant tumours [27, 42]. Some investigators [1, 49] reported that no p53 protein immuno-staining was obtained with Ab-7 (anti-wild type and mutant proteins) in dogs, cats, sheep, horse and cattle whereas the anti-wild type and mutant human p53 DO7 antibody was able to recognize the ovine, equine and bovine p53 proteins but not the feline and canine proteins. These discrepancies could be related to structural differences (especially located on the surface of the molecule) of the wild type and mutant p53 proteins according to species [1, 45]. Moreover, the cellular
p53 protein accumulation is not absolutely linked to the occurrence of gene mutations [5, 13, 32, 39, 44, 52]: several mechanisms of cytoplasm p53 sequestration and inactivation have been described including interactions with viral or cellular proteins (mdm2 also often over-expressed in mammary tumours, bcl2, hsc70) or nuclear transport deregulation [4, 5, 13, 16, 32, 39, 44]. On the other hand, some gene alterations (nonsense mutations, deletions or intronic mutations) can generate unstable mutant proteins and structural modifications due to slide processing can abrogate antigen reactivity [52]. In canine mammary tumours, p53 protein positivity was observed in the nucleus as 20% and in the cytoplasm as 80% [20]. In this study, all cases showing p53 protein positivity displayed staining in the cytoplasm and/or nucleus or both.

The metallothionein (MT) over-expression is also considered as another criterion of malignancy and poor prognosis, particularly in human and animal invasive ductal carcinomas and malignant melanomas, because of strong correlations found between MT accumulation and tumour types and grades, the invasive characteristics (local recurrence, lymphatic or distant metastasis) and reduced survival [10, 11, 21, 22, 30, 35, 48]. In pre-neoplastic liver lesions, it is accepted as a positive prognostic factor [3, 6] and MT expression is associated with a poor prognosis in ductal breast carcinoma in humans [48]. However, the increase or decrease of MT expression may differ according to the species or to the tumour types of the living organism [10]. However, the mechanisms of MT induction and their role in tumorgenesis have not been fully elucidated [53]. In mouse mammary carcinoma, the intensity of MT expression is thought to be correlated with the tumour malignancy [53] and reflected a poor prognosis [48], but some researchers reported not uniform results about MT expression immediately occurs [21, 23]. Metallothioneins are proteins able to selectively bind Zn, Cu, and other group II heavy metals, and consequently are particularly involved in the Zn and Cu metabolisms and in heavy metal detoxification and transport [6, 23]. YONISH-ROUACH et al. [54] have reported increased cellular Zn and Cu contents in some benign and malignant tumours including breast carcinomas. The tissue accumulation of the divalent ions could be due to fixation with metallothioneins. Furthermore, as Zn2+ is a cofactor of the p53 protein, it would be possible that MTs interact and inactivate the tumour suppressive protein by chelating the divalent ion, amplifying in the way the loss of function of p53 and promoting the tumoral expansion [11].

In conclusion, positive and intense p53 protein and MT immunostaining preferentially occurs in malignant mammary tumours in dog, confirming the poor prognosis value of these 2 biochemical markers in this way. The occasional labelling of 2 benign mixed tumours seems to be associated with a malignancy risk. However, in order to confirm this, detailed and long-term studies should be performed on mammary and other solid tumours, eventually using multiple anti-p53 antibody types, for establishing correlations between p53 and/or MT over-expression and tumour type and grade, metastasis potential and survival duration. Moreover, the possible interactions between the p53 protein and metallothioneins and complex formation leading to the loss of tumour suppressive function would be investigated using double immunolabelling.

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References


