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Short Communication

Expression of steroidogenic factor 1 in canine cortisol-secreting adrenocortical tumors and normal adrenals

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ABSTRACT

We report on a screening for the relative messenger RNA (mRNA) and protein expression of steroidogenic factor 1 (SF-1) in normal canine adrenals (n = 10) and cortisol-secreting adrenocortical tumors (11 adenomas and 26 carcinomas). The relative mRNA expression of SF-1 was determined by quantitative real-time polymerase chain reaction analysis and revealed no differences between normal adrenals, adenomas, and carcinomas. Immunohistochemistry demonstrated SF-1 protein expression in a nuclear pattern throughout the normal adrenal cortex and a predominantly nuclear staining pattern in adrenocortical tumors. Of the 15 dogs available for follow up, 7 dogs developed hypercortisolism within 2.5 yr after adrenalectomy, with metastatic disease in 6 dogs and adrenocortical tumor regrowth in 1 dog. The relative SF-1 mRNA expression in dogs with early recurrence was greater (2.46-fold, $P = 0.020$) than in dogs in remission for at least 2.5 yr after adrenalectomy. In conclusion, we demonstrated the presence of SF-1 expression in normal canine adrenals and adrenocortical tumors. The high SF-1 mRNA expression in carcinomas with early recurrence might indicate its value as a prognostic marker, as well as its potential for therapeutic development.

1. Introduction

Canine cortisol-secreting adrenocortical tumors (ATs) underlie adrenocorticotropin (ACTH)-independent hypercortisolism and are characterized by uncontrolled growth and hormone secretion [1]. Their pathogenesis is largely unknown. Potential explanations for ACTH-independent hypersecretion of cortisol and growth of canine ATs can be derived from the current understanding of adrenal growth biology.

Adrenal development and steroidogenesis depend greatly on the expression of steroidogenic factor 1 (SF-1) [2]. Mice with homozygous null mutations in SF-1 are born without adrenal glands and gonads and die within hours after birth because of adrenal insufficiency [3,4]. In the adult adrenal cortex, SF-1 plays a prominent role in the regulation of steroidogenesis, by being an obligate activator of most of the cytochrome P450 steroid hydroxylases and steroidogenic acute regulatory (StAR) protein [5,6]. The growth-promoting effect of SF-1 in the adult adrenal gland is dosage dependent. For the compensatory growth of the contralateral adrenal gland following unilateral adrenalectomy, physiologic SF-1 expression is sufficient [7], whereas an increased dosage stimulates proliferation and decreases apoptosis in human adrenocortical cells and triggers adrenal tumorigenesis in mice [8]. In childhood ATs, SF-1 gene amplification and protein overexpression are the most consistent findings [9]. In adult humans with an adrenocortical carcinoma, SF-1 staining intensity is negatively correlated with survival, and SF-1 is considered a tumor stage-independent prognostic factor [10]. Taken together, this indicates that SF-1 plays an important role in the pathogenesis of ATs.

In dogs, the expression of SF-1 has been studied in sex-reversal syndrome only [11]. The aim of the present study was to determine the expression of SF-1 messenger RNA (mRNA) and protein in normal adrenals and cortisol-secreting ATs of dogs.

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2. Materials and methods

2.1. Animals and tissues

The study was approved by the Ethical Committee of Utrecht University. Cortisol-secreting ATs were obtained from 37 dogs that underwent adrenalectomy at the Department of Clinical Sciences of Companion Animals and permission to use the AT tissue was obtained from all patient owners. The dogs' ages at the time of surgery ranged from 6 to 14 yr (mean, 9 yr). Twelve dogs were mongrels and the others were of 10 different breeds. Eighteen of the dogs were male (8 castrated) and 19 female (15 neutered). The diagnosis of ACTH-independent hypercortisolism was made as described previously [1,12]. Ten normal adrenal glands (whole tissue explants) of healthy beagle dogs served as control tissue. The dogs were euthanized for reasons unrelated to the present study. Their ages ranged from 2 to 5 yr, there were 5 males and 5 females, all intact.

From 15 of 37 dogs, follow up information was available. When reoccurrence of ACTH-independent hypercortisolism was suspected, the diagnosis was confirmed by endocrine testing, measurements of the basal plasma ACTH concentration and diagnostic imaging [1,12]. The dogs were categorized in (1) a group with relapse of ACTH-independent hypercortisolism within 2.5 yr after surgery; and (2) a group in remission for at least 2.5 yr after surgery.

2.2. Histopathology

All tissues were fixed in 4% buffered formalin, embedded in paraffin after at least 24 h and maximally 48 h of fixation, cut into 5-μm sections, and mounted on SuperFrost Plus microscope slides (Menzel-Gläser, Braunsweig, Germany). On histopathology, there were 26 carcinomas and 11 adenomas. The diagnosis was made by a single pathologist. In case of doubt, a second pathologist was consulted and slides were reviewed at a multihead microscope, and a consensus was reached. In all carcinomas there was evidence of invasion of neoplastic cells into blood vessels and/or capsular invasion. Additional characteristics for carcinomas were trabecular growth pattern and peripheral fibrosis. In agreement with the criteria published before [13], none of the adenomas exceeded 2 cm diameter at the major axis width, whereas carcinomas were generally larger. Typical histologic characteristics for adenomas were hematopoiesis, fibrin thrombi, and cytoplasmic vacuolization, whereas hemorrhage, necrosis, and single cell necrosis were detected in both, adenomas and carcinomas.

2.3. Total RNA extraction and reverse transcription

Tissue fragments for RNA isolation were snap frozen in liquid nitrogen within 10 to 20 min after surgical removal and stored at −70°C until RNA isolation. Total RNA isolation and complementary DNA synthesis were performed, as described previously [12].

2.4. Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction primers for SF-1 (XM_846937, forward: AGGGCTGCAAGGGGTTTTTCAA, reverse: CATCCCCACTGTCAGGCACTTCT, T_a: 59°C) were designed using Perl-primer v1.1.14 according to the Bio-Rad iCycler parameters, and ordered from Eurogentec (Maastricht, The Netherlands). Polymerase chain reaction optimization and confirmation of primer specificity were performed as described previously [12].

The mRNA expression abundances of SF-1 were measured in 10 normal adrenals and 37 cortisol-secreting ATs (26 carcinomas and 11 adenomas). All reactions were performed on a MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA), according to a previously described protocol [12]. Ribosomal protein S5 (RPS5), ribosomal protein S19 (RPS19), and hypoxanthine-guanine phosphoribosyltransferase (HPRT) were used as reference genes [14]. Analysis of the relative expression abundances of the reference genes revealed no significant differences between groups and their expression was shown to be stable using GeNorm software, justifying their use as reference genes.

2.5. Immunohistochemistry

For IHC staining of SF-1, tissue slides were rehydrated in a series of xylene-alcohol baths. Antigen retrieval was performed using 10 mM sodium citrate buffer pH 6, for 20 min at 95°C. To block endogenous peroxidase activity, slides were incubated with peroxidase block (S2003, Dako, Glostrup Denmark) for 5 min. Aspecific binding sites were blocked with 10% normal goat serum in phosphate-buffered saline for 20 min. Slides were incubated overnight at 4°C with a polyclonal rabbit-anti-human anti SF-1 antibody (LS- A5534, MBL International, USA), in a 1:200 dilution in 1% normal goat serum in phosphate-buffered saline. Subsequently, all slides were incubated with anti-rabbit HRP conjugated secondary antibody (Dako K4003) for 45 min at room temperature. Antibody detection was performed using Dako K3468 HRP substrate. All slides were incubated with 3,3′-diaminobenzidine (Dako liquid DAB + substrate chromogen system, K3468, Dako) for 4 min and subsequently counterstained with hematoxylin, dehydrated, and mounted. To confirm the specificity of the reaction, blocking peptides against SF-1 were used (LS-P5534, MBL in a concentration of 1 mg/mL and a dilution of 1:400). Preincubation of the antibody with those blocking peptides abolished all staining. IHC analysis was performed using light microscopy. The presence and localization (membranous, cytoplasmic, or nuclear) of staining in ATs and normal adrenals were described.

2.6. Statistical analyses

Statistical analyses were performed with SPSS20 (IBM, Armonk, NY, USA). Relative mRNA expression abundances were calculated using the ΔΔ-Ct method [15]. A Mann-Whitney U test was used to compare the relative expression abundances of SF-1 between normal adrenals, adenomas and carcinomas, and between dogs with recurrence of hypercortisolism within 2.5 yr and in dogs in remission for at least 2.5 yr after adrenalectomy. For the first comparison, a Bonferroni correction was applied and