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[*Análise de mutação germinativa do* Tpit *em cães da raça Poodle com hiperadrenocorticismo ACTH-dependente*]

V. *De Marco*[1,2,3], L.R. *Carvalho*[1], A.E.C. *Billerbeck*[1], B.B. *Mendonça*[1]

[1]Faculdade de Medicina - Universidade de São Paulo, São Paulo, SP
[2]Faculdade de Medicina Veterinária - UNISA - São Paulo, SP
[3]NAYA Especialidades Veterinárias - São Paulo, SP

ABSTRACT

There is a high incidence of pituitary-dependent hyperadrenocorticism (PDH) in Poodle dogs, with family members being affected by the disease, suggesting a genetic involvement. *Tpit* is an obligate transcription factor for the expression of pro-opiomelanocortingene and for corticotroph terminal differentiation. The aim of the present study was to screen the *Tpit* gene for germline mutations in Poodles with PDH. Fifty Poodle dogs (33 female, 8.71±2.8 years) with PDH and 50 healthy Poodle dogs (32 females, 9.4*241* 2.8 years) were studied. Genomic DNA was isolated from peripheral blood, amplified by PCR and submitted to automatic sequence. No mutation in the coding region of *Tpit* was found, whereas the new single nucleotide polymorphism p.S343G, in heterozygous state, was found in the same frequency in both PDH and control groups. We concluded that Tpit gain-of-function mutations are not involved in the etiology of PDH in Poodle dogs.

Keywords: dog, ACTH-dependent hyperadrenocorticism, Tpit, mutation

RESUMO

O hiperadrenocorticismo ACTH-dependente (HACAD) apresenta elevada incidência em cães da raça Poodle, sendo que membros da mesma família têm sido acometidos pela doença, sugerindo envolvimento genético. Tpit *é um fator de transcrição obrigatório para a expressão do gene da pro-opiomelanocortina e para a diferenciação terminal dos corticotrofos. O objetivo deste trabalho foi pesquisar mutações germinativas no gene* Tpit *em Poodles com HACAD. Cinquenta cães da raça Poodle, 33 fêmeas, média de idade de 8,71±2,8 anos, com HACAD, e 50 cães Poodles saudáveis, 32 fêmeas, média de idade de 9,4±2,8 anos, foram estudados. Mutações na região codificadora do gene* Tpit *não foram identificadas. Foi observado um novo polimorfismo em heterozigose, p.S343G, com a mesma frequência no grupo de cães com HACAD e no grupo-controle. Conclui-se que a mutação ativadora no gene* Tpit *não está envolvida na patogênese do hiperadrenocorticismo ACTH-dependente em cães da raça Poodle.*

Palavras-chave: cão, hiperadrenocorticismo ACTH-dependente, Tpit, *mutação*

INTRODUCTION

Cushing's syndrome is a very common endocrinopathy in dogs, characterized by chronic exposure to hypercortisolism leading to a classic phenotype characterized by polydipsia, polyuria, polyphagia, abdominal enlargement, alopecia, pyoderma, panting and muscle weakness (Behrend and Kemppainen, 2001; Feldman and Nelson, 2004; Peterson, 2007). ACTH-dependent hyperadrenocorticism (ADHAC) is the most common cause of naturally occurring hypercortisolism in dogs, accounting for 80 to 85% of cases (Peterson *et al*., 1982; Behrend and Kemppainen, 2001; Peterson, 2001; Peterson, 2007). ADHAC mainly affects dogs older than 6 years with no sexual predisposition

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E-mail: vivianidemarco@terra.com.br

(Guptill *et al*., 1997; Feldman and Nelson, 2004). Poodle, Dachshunds, Beagles, various Terrier breeds and Boxers appear to be at great risk of developing hyperadrenocorticism (HAC) (Behrend *et al*., 2002; Feldman and Nelson 2004; Peterson, 2007).

Several putative transcription-regulating proteins have been identified in the adenohypophysis and have been implicated as key elements in the definition of cell-specific phenotypes and the regulation of hormone gene expression (Asa and Ezzat, 1998). *Tpit* is a highly cell-restricted transcription factor that is only present in the two pituitary POMC-expressing lineages, the corticotrophs and melanotrophs. This protein activates the transcription of the proopiomelanocortin (POMC) gene and it has an important role in the late differentiation of the corticotrophs and in the maintenance of both corticotroph and melanotroph cells (Lamolet *et al*., 2001; Pulichino *et al*., 2003). POMC is processed into adrenocorticotropin (ACTH) by pro-convertase 1 (PC1) in anterior pituitary corticotrophs and into melanocyte-stimulating hormone (MSH) by PC2 in rodent and dog intermediate lobe melanotrophs (Seidah *et al*., 1999).

Tpit, also described as *Tbx19*, belongs to the T-box family of transcription factors that have a highly conserved DNA-binding domain in common, the T-box, and are present in all vertebrates (Yi *et al*., 1999). The canine *Tpit* gene is situated in chromosome 7, contains 8 exons that codify a protein of 445 aminoacids, and is 93.5% identical to human TPIT and 90.8% identical to murine Tpit (Hanson *et al*., 2008).

Recently, several *Tpit* mutations leading to loss-of-function have been reported in isolated neonatal cases of ACTH deficiency in humans, supporting the essential role of *Tpit* for corticotrophs terminal differentiation and POMC regulation (Lamolet *et al*., 2001; Pulichino *et al*., 2003; Atasay *et al*., 2004; Metherell *et al*., 2004; Pulichino *et al*., 2004; Drouin *et al*., 2007). Otherwise, *Tpit* gain-of-function mutations induce POMC expression in undifferentiated pituitary cells (Lamolet *et al*., 2001).

Given its pivotal role in corticotroph development, *Tpit* involvement in pituitary tumorigenesis has been investigated. *Tpit* somatic mutations studies performed in canine and human pituitary tumors cDNA shave showed negative results (Hanson *et al*., 2008). However, the mutation analysis of genomic DNA in a large sample of dogs of the same breed highly predisposed to hyperadrenocorticism has not been reported yet. The aim of this study was to investigate the presence of germline mutations in the coding region of the *Tpit* gene in Poodle dogs with ACTH-dependent hyperadrenocorticism.

MATERIALS AND METHODS

The study included 50 Poodle dogs, of which 33 were females, with a mean age of 8.7±2.8 years (ranging from 1.5 to 14 years) with ACTH-dependent hypercortisolism, presented to a Veterinary Teaching Hospital between January, 2007 and February, 2009. Among these 50 dogs, three cases had other affected familial members with ADHAC (Figure 1). A tentative diagnosis of HAC was based on the presence of clinical signs associated to hypercortisolism (polydipsia-polyuria, polyphagia, pendulous abdomen, dermatologic problems and anoestrus) and the results of haematological and biochemical analyses of blood and urinalysis. A low dose of dexamethasone suppression (LDDS) test was carried out in each case to confirm the diagnosis. ADHAC was confirmed on the basis of a bilateral symmetric appearance and enlargement of the adrenal glands and an increased plasmatic ACTH concentration (Behrend and Kemppainen, 2001; Gould *et al*., 2001; Peterson, 2007). Informed consent was obtained from the owners of all dogs.

The control group was comprised of 50 Poodle dogs, of which 32 were females, mean age of 9.4±2.8 years (ranging from 6 to 16 years) with no evidence of adrenal disease, also presented to a Veterinary Teaching Hospital between March, 2008 and February, 2009, and also normal dogs owned by hospital employees and students. Adrenal disease was ruled out in each dog on the basis of history, physical examination, and biochemical analysis. Control dogs were matched to ADHAC dogs on the basis of age (≥6 years old) and breed (100% Poodles).

The LDDS test consisted of measuring cortisol levels before and 8 hours after dexamethasone (Azium, dexamethasone phosphate, 2mg/mL, Intervet – Shering Plough, Beaucouzé,

France) administration (0.01mg/kg/IV). Failure to suppress cortisol concentration adequately 8 hours after dexamethasone (cortisol ≥1.4µg/dL; reference value <1.0µg/dL) was considered compatible with hyperadrenocorticism. Serum cortisol concentrations for the dynamic endocrine test were performed (BET Laboratories, Rio de Janeiro, RJ, Brazil) using a commercial radioimmunoassay kit (Coat-A-Count, Cortisol, Radioimmunoassay Kit, Diagnostic Products, Los Angeles, USA), previously validated for canine cortisol (Watson *et al*., 1993). The intra-assay coefficient variation for cortisol was less than 5.1% and the inter-assay was less than 6.4%. Blood samples for the measurement of plasma ACTH were collected in ethylenediaminetetraacetic acid-coated (EDTA) tubes, centrifuged immediately and the plasma promptly stored in plastic tubes and frozen at -70°C until assay. Plasma ACTH concentrations were measured by immunoradiometric assay (IRMA) using a commercial kit (ELSA-ACTH, CIS Bio international, Gif Sur Yvette, France) at Hormone and Molecular Genetics Laboratory LIM/42, Hospital das Clínicas, Faculdade de Medicina, São Paulo University (FMUSP), Brazil. The ACTH intra-assay and inter-assay coefficients of variation were less than 6.1% and 5.3%, respectively. Dogs with ADHAC were identified by unsuppressed basal ACTH plasma concentrations (>17pg/mL).

Genomic DNA was harvested from EDTA-anticoagulated blood obtained from 50 ADHAC and 50 control Poodle dogs using standardized protocols (Miller and Dykes *et al*., 1988). Genomic DNA was amplified through the reaction of chain polymerization (PCR) in an automatic Thermal Cycler (Applied Biosystems PCR System 9700, Foster City, CA), using specific primers to ensure the assessment of all coding areas of *Tpit* gene (Table 1). PCR reaction was performed on 2µL genomic DNA in a 50µL volume containing 200µM dNTPs, 0.5mM (20 pmol) of each *primer*, 1.5U Go TaqPolimerase (Promega), 5x PCR reaction buffer (Promega) with 1.5mM $MgCl_2$. The PCR programs consisted of an initial activation at 98°C for 5min followed by 40 cycles of 45s each at 98°C, 30s at 50-54°C (*annealing temperature* depending on the primer), 1min at 72°C, followed by a final extension at 72°C for 5min. The products were visualized in 1.5% agarose gel containing ethidium-bromide.

Table 1. Forward (F) and reverse (R) primers for canine *Tpit* amplification, product size (bp) and annealing temperature (T_a)

Primer	Sequence	Product size (bp)	T_a(°C)
1F *Tpit*	5' GAA CGC TTC TCC GCC AAG TTT 3'	341	50
1R *Tpit*	5' CCT CTG AGT GAA GAA GGG CA 3'		
2F *Tpit*	5' GCC ACC CAG GGA TCC CCT 3'	392	55
2R *Tpit*	5' GGA GAA GAG GCC GGT GAG GA 3'		
3F *Tpit*	5' TGG TTA TCA CCC CTG CTA CCA 3'	232	52
3R *Tpit*	5' GTT TAC ACA CAC GCA GCC AC 3'		
4F *Tpit*	5' ACT AAT GCT TCT TGT CTT TG 3'	122	50
4R *Tpit*	5' AGA AGA TTT TAC TGA CAT T 3'		
5F *Tpit*	5'CAG GAG CAA TAA GTG CAA GCC A 3'	370	53
5R *Tpit*	5' TGT GGC CCA CCC AGT GTT CA 3'		
6F *Tpit*	5' CGA CAT TCC GTG TCG TTC ACA 3'	328	51
6R *Tpit*	5' TAC ACC CAA GGG TTT AAT TA 3'		
7F *Tpit*	5' TGT GGC ATA TGG TTG ACA TGG TA 3'	329	50
7R *Tpit*	5' ACC AAT GAG GAA GTT GCT GGA 3'		
8F *Tpit*	5' GTC AGG ACA GCT ATT GTA CGC T 3'	515	50
8R *Tpit*	5' CCT GCC CTA GGA TCC TGC CT 3'		

Primers were designed in a sequence that was derived from the canine boxer genomic DNA sequence available at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/genome/seq/BlastGen/BlastGen.cgi?taxid=9615). Eight pairs of intron-flanking primers were designed based on the intron/exon information using the Primer3 core program (http://frodo.wi.mit.edu/cgi_bin/primer3/primer3_www.cgi) (Table 1). Amplification products were submitted for pre-enzyme purification, using the Exo-SAP commercial

product containing Shrimp Alkaline Phosphatase and exonuclease I enzimes (Amersham Science, USB, Cleveland, Ohio, EUA) according to manufacturer as follow: 2µL of exosap to 5µL of PCR product at 37°C for 15 min. The sequencing reaction was performed using the commercial product ABI PrismTMBigDye Terminator (Perkin Elmer, Foster City, CA, EUA) followed by biotreatment columns Centri-SEP (Princenton separations, Adelphia, NJ, USA). The items on this reaction were submitted to capillary electrophoresis in automatic ABI3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The canine sample coding sequence for Tpit was compared with the normal dog sequence from Ensembl database (www.ensembl.org.com) under number ENSCAFT00000024243 which was derived from the NCBI contigNC_006589.

The allelic variant p.S343G found in exon 7 of the *Tpit* gene creates an enzyme *MSP I* cleavage. The WT sequence leads to one fragment of 329bp and the mutant leads to fragments of 82 and 247bp. Enzymatic digestion reaction was performed on the entire control group in a 20µl volume containing 10µl PCR product of exon 7, 1µl *Msp I* enzyme, 2 µl specific buffer, for 4h at 37°C. Digestion products were visualized in ethidium-bromide containing 4% agarose gel and DNA fragments were viewed in ultraviolet light using 1KB molecular weight marker.

RESULTS

PCR assays were performed on DNA samples isolated from one hundred Poodle dogs, 50 with ADHAC and 50 control dogs. No *Tpit* mutation was found to be responsible for the Cushing's disease phenotype in our cohort.

The new single nucleotide polymorphism p.S343G was found in exon 7 of the Tpit gene at the same frequency in both ADHAC and control groups. This allelic variant is characterized by an exchange of adenine by guanine, in a heterozygous state, at position 1027 from the cDNA sequence, leading to a substitution of serine with glycine in codon 343 (p.S343G) (Figure 2). The exchange of serine (AGC) with glycine (GGC) alters amino acid physico-chemical properties, since serine is a polar amino acid (hydrophilic) and glycine is apolar (hydrophobic). However, S343G is located out of the T-box (Figure 3), a conserved DNA region which encodes the DNA binding domain and is not conserved among several species. The heterozygous allelic variation p.S343G was also found in 2 dogs from the control group (n = 2/50). In both groups the allelic and genotypic frequency of p.S343G was 2% and 4%, respectively.

DISCUSSION

ACTH-dependent hyperadrenocorticism is the most common cause of endogenous hyperadrenocorticism in dogs, accounting for 80 to 85% of the cases (Behrend and Kemppainen, 2001; Feldman and Nelson, 2004). The high incidence of this disease in dogs, the high breed predisposition, especially in Poodles and the description of familial ADHAC (Scholten-Sloof *et al*., 1992; Stritzel *et al*., 2008) have heightened the attention to the molecular basis of Cushing's disease.

The Poodle was chosen for molecular studies because it is the breed which is most prone to HAC in several studies (Guptill *et al*., 1997; Feldman and Nelson, 2004; Peterson, 2007) and also because three of our patients presented affected familial members. One of our familial cases (case 2, family 1, Figure 1) developed ADHAC with only 1 year and 5 months of age. Until this study, only 5 dogs younger than 2 years of age have been reported with ADHAC (Feldman and Nelson; 2004). Most of the cases are older than 6 years, with an average age of 11 years (Ling *et al*., 1979; Guptill *et al*., 1997; Peterson, 2001; Feldman and Nelson, 2004). Similarly, in this series the patients' mean age was 8.7±2.8 years, but seven dogs (14%) were younger than 6 years.

In addition to our three familial cases, there are only two other case reports in veterinary literature describing familial Cushing's disease in dogs involving the Dandie Dinmont terrier and the wire-haired Dachshund (Scholten-Sloof *et al*., 1992; Stritzel *et al*., 2008).

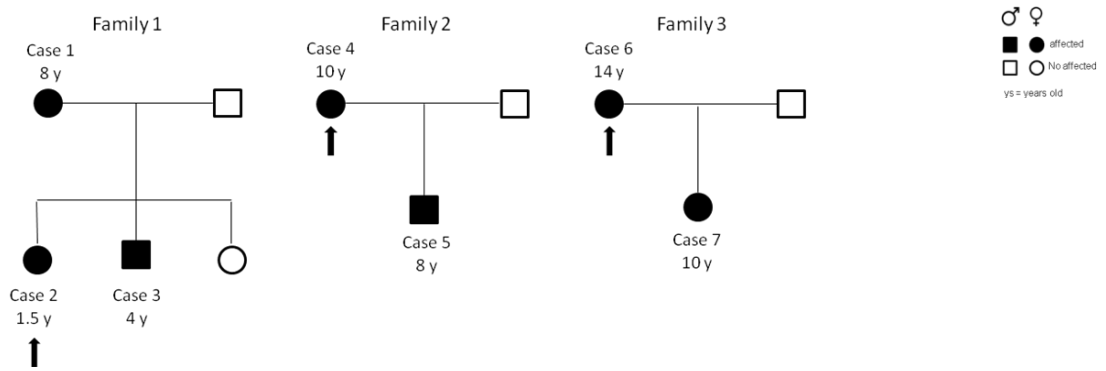

Figure 1. Pedigree of three families of Poodle dogs with ACTH-dependent hypercortisolism (y= years old).

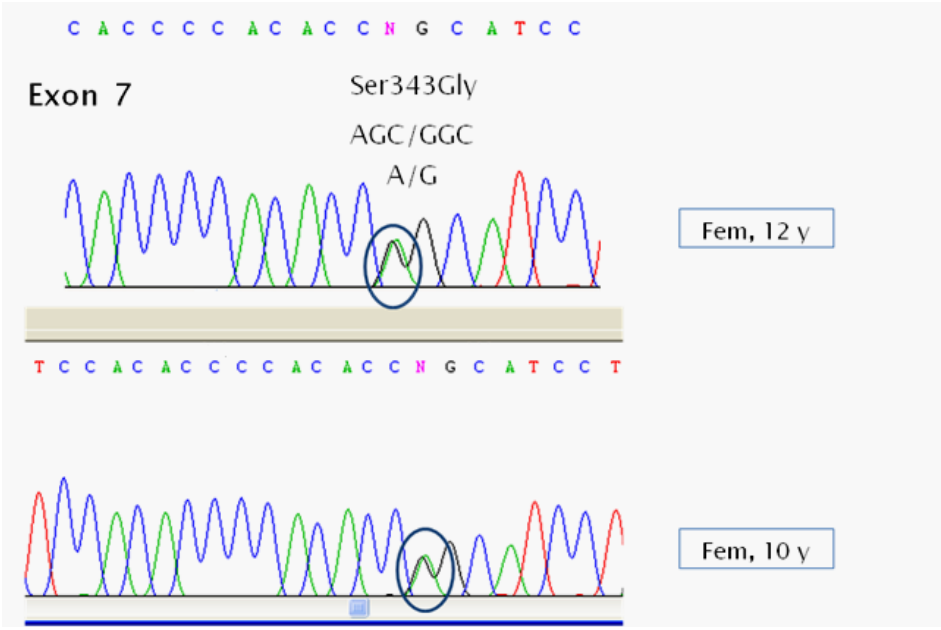

Figure 2.Heterozygous allelic variant, p.S343G, in the Tpit gene in two unrelated dogs with ACTH-dependent hyperadrenocorticism. (Fem = female; y = years old).

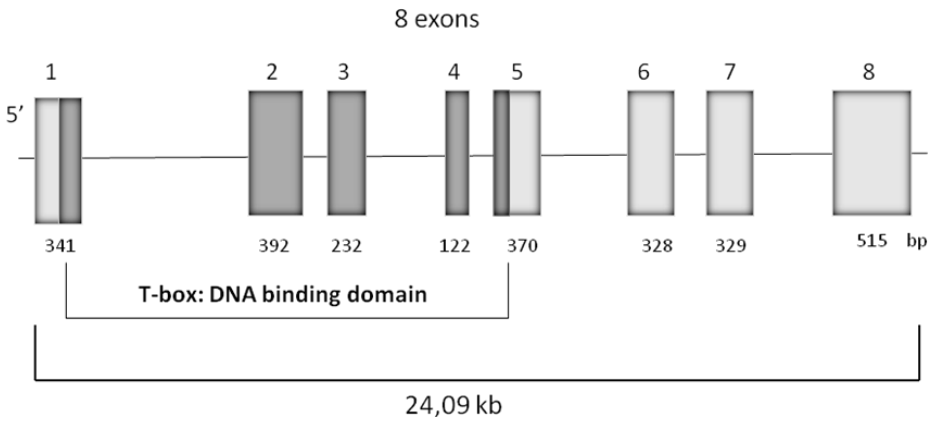

Figure 3.The gray-boxed region corresponds to the T-box region of canine *Tpit*.

In the pituitary gland, *Tpit* is an obligate transcription factor for the expression of the pro-opiomelanocortin (POMC) gene and for terminal differentiation of the corticotroph lineage. *Tpit* loss of function mutations have been reported in cases of early onset isolated corticotrophin deficiency (Lamolet *et al*., 2001; Pulichino *et al*., 2003; Atasay *et al*., 2004; Metherell *et al*., 2004; Pulichino *et al*., 2004; Drouin *et al*., 2007). *Tpit* knock out homozygous mutant mice (*Tpit* -/-) present almost complete loss of POMC-expressing cells in pituitary, intermediate lobe and adrenal glands hypoplasia and very low plasma ACTH and corticosterone levels (Pulichino *et al*., 2003).

It has been shown that *Tpit* mRNA is present and co-expressed with POMC mRNA in normal and adenomatous corticotroph cells in humans and in dogs, but it is most abundantly expressed in ACTH-secreting pituitary tumors causing Cushing's disease (Tateno *et al*., 2007; Hanson *et al*., 2008).

Considering the importance of *Tpit* in the development of corticotrophs and the transcription of POMC, we hypothesized that the *Tpit* gain-of-function mutations would be involved in the pathogenesis of ACTH-dependent hyperadrenocorticism in Poodle dogs. However, no germline mutations were found in the *Tpit* from genomic DNA from 50 dogs with ADHAC. This finding is in agreement with two other studies that did not find *Tpit* somatic mutations in 8 human corticotrophmacroadenomas and in 23 canine ACTH-secreting pituitary tumors (Bucciarelli *et al*., 2005; Hanson *et al*., 2008). Nevertheless, we found the new polymorphism p. S343G in exon 7 of the *Tpit* in ADHAC dog groups as well as in the control group with the same frequency (4%).

CONCLUSIONS

Germline mutation into the coding region of the *Tpit* gene is not involved in the pathogenesis of pituitary-dependent hyperadrenocorticism in this cohort of Poodle dogs. Future studies should include other genes to elucidate the genetic basis of Cushing's disease.

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