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Prognostic Value of Occult Isolated Tumour Cells within Regional Lymph Nodes of Dogs with Malignant Mammary Tumours

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Summary

Canine mammary tumours (CMTs) are the most common type of neoplasm in bitches. As in women, the presence of metastasis in regional lymph nodes is an important prognostic factor in bitches with mammary carcinomas, but the clinical significance of occult isolated tumour cells (ITCs) within lymph nodes is still undefined in this species. The effectiveness of immunohistochemistry (IHC) in identifying occult ITCs and micrometastasis (MIC) was compared with that of the conventional haematoxylin and eosin staining technique. The relationship between tumour size, histological type, histological grade and the presence of metastasis was evaluated. The overall survival (OS) of female dogs with occult mammary carcinomas and ITCs within lymph nodes was analysed. Fragments of mammary carcinoma and regional lymph nodes of 59 female dogs were also evaluated. Histological sections of mammary carcinoma and lymph node samples were studied for tumour diagnosis and lymph node samples were tested by IHC using a pan-cytokeratin antibody. It was found that 35.2% of occult ITCs and 2.8% of hidden MIC were detected when IHC was used. There was a good correlation between the size of the tumour and metastasis to the lymph nodes ($P = 0.77$). ITCs were observed more frequently in the medullary region (60.7%) and metastases in the cortical region (44.4%). There was no significant difference in the OS between female dogs with occult ITCs and lymph nodes without ITCs. IHC can detect occult tumour cells in lymph nodes that are negative by histopathological examination. Female dogs with nodal ITCs do not have lower survival.

Keywords: dog; immunohistochemistry; mammary tumour; metastasis

Introduction

Canine mammary tumours (CMTs) are the most common type of neoplasm in bitches and the frequency of malignant tumours is high (>50%) (Carvalho *et al.*, 2016). Several factors have been used to determine the prognosis of CMTs, including tumour size (Sorenmo *et al.*, 2009), histological type (De Araújo *et al.*, 2015), histological grade (Gundim *et al.*, 2016) and the 'tumour: node: metastasis' (TNM) classification of malignant tumours (Owen, 1980). The TNM system considers the presence of tumour cells in regional lymph nodes as a parameter for the clinical staging of CMTs in female dogs.

The American Joint Committee on Cancer Staging (AJCC) defined macrometastasis (MAC) in man as parenchymal or sinusoidal clusters of epithelial cells >2 mm at their largest diameter. Micrometastasis (MIC) ranged from 0.2 to 2 mm, and isolated tumour cells (ITCs) were <0.2 mm (Edge *et al.*, 2010). However, the clinical significance of occult ITCs and MIC is unclear in human medicine (Ahmed *et al.*, 2014).

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In dogs, the diagnosis of mammary carcinoma with metastasis to lymph nodes has a negative impact on survival (Szczubial and Lopuszynski, 2011; De Araújo *et al*., 2015; Goldschmidt *et al*., 2017), but the clinical significance of occult ITCs within lymph nodes is still unknown in veterinary medicine (Matos *et al*., 2006).

Routinely, the evaluation of lymph nodes for metastasis is performed by conventional histopathology (i.e. using haematoxylin and eosin [HE] staining), which has low sensitivity in identifying ITCs and MIC. Immunohistochemistry (IHC) has been shown to be a highly sensitive technique for the detection of occult ITCs and MIC in previously negative lymph nodes that had been evaluated by conventional histopathology (Matos *et al*., 2006; Beserra *et al*., 2016).

In the current study, we examined the effectiveness of IHC in identifying occult ITCs and MIC in comparison with conventional histopathology, as well as the survival of female dogs with occult ITCs in regional lymph nodes. We also ascertained the relationship between tumour size, histological type, histological grade and the presence of metastasis, and described the microanatomical location of neoplastic cells in lymph nodes.

Materials and Methods

Animals

The 59 female dogs included in this study were presented between April and July 2016 with primary spontaneously arising mammary tumours and no pulmonary metastasis. The dogs were of different breeds and ages, entire or neutered, and had no history of adjuvant treatment for mammary tumours. All animals included in the study were clinically and radiologically free from distant metastasis. After clinical evaluation, the dogs were subjected to unilateral or bilateral total mastectomy with the removal of regional lymph nodes.

In order to evaluate the lymph nodes, the following criteria were used to include animals in the study: female dogs with mammary carcinomas for which respective draining lymph nodes were excised during the surgical procedure, according to the method of MacPhail (2015). When the dog had only one malignant neoplastic nodule, it was accompanied by the draining lymph node. When the dog had more than one malignant neoplastic nodule of different histological types, the most aggressive histological type was considered, according to Misdorp *et al*. (1999), and the nodule was accompanied by the draining lymph node.

The clinical stages of all animals were established using the TNM system (Sorenmo *et al*., 2013). After obtaining IHC results, when necessary, the dogs were assigned a new clinical stage.

Tumour Samples

Immediately after surgical excision, the mammary chain was examined to identify neoplastic nodules, which were measured using a pachymeter (Zaas, Jabalpur, India) and classified according to size: T1 (<3 cm), T2 (3–5 cm) or T3 (>5 cm). Samples were collected from all mammary glands and lymph nodes. Mammary glands were identified as thoracic (M1 and M2), abdominal (M3 and M4) or inguinal (M5).

For histopathological study, tumour specimens were fixed in 10% neutral buffered formalin, processed routinely and embedded in paraffin wax. Sections (4 μm) were stained with HE. The histopathological examination followed the World Health Organization (WHO) classification system for CMTs (Misdorp *et al*., 1999). Histological grade was attributed according to the Nottingham system modified by Elston and Ellis (1991).

Immunohistochemistry

An average of three (minimum of one and maximum of 12) sections per lymph node were evaluated. The number of lymph node sections was dependent on lymph node size. If the lymph nodes were small, they were evaluated in a single longitudinal section in the direction of the largest diameter to sufficiently capture the largest surface area (Stromberg and Meuten, 2017).

To ensure correlation between the features seen on HE-stained sections with those used for pan-cytokeratin IHC, serial sections were obtained. Serial section 1 was stained with HE and serial section 2 was used for pan-cytokeratin IHC. The microtome was advanced 200 μm, and the process was repeated twice for a total of two HE-stained sections and two sections for IHC (Casey *et al*., 2016).

IHC was performed on 4 μm sections mounted on slides following a modification of the method of Hsu *et al*. (1981). Sections were dewaxed through xylene to 100% alcohol and treated with H_2O_2 0.3% in 100% methanol for 30 min. Sections were rehydrated to water through 95% and 70% alcohols. Antigen retrieval was performed using pressure cooking in a 0.01 M citrate buffer (pH 6.0). Following antigen retrieval, slides were rinsed in deionized water and placed in 0.1 M phosphate buffered saline (PBS, pH 7.4). Sections were blocked for 30 min with protein block (Dako, Carpinteria, California, USA). The