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NEOPLASTIC DISEASE

Evaluation of Clinicopathological Characteristics and Oestrogen Receptor Gene Expression in Oestrogen Receptor-negative, Progesterone Receptor-positive Canine Mammary Carcinomas

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Summary

The existence of the oestrogen receptor-negative (OR^-)/progesterone receptor-positive (PR^+) phenotype in canine mammary carcinomas (CMCs) is not well understood, although this phenotype was reported consistently in previous studies. In the present study, immunohistochemistry (IHC) was performed to categorize CMCs with the OR^-/PR^+ phenotype and compare their clinicopathological features with OR^+/PR^+ tumours. Of a total of 305 CMCs, 36 (11.8%) were categorized as OR^-/PR^+ and showed intermediate characteristics between those of OR^+/PR^+ and OR^-/PR^- cases. OR mRNA levels were measured in formalin-fixed, paraffin wax-embedded samples using a novel branched-chain DNA assay method. Similar to the IHC result, one-way analysis of variance showed that the mean normalized OR mRNA level of OR^-/PR^+ tumours (11.4 ± 16.34) was between that of the OR^-/PR^- (mean 4.7 ± 6.35) and OR^+/PR^+ (mean 15.8 ± 11.95) ($P = 0.033$) tumours. Only three of the 36 OR^-/PR^+ tumours completely lacked OR mRNA expression. The OR^-/PR^+ tumours were not categorized as an independent group nor were they included in the other groups on post-hoc analysis. OR^-/PR^+ tumours were associated with factors related to poor prognosis compared with OR^+/PR^+ tumours, but OR^-/PR^- tumours were associated with the worst prognostic indicators. Further studies are required in order to determine the clinical significance of the OR^-/PR^+ phenotype.

Keywords: branched-chain DNA assay; canine mammary carcinoma; steroid receptors

Introduction

Canine mammary gland tumours are the most common neoplasms in female dogs (Misdorp *et al.*, 1999), but these tumours have heterogeneous features (Rutteman *et al.*, 2001; Misdorp, 2002). Clinical and pathological factors including histological type and grade, lymph node status, distant metastasis, tumour size and age are known to affect the prognosis for canine mammary gland tumours (Yamagami *et al.*, 1996; Rutteman *et al.*, 2001; Misdorp, 2002; Sarli *et al.*, 2002). Research on the molecular mechanisms underlying tumour development has identified other factors that may be associated with tumour growth and progression, although much remains unclear (Pena *et al.*, 1998; Wakui *et al.*, 2001; Sarli *et al.*, 2002).

The steroid receptors, oestrogen receptor (OR) and progesterone receptor (PR) are members of the nuclear receptor superfamily and play important roles as transcription factors and in signal transduction of steroid hormones. OR and PR are considered potent prognostic factors for human breast cancer (Murphy and Watson, 2002) and canine mammary

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carcinomas (CMCs) (Sartin *et al.*, 1992; Nieto *et al.*, 2000; Chang *et al.*, 2009). In particular, OR expression is known to be closely related to mammary tumourigenesis, while PR expression is primarily an indicator of intact OR function (Murphy and Watson, 2002).

In human breast cancer, the expression of steroid receptors has prognostic value and predictive value for the response to endocrine therapy (Pichon *et al.*, 1996; Yamashita *et al.*, 2006). Breast cancers with low or negative expression of OR and PR are not sensitive to endocrine therapy and are associated with a poor clinical outcome (Osborne *et al.*, 2005), while steroid receptor-positive tumours have a relatively favourable prognosis (Lester, 2004; Dowsett *et al.*, 2006). In CMCs, a number of studies of steroid receptor expression have found similar results to those reported for human breast cancers (Sartin *et al.*, 1992; Nieto *et al.*, 2000); however, few of these reports have focused on the combined expression of both OR and PR. Martin de Las Mulas *et al.* (2005) showed that tumours with expression of both ORα and PR were associated with a longer disease-free period than tumours that lacked expression of both receptors, and Chang *et al.* (2009) showed that tumours with the OR^+/PR^+ phenotype had the highest survival rates when compared with other phenotypes.

Breast cancers that express both OR and PR have been shown to have favourable outcomes, but the characteristics of tumours that express only one of these steroid receptors are not well defined (Rakha *et al.*, 2007). Tumours with an OR^+/PR^- phenotype are believed to be a distinct subset of tumours that are resistant to endocrine therapy despite OR positivity (Thakkar and Mehta, 2011). Since the loss of PR expression implies that normal OR function is impaired, there is decreased response to the OR antagonist tamoxifen (Belleine *et al.*, 2000; Cui *et al.*, 2005). The existence of OR^-/PR^+ tumours is controversial because PR is typically known to be expressed under OR^+ conditions (De Maeyer *et al.*, 2008; Cserni *et al.*, 2011) and the specific characteristics of OR^-/PR^+ CMCs have not been discussed in the veterinary field.

The aim of the present study was to categorize CMCs by their OR/PR phenotype using immunohistochemistry (IHC) and to evaluate the clinicopathological features of the different tumour phenotypes. A further aim was to determine whether the OR^-/PR^+ phenotype is truly OR^- by measuring OR gene expression with a branched-chain DNA assay and comparing these results with those from OR^+/PR^+ and OR^-/PR^- tumours.

Materials and Methods

Tissue Samples

In total, 305 samples from cases of primary CMC were categorized for OR and PR expression by IHC. Mesenchymal tumours of the mammary gland (e.g. fibrosarcoma and osteosarcoma) were excluded. All samples were obtained from the histopathological database of the Department of Veterinary Pathology, Veterinary Medical Teaching Hospital of Konkuk University, Seoul, Korea, between 2010 and 2012.

Immunohistochemistry

Sections (4 μm) were cut from formalin-fixed and paraffin wax-embedded (FFPE) tissue samples, dewaxed in xylene, rehydrated in graded ethanols and then washed in phosphate buffered saline (PBS, pH 7.4; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4). Endogenous peroxidase activity was blocked in H_2O_2 3% in PBS for 20 min at room temperature. Antigen retrieval was performed by boiling for 15 min in tris−EDTA (pH 9.0) for OR or citric acid (pH 6.0) for PR in a microwave oven. After washing three times in PBS, primary antibodies were applied to the slides. Anti-OR antibodies (Clone ER88, Biogenex, San Ramon, California, USA) were diluted 1 in 60 and incubated for 3 h at room temperature. Anti-PR antibodies (Clone 10A9, Immunotech SAS, Marseille, France) were diluted 1 in 500 and incubated at 4°C overnight. For the anti-OR antibodies only, 5% normal goat serum was used as a blocking agent. All slides were washed four times in PBS, and were then incubated with horseradish peroxidase-conjugated secondary antibodies (DAKO REAL™ Envision kit; DAKO, Glostrup, Denmark) for 40 min. Mouse IgG_1 (eBioscience, San Diego, California, USA) and mouse IgG_{2a} (Biolegend, San Diego, California, USA) were used as isotype controls. A tumour was considered positively labelled when OR or PR were expressed by >10% of the nuclei in the entire section of the tumour.

Tumour Classification

The CMCs were evaluated based on the classification proposed by Goldschmidt *et al.* (2011) and were subcategorized into three groups: epithelial tumours, special types and mixed tumours. Three histological grades according to Elston and Ellis (1991) were used: grade I, well differentiated; grade II, moderately differentiated; and grade III, poorly differentiated. The cases were also categorized by

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