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Expression and clinical relevance of paired box protein 7 and sex determining region Y-box 2 in canine corticotroph pituitary adenomas

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ABSTRACT

Pituitary-dependent hypercortisolism is a common endocrinopathy in dogs, caused by an adrenocorticotrophic hormone secreting pituitary tumour of the anterior or intermediate lobe. The prognosis of intermediate lobe adenomas is worse than that of anterior lobe adenomas, indicating the possible usefulness of melanotropic markers as prognosticators. Another possible origin of pituitary adenomas is found in cancer stem cells. The aim of the present study was to investigate the expression of melanotroph specific transcription factor paired box protein 7 (Pax7) and stem cell marker and reprogramming factor sex determining region Y-box 2 (Sox2) and to relate their expression to clinical parameters.

The mean ± SD of labelling index (LI) for Pax7 was 8.6% ± 21.7% in the adenomas; 1/6 controls had positive staining (LI, 15.2%). For Sox2, the LI in the adenomas was 16.9% ± 15.2% and 19.5% ± 11.6% in the controls. Pax7 expression was significantly higher in enlarged pituitaries, compared to non-enlarged pituitaries ($P = 0.05$), but Pax7 or Sox2 immunopositivity did not correlate to other clinical parameters such as histological diagnosis, survival time or disease-free interval. Gene expression of Pax7 target genes, such as *proconvertase 2* (*PC2*), *pro-opiomelanocortin* (*POMC*), and *dopamine D2 receptor* (*DRD2*), was significantly lower in the adenoma samples compared to normal tissue, indicating that Pax7 signalling was not activated in adenomas. It was suggested that Pax7 and Sox2 remain interesting targets for molecular investigations into their role in pituitary tumorigenesis, but were unsuitable as clinical prognosticators in dogs.

Introduction

Pituitary-dependent hypercortisolism (PDH) is a common endocrinopathy in dogs caused by an adrenocorticotrophic hormone (ACTH) secreting tumour in the anterior or intermediate lobe of the pituitary gland (Galac et al., 2010). The estimated incidence is 1–2 in 1000 dogs/year (Willeberg and Priester, 1982). Research into reliable prognostic factors focuses on pituitary tumorigenesis. Tumours in the intermediate lobe tend to be larger, and related to a worse prognosis than anterior lobe adenomas (Peterson et al., 1982; Hanson et al., 2007; Kooistra and Galac, 2012). This indicates the possible usefulness of melanotropic markers as prognosticators. Given that stem cells can also give rise to pituitary adenomas, stem cell markers may be interesting research targets as prognosticators (Gleiberman et al., 2008; Florio, 2011; van Rijn et al., 2013).

Previous studies showed that a subset of canine and human corticotroph pituitary adenomas express paired box protein 7 (Pax7), an essential regulator for the melanotroph fate, the predominant cell type of the intermediate lobe (Hosoyama et al., 2010; Budry et al., 2012). Pax7 is a member of the Pax transcription factor family, which is essential in embryonic patterning and postnatal stem cell renewal in many organs (Hill et al., 1991; Seale et al., 2000). It was hypothesised that expression of Pax7 could be related to clinical parameters and as such can be used as prognosticator in dogs with PDH.

Pax7 has several melanotroph specific target genes. In mice, *PAX7* inactivation results in a decreased expression of the genes encoding for pro-opiomelanocortin (POMC), proconvertase 2 (PC2) and dopamine D2 receptor (DRD2) (Budry et al., 2012). The corticotroph and melanotroph cells in the anterior and intermediate lobe of the pituitary gland are derived from the POMC cell lineage, which

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develops under the influence of the pituitary T-box transcription factor Tpit (Lamolet et al., 2001). The prohormone POMC is processed into ACTH in both melanotroph cells and corticotroph cells, followed by further cleavage into α-melanocyte stimulating hormone (α-MSH) by PC2 in melanotroph cells (Miller et al., 2003). In human corticotroph adenomas, 47% stained positive for PC2, whereas healthy pituitaries did not (Iino et al., 2010). Drd2 is expressed in melanotroph cells and inhibits α-MSH secretion (Garcia-Tornadu et al., 2010). Approximately 75% of the human corticotroph adenomas express Drd2 (Pivonello et al., 2004). Up regulation of *DRD2* in a pituitary cell line led inhibited cell proliferation and ACTH secretion without a switch to a melanotrophic cell type (Occhi et al., 2014).

It is also thought that undifferentiated cells, i.e. stem cells, play a role in tumorigenesis in the pituitary gland (Gleiberman et al., 2008; Florio, 2011; van Rijn et al., 2013). A possible pituitary stem cell marker is sex determining region Y-box 2 (Sox2), a transcription factor that plays an important role in pituitary development (Alatzoglou et al., 2009). Mutations in *SOX2* led to developmental abnormalities of the pituitary gland (Kelberman et al., 2008). During organogenesis, Sox2 positive ($Sox2^+$) cells are found in Rathke's pouch. Sox2 expression is down regulated in the postnatal pituitary, but a residual level of Sox2 positivity persists and these $Sox2^+$ cells show several stem cell characteristics (Fauquier et al., 2008; Andoniadou et al., 2013; Rizzoti et al., 2013). $Sox2^+$ cells do indeed give rise to pituitary adenomas in transgenic mice (Andoniadou et al., 2013), suggesting Sox2 as a prognostic marker in patients with pituitary adenomas.

The aims of the present study were to analyse the protein and mRNA expression of Pax7 and Sox2 in pituitary tissue removed during hypophysectomy of patients treated for PDH and to relate these to clinical parameters, in order to determine whether their expression could serve as a prognostic factor.

Materials and methods

Animals

Pituitary adenoma tissues from client-owned dogs that had undergone transsphenoidal hypophysectomy as treatment for PDH (Meij et al., 1997) were included in the study ($n = 58$). The clinical characteristics of the dogs are shown in Appendix: Supplementary Tables S1 and S2. The diagnosis of hypercortisolism was based on an elevated ($\geq 10 \times 10^{-6}$) urinary corticoid-to-creatinine ratio (UCCR), combined with a high dose dexamethasone suppression test and measurement of plasma ACTH concentrations. The diagnosis was further supported by visualisation of the adrenals by ultrasonography and pituitary imaging as previously described (Galac et al., 1997; Hanson et al., 2005, 2007).

In dogs with PDH, enlarged pituitaries were distinguished from non-enlarged pituitaries by their pituitary height/brain area (P/B) value (Kooistra et al., 1997). Remission was defined as UCCR $< 10 \times 10^{-6}$ and resolution of clinical signs of hypercortisolism. Recurrence was defined as UCCR $\geq 10 \times 10^{-6}$ and reappearance of clinical signs of hypercortisolism after initial remission.

As control tissue, anterior lobes of normal pituitary glands were obtained from five healthy Labrador retrievers, 11 Beagles and nine crossbred dogs, all euthanased in other, unrelated experiments approved by the Ethics Committee on Animal Experimentation, Utrecht University, The Netherlands, in accordance to the 3R-policy (Dier Ethische Commissie number 2007.III.06.080, project 080, approval date 24 October 2007). One normal pituitary gland was obtained from a healthy 8 month old male dog. This dog was a client-owned Bouvier des Flandres euthanased because of spinal trauma and an autopsy was performed with owner consent.

Immunohistochemistry

Specimens of pituitary tissue removed during surgery ($n = 44$) or autopsy ($n = 6$) were fixed in 4% neutral buffered formaldehyde, embedded in paraffin, and consecutive sections were used for histology and immunohistochemistry. The diagnosis of pituitary adenoma was made on haematoxylin and eosin (HE) stained tissue sections by a board certified veterinary pathologist. The diagnosis of corticotroph adenoma was confirmed by immunostaining for ACTH, α-MSH, and growth hormone (GH) as previously described (van Rijn et al., 2010).

For the detection of Pax7 and Sox2, 4 μm thick paraffin embedded tissue sections were mounted on poly-L-lysine-coated slides. After deparaffinisation, antigen retrieval was performed by incubation in a citrate bath during 60 min for Pax7 immunostaining and 30 min for Sox2 immunostaining. Endogenous peroxidase activity was blocked by 30 min incubation in 0.3% H_2O_2 in methanol followed by 30 min treatment with 10% normal goat serum. Incubation with the primary antibodies took place overnight at 4 °C (anti-Pax7 monoclonal mouse antibody, Developmental Studies Hybridoma Bank, isotype IgG1, 1:20; anti-Sox2 monoclonal rabbit antibody, Cell Signaling, isotype IgG, 1:800), followed by a 30 min incubation with secondary antibodies (polymer-HRP labelled anti-mouse for Pax7 and anti-rabbit for Sox2, Dako). Staining was detected with DAB-substrate (Vector Laboratories) and slides were counterstained for 10 s with haematoxylin staining.

Antibodies were tested for specificity against canine tissue according to the manufacturer's product information. As a positive control, normal canine pituitary tissue was used. Negative controls were obtained by replacing the primary antibodies with specific isotype controls (mouse IgG1 isotype control, PE labelled, Biolegend, for Pax7 immunostaining; rabbit IgG isotype control, PE labelled, Antibodies online, for Sox2 immunostaining, see Appendix: Supplementary Figs. S1 and S2).

For the detection of PC2, an immunohistochemistry protocol was optimised on canine testis tissue (anti-PC2 polyclonal rabbit antibody, Bioss, isotype IgG, 1:400).

Scoring of Pax7 and Sox2 immunopositivity

The location of the pituitary corticotroph adenoma in the tissue sections was determined using the HE slides and slides from consecutive sections stained for ACTH, α-MSH, and GH. The adenomatous tissue usually immunostained positive for ACTH and/or α-MSH but negative for GH. In similar tissue sections, the Pax7 and Sox2 positive cells were identified and quantified in digital images of the immunostained adenomatous areas made by a Color View III camera connected to an Olympus BX41 microscope and Cell^B 3.0 software (Olympus Soft Imaging Systems). In the control samples, a representative section of the anterior lobe was photographed and analysed. On average, 1000 cells/sample were counted at 200× magnification, using the cell counter plug-in of the ImageJ Software package (National Institute of Mental Health). The labelling index (LI) of Pax7 and Sox2 was obtained by dividing the number of cells staining positive for, respectively, Pax7 and Sox2, by the total number of cells counted.

Gene expression analysis

Gene expression analysis was performed in order to determine whether Pax7 signalling was activated. Total RNA was isolated from snap frozen samples (pituitary adenoma tissue, $n = 20$; anterior lobe tissue of healthy controls, $n = 20$), using Qiagen RNeasy Mini Kit (Qiagen). RNA quantity and quality was measured with the Agilent BioAnalyzer 2100 (Agilent). The RNA integrity number (RIN) values were >7.0, indicating for sufficient RNA quality to perform quantitative polymerase chain reaction (qPCR).

cDNA was synthesised using the iScript cDNA Synthesis Kit (Bio-Rad). The qPCR reaction was performed in duplicate on a Bio-Rad I-Cycler (Bio-Rad) using IQ SYBR green SuperMix (Bio-Rad) and both forward and reverse primers (Table 1). To normalise gene expression the reference genes *TATA box binding protein* (*TBP*), *tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide* (*YWHAZ*) and *hydroxymethylbilane synthase* (*HMBS*) were used, previously identified as the most stable reference genes for pituitary and adenoma samples (van Rijn et al., 2014) .

Statistical analysis

Statistical analysis was performed with IBM SPSS Statistics for Windows (Version 20.0, IBM). Groups were created based on pituitary size (normal pituitary tissue, non-enlarged pituitary, and enlarged pituitary), histological diagnosis (normal pituitary tissue, anterior lobe adenoma, and intermediate lobe adenoma) and recurrence status (recurrence or no recurrence). Normality of data was assessed using a Shapiro–Wilk test and parametric or non-parametric tests were used accordingly. To compare LIs and gene expression levels between different groups of adenomas Student's t test or Mann–Whitney U test was used. Correlation coefficients were calculated using a Spearman ρ test. Furthermore, in order to determine the prognostic value of the selected markers, survival analysis was performed using Kaplan–Meier curves and the log rank test was used to assess significance. Bonferroni corrections for multiple comparisons were applied when appropriate. Significance was set at $P < 0.05$.

Results

Histology and immunohistochemistry for ACTH, α-MSH, and GH

In all dogs with PDH, histological examination of the surgical specimen revealed a corticotroph adenoma staining positive for ACTH and/or α-MSH and mostly negative for GH (See Appendix: