TITLE: Feline hypersomatotropism and acromegaly tumorigenesis: A potential role for the AIP gene

AUTHORS: B. C.J. Scudder, S.J. Niessen, B. Catchpole, R.C. Fowkes, D.B. Church, Y. Forcada

JOURNAL: Domestic Animal Endocrinology

PUBLISHER: Elsevier

PUBLICATION DATE: April 2017

DOI: 10.1016/j.domaniend.2016.11.005

DAE 16-99

Feline hypersomatotropism and acromegaly tumorigenesis: A potential role for the AIP gene

C.J. Scudder[a], S.J. Niessen[a], B. Catchpole[b], R.C. Fowkes[c], D.B. Church[a], Y. Forcada[a]

[a] *Clinical Science and Services, Royal Veterinary College, Hawkshead Lane, North Mymms, Hertfordshire, United Kingdom, AL9 7TA*

[b] *Department of Pathology and Pathogen Biology, Hawkshead Lane, North Mymms, Hertfordshire, United Kingdom, AL9 7TA*

[c] *Department of Comparative and Biomedical Sciences, Royal Veterinary College, Royal College Street, London, United Kingdom NW1 0TU*

Corresponding author: C.J. Scudder

email: cscudder@rvc.ac.uk, Tel: +44 1707 666 801

Clinical Science and Services, Royal Veterinary College, Hawkshead Lane, North Mymms, Hertfordshire, United Kingdom, AL9 7TA

Abstract

Acromegaly in humans is usually sporadic, however up to 20% of familial isolated pituitary adenomas are caused by germline sequence variants of the aryl-hydrocarbon-receptor interacting protein (*AIP*) gene. Feline acromegaly has similarities to human acromegalic families with AIP mutations. The aim of this study was to sequence the feline *AIP* gene, identify sequence variants and compare the *AIP* gene sequence between feline acromegalic and control cats, and in acromegalic siblings. The feline *AIP* gene was amplified through PCR using whole-blood genomic DNA from 10 acromegalic and 10 control cats, and three sibling pairs affected by acromegaly. PCR products were sequenced and compared to the published predicted feline *AIP* gene. A single non-synonymous SNP was identified in exon 1 (AIP:c.9T>G) of two acromegalic cats and none of the control cats, as well as both members of one sibling pair. The region of this SNP is considered essential for the interaction of the AIP protein with its receptor. This sequence variant has not previously been reported in humans. Two additional synonymous sequence variants were identified (AIP:c.481C>T and AIP:c.826C>T). This is the first molecular study to investigate a potential genetic cause of feline acromegaly and identified a non-synonymous *AIP* single nucleotide polymorphism in 20 % of the acromegalic cat population evaluated, as well as in one of the sibling pairs evaluated.

Keywords: feline acromegaly hypersomatotropism genetic SNP AIP

1. Introduction

Feline acromegaly is an increasingly recognised endocrinopathy which is predominantly caused by a growth hormone producing adenoma in the anterior pituitary gland [1–3]. Chronic excessive growth hormone secretion results in increased insulin-like growth factor-1 (IGF-1), soft tissue and bone growth, increased risk of diabetes mellitus and cardiovascular disease.

The majority of human pituitary adenomas that cause acromegaly are sporadic but some occur in a familial setting by genetic inheritance of disease causing gene sequence variants [4]. Familial causes of acromegaly include sequence variants within *MEN1*, protein kinase A regulatory subunit-1 alpha, *GNAS1* and aryl hydrocarbon receptor interacting protein (*AIP*) genes [5]. Feline acromegaly is clinically most similar to human *AIP*-associated acromegaly demonstrating a male predominance, macroadenomas and poor biochemical response to octreotide or lanreotide therapy [1,3,6–9]. Genomic variants of the *AIP* gene account for 20 % of human familial isolated pituitary adenomas (FIPA), of which 30 % are functional somatrophinomas. Disease onset is typically at a younger age in *AIP* gene variant human acromegalics compared to other causes of acromegaly [10]. *AIP*-variant acromegaly has also been identified in patients with non-familial human acromegaly [11,12].

The human AIP gene is located on chromosome 11q13 and containing six exons which encode for a 330 amino acid protein. The AIP protein is thought to act as a tumour suppressor by mediating gene transcription via interaction with the aryl-hydrocarbon receptor (AhR), and modulates oestrogen and androgen receptors and response to xenobiotics[13–15]. The latter is of extra interest since cats with acromegaly demonstrate increased circulating concentrations of organohalogenated contaminants [16]. The tertiary structure of the C-terminal region of the AIP protein is a tetratricopeptide double helix motif and a terminal seven amino acid helix known as the TPR domain [17]. Sequence variants within the TPR domain may affect the binding properties of the AIP protein and the importance of this region is highlighted by the finding that 70% of clinically relevant genomic sequence variants in humans occur within this region [13].

62 This aim of the study was to sequence the feline *AIP* gene, identify any genomic sequence variants

63 and compare germline *AIP* sequences of acromegalic cats and controls, as well as affected siblings.

2. Materials and methods

2.1 Animals

This study was approved by the Ethics and Welfare Committee at the Royal Veterinary College (RVC), ethical approval number URN 2014 1306.

Medical records of client owned cats who presented to the RVC Acromegalic Cat Clinic from first opinion veterinary practices between 2005 to 2013 were searched for cats with a diagnosis of acromegaly (inclusion criteria were serum IGF-1 > 1000 ng/mL and pituitary mass identified using contrast-enhanced pituitary computed tomography or at necropsy). Total serum IGF-1 was measured by a commercially available radioimmunoassay previously validated for cats (Nationwide Laboratories, Cambridge, UK) [3]. The intra- and inter-assay coefficient of variation (CV) has previously been reported: inter-assay CV 4.6 % for a cat sample of 519 ng/mL; 9.3 % for a standard sample of 216 ng/mL; 12.1 % for a standard sample of 62 ng/mL; intra-assay CV 7.9 % for a cat sample of 172 ng/mL run 18 times [3]. All cats had whole blood stored in EDTA anticoagulant from residual clinical samples frozen at -80 ^{o}C. The youngest ten cats were selected in an attempt to increase chances of detecting a feline *AIP*-variant (*AIP*-variant associated acromegaly in people typically affects humans at a younger age than non *AIP*-variant associated acromegaly). Control cats were selected from the RVC Genetic Archive using residual whole blood samples stored in EDTA anti-coagulant from cats who were presented to RVC as a referral patient from first opinion veterinary practices. All control cats were considered unlikely to have acromegaly on the basis of no history or clinical signs suggestive of acromegaly and were greater than 15 yr of age. This older age was chosen to minimise the chances of including cats that could have developed acromegaly at a later age. Residual whole blood samples stored in EDTA anti-coagulant from sibling pairs of cats, all diagnosed with acromegaly using the criteria above, were recruited and analysed. This was a further attempt to increase the chances of detecting *AIP*-variants (should they exist) because *AIP*-variant associated acromegaly is most commonly encountered in a familial setting.

2.2 Identification of the feline AIP gene sequence

The feline genome was searched for nucleotide similarity to the coding sequence of the human *AIP* gene using a BLAST search and Felis catus (domestic cat) nucleotide database (*https://blast.ncbi.nlm.nih.gov*). This revealed a six exon, 1250 base pair sequence located on chromosome D1 (NCBI Reference Sequence: NW_004065058.1, Assembly Felis_catus_6.2). Primers for cDNA were designed using Primer3Plus *(http://www.primer3plus.com)* and NCBI PrimerBLAST *(http://www.ncbi.nlm.nih.gov/tools/primer-blast)* for DNA polymerase. The nucleotide sequence of the sense primer was 5'-3' TAG AAG TTG CCG AAG CAG GT and anti-sense primer was 5'-3' GGG AGA GAT AAA TAC GGC CTT T. Polymerase chain reactions (PCRs) were performed using 1 µL of cDNA derived from the pituitary of an acromegalic cat (tissue obtained during necropsy), 13 µL of water, 5 µL of 5xHispec (Bioline, London, UK), 2.5 µL of PCR buffer (Roche, Welwyn Garden City, UK), 1.25 µL of $MgCl_2$ (5 nM) (Bioline, London, UK), 0.25 µL of 250 µM dNTPs (Bioline, London, UK), 1 µL of each sense and anti-sense primer (each at 200 pmol/µL) and 0.1 µL of Immolase (Bioline, London, UK).

PCR amplification cycles (n = 35) were performed using PCR thermal cycler (G-Storm GS1 thermal cycler, Somerton, UK) according to the following protocol: denaturation at 95 ^{o}C for 10 min followed by 94 ^{o}C for 40 s, annealing at 55 ^{o}C for 30 s, followed by elongation at 72 ^{o}C for 2 min. The final cycle was followed by a final elongation step at 72 ^{o}C for 10 min. Agarose gel electrophoresis was performed for 30 min followed by visualisation using 590 nm UV light then DNA purification using a commercially available kit (GenElute Gel Extraction Kit, Sigma-Aldrich. Dorset, UK). The extracted DNA was submitted for standard Sanger sequencing (Source BioScience LifeSciences, Nottingham, UK) and compared to the reference feline sequence using sequence analysis software (CLC Main Workbench 7, Qiagen Aarhus, Waltham, MA, USA).

2.3 SNP discovery and assessment of siblings

Once the complete coding sequence of the feline AIP gene was identified, whole blood origin genomic DNA from case and control samples, and feline acromegalic siblings, was extracted from whole blood stored in EDTA anticoagulant using a commercially available DNA extraction kit (DNeasy blood and tissue kit, QUIAGEN, Manchester, UK) according the manufacturer's instructions. Sense and anti-sense gDNA specific primers were designed to amplify exons 1, 2, 3 and 4 to 6. The optimum primer set and PCR conditions were determined for each primer pair (Table 1).

Amplicon gel electrophoresis, gel excision and purification were performed using the same protocol as for AIP cDNA identification. Standard Sanger sequencing was performed to determine the exon sequences. Amplicons were compared to the reference feline genome and to each other using commercially available gene analysis software (CLC Main Workbench 7, Qiagen Aarhus, Waltham, MA, USA).

2.4 Structural effect assessment

The structural and functional effect of the identified non-synonymous SNP was estimated using protein modelling software (Pyhre2 version 2.0 [18], PyMOL Molecular Graphics System Version 1.7.4.4 Schrödinger LLC, Sorting Tolerant from Intolerant [SIFT] [http://sift.jcvi.org/www/SIFT_seq_submit2.html] and Polyphen-2 [http://genetics.bwh.harvard.edu/pph2/] programmes).

2.5 Statistics

Statistical analysis was performed using Windows Excel 2010 and SPSS (IBM Statistics SPSS 21). Statistical significance was established using $P < 0.05$. Normality testing was performed visually using

histograms and Shapiro-Wilk tests. Groups were compared using the Student's t test where appropriate and Fisher's exact test was used to compare SNP frequency between groups.

3 Results

3.1 Animals

The mean age of control cats (19.2 ± 2.4 yr) was greater than acromegalic cats (10.7 ± 2.7 yr; $P < 0.001$). There were eight domestic short hair (DSH) cats, one British short hair and one Maine Coon cat in the acromegalic group and nine DSH and one domestic long hair cat in the control group. There were six male and four female cats in the acromegalic group and three male and seven female cats in the control group.

Genomic DNA of three pairs of sibling cats (all with a diagnosis of acromegaly) was acquired; all were DSH with a median age of 11 yr (range 9 to 12), four were male and two were female, all were neutered. These siblings had a mean serum IGF-1 of 1640 ng/mL (range 1460 to 2000).

3.2 Feline AIP coding sequence identification

A single amplicon was identified using the pituitary cDNA template and primers designed for sense and anti-sense AIP primers. Sanger sequencing of an acromegalic case revealed an 1181 base pair amplicon, coding for a 330 amino acid protein. The coding sequence identified from the amplicon shared 100 % homology to the predicted mRNA transcript variant X1 of feline *AIP* gene (XM_003993700.2, Assembly: GCF_000181335.2). The feline *AIP* nucleotide and predicted amino acid sequences were compared to the human AIP nucleotide and amino acid sequences and were found to be 91 % and 96 % homologous, respectively (Figure 1).

3.3 SNP discovery and assessment of acromegalic siblings

Two female DSH cats in the acromegalic group had a heterozygote non-synonymous SNP in exon 1, position 9 of the coding sequence (AIP:c.9T>G) changing the third amino acid from aspartic acid to glutamic acid (Figure 2). Two additional heterozygote synonymous SNPs were identified; AIP:c.481C>T in exon 4 of two female DSH cats in the control group and AIP:c.826C>T in exon 6 of one male DSH cat in the acromegalic and one female DSH cat in the control group. The AIP:c.481C>T SNP has previously been reported in cats (rs783758897, http://www.ncbi.nlm.nih.gov/snp).

The results of 3-D protein modelling predicted that the AIP:c.9T>G SNP resulted in a minor effect on the tertiary structure of the protein at the N-terminal. The SIFT score was 0.00, indicating the amino acid change could affect the protein function (scores > 0.05 are not predicted to have deleterious effects). Nevertheless, the prediction was deemed to be of low confidence. ThePolyPhen-2 report described the predicted mutation to be benign with a score of 0.003 (sensitivity 0.98 and specificity 0.44).

The AIP:c.9T>G SNP was also identified in one pair of acromegalic siblings (both male cats) and these two cats also had the AIP:c.481C>T SNP. No additional SNPs were identified among the other two pairs of siblings.

4 Discussion

The feline *AIP*-gene was sequenced and showed homology with the human equivalent. The sequencing results revealed three SNPs in the coding sequence of the *AIP* gene. A non-synonymous SNP was not detected in the control cats, whereas two of the ten initially assessed acromegalic cats displayed a non-synonymous SNP in exon 1 (AIP:c.9T>G). This SNP was predicted to result in a minor structural change, suggesting a potential relevance, and was also detected in both members of one of the three subsequently assessed sibling pairs.

The majority of functionally important *AIP* SNPs identified in humans affect the C-terminus of the protein [19]. This region is essential for the binding of AIP to the AhR, which is thought to be required for tumour suppressor activity [20]. The only non-synonymous nucleotide variant that was identified in this study affects the N-terminal region. The AIP:c.9T>G SNP encodes for an amino acid change from aspartic acid to glutamic acid. The likely structural effect of the aspartic acid to glutamic acid was estimated to be minor. Nevertheless, minor changes may affect spatial preferences and amino acid interactions [21,22]. The AIP N-terminal is important because it is required for the stability of the AIP-AhR-receptor complex and essential for the regulation of the intracellular localization AhR [23]. It is possible that the described amino acid change could affect AIP interaction with the AhR and downstream tumour suppressor activity, even if it causes a minor structural change to the protein. *AIP*-variant associated acromegaly is most commonly identified in a familial setting in human medicine [7], which explains our additional interest in assessing acromegalic sibling cats. Recruitment of this subset of cats was difficult, resulting in a low number of siblings assessed. Nevertheless, one of the three assessed siblings pairs had the AIP:c.9T>G SNP.

The AIP:c.9T>G SNP was heterozygous in all cats. The expected heterozygosity in an individual human genome estimates a SNP will occur once every 300 nucleotides and one study describing SNPs within the feline genome reported a SNP rate around one every 500 nucleotides [24–27]. Additionally, only 20 to 30% of heterozygous SNPs are estimated to affect protein function [28–30]. The identified AIP:c.9T>G SNP may not be clinically significant. Further functional studies would be beneficial to determine the significance of a change of the third amino acid from glutamine acid to aspartic acid. Additionally it is possible that homozygosity proves lethal or is associated with more severe disease leading to premature death, thus precluding eventual development of acromegaly later in life.

The clinical records of all four of the AIP:c.9T>G variant cats (two cats from the original study and both members of one pair of sibling cats) revealed these cats had the following pituitary tumour

sizes (dorsoventral height): 8.3 mm, 8.4 mm, 4.6 mm, 16 mm. The median pituitary adenoma height of the largest reported group of acromegalic cats (n=68) was 6.1 mm (interquartile range 5.2 to 7.6, range 4.2 to 16) [31].Therefore three of the four cats had a pituitary height in the upper quartile of reported pituitary heights in acromegalic cats. Human *AIP*-variant-associated pituitary adenomas are frequently also larger adenomas than those not associated with *AIP*-variant. This study raises the possibility that, like in humans, the identified feline *AIP*-variant may also be associated with a more expansive behaviour of the tumour, though more cases need to be assessed to ascertain.

Acromegaly in humans due to a germline *AIP* mutation develops at a younger age compared to the general population of acromegalics [32]. Ten young acromegalic cats were purposely selected for this study in order to maximise the chances of identifying a feline *AIP*-variant acromegalic population. In doing so, however, we might have biased our investigations, should no such correlation between age and this type of acromegaly exist in the cat, or should an opposite correlation exist.

One of the limitations of the study is the small number of patients in each group. Preferentially we would have larger case and control numbers. This study was designed as a preliminary investigation of the feline *AIP* gene and its possible association with acromegaly in cats. The results imply an extension of this study would be worthwhile.

In conclusion, we have identified a single non conservative SNP in exon 1 in 4 / 16 acromegalic cats investigated. This SNP has not been previously identified in human acromegalics. The SNP affects a region of the protein which might impact AIP protein function predisposing to acromegaly in affected cats. Larger screening studies, as well as functional studies would be required to assess this possibility further.

References

[1] Niessen SJM, Petrie G, Gaudiano F, Khalid M, Smyth JBA, Mahoney P, Church DB. Feline acromegaly: an underdiagnosed endocrinopathy? J. Vet. Intern. Med. 2007;21(5):899–905.

[2] Peterson ME, Taylor RS, Greco DS, Nelson RW, Randolph JF, Foodman MS, Moroff SD, Morrison SA, Lothrop CD. Acromegaly in 14 cats. J. Vet. Intern. Med. 1990;4(4):192–201.

[3] Niessen SJM, Forcada Y, Mantis P, Lamb CR, Harrington N, Fowkes R, Korbonits M, Smith K, Church DB. Studying cat (Felis catus) diabetes: beware of the acromegalic imposter. PLoS One 2015;10(5):e0127794.

[4] Xekouki P, Azevedo M, Stratakis CA. Anterior pituitary adenomas: inherited syndromes, novel genes and molecular pathways. Expert Rev. Endocrinol Metab 2010;5(5):697–709.

[5] Dworakowska D, Grossman AB. The pathophysiology of pituitary adenomas. Best Pract. Res. Clin. Endocrinol. Metab. 2009;23(5):525–41.

[6] Slingerland LI, Voorhout G, Rijnberk A, Kooistra HS. Growth hormone excess and the effect of octreotide in cats with diabetes mellitus. Domest. Anim. Endocrinol. 2008;35(4):352–61.

[7] Beckers A, Aaltonen LA, Daly AF, Karhu A. Familial isolated pituitary adenomas (FIPA) and the pituitary adenoma predisposition due to mutations in the aryl hydrocarbon receptor interacting protein (AIP) gene. Endocr. Rev. 2013;34(2):239–77.

[8] Niessen SJ, Church DB, Forcada Y. Hypersomatotropism, acromegaly, and hyperadrenocorticism and feline diabetes mellitus. Vet. Clin. North Am. Small Anim. Pract. 2013;43(2):319–50.

[9] Leontiou CA, Gueorguiev M, van der Spuy J, Quinton R, Lolli F, Hassan S, Chahal HS, Igreja SC, Jordan S, Rowe J, Stolbrink M, Christian HC, Wray J, Bishop-Bailey D, Berney DM, Wass JAH, Popovic V, Ribeiro-Oliveira A, Gadelha MR, Monson JP, Akker SA, Davis JRE, Clayton RN, Yoshimoto K, Iwata T, Matsuno A, Eguchi K, Musat M, Flanagan D, Peters G, Bolger GB, Chapple JP, Frohman LA, Grossman AB, Korbonits M. The role of the aryl hydrocarbon receptor-interacting protein gene in familial and sporadic pituitary adenomas. J. Clin. Endocrinol. Metab. 2008;93(6):2390–2401.

[10] Daly AF, Tichomirowa MA, Petrossians P, Heliövaara E, Jaffrain-Rea ML, Barlier A, Naves LA, Ebeling T, Karhu A, Raappana A, Cazabat L, De Menis E, Montañana CF, Raverot G, Weil RJ, Sane T, Maiter D, Neggers S, Yaneva M, Tabarin A, Verrua E, Eloranta E, Murat A, Vierimaa O, Salmela PI, Emy P, Toledo RA, Sabaté MI, Villa C, Popelier M, Salvatori R, Jennings J, Longás AF, Labarta Aizpún JI, Georgitsi M, Paschke R, Ronchi C, Valimaki M, Saloranta C, De Herder W, Cozzi R, Guitelman M, Magri F, Lagonigro MS, Halaby G, Corman V, Hagelstein MT, Vanbellinghen JF, Barra GB, Gimenez-Roqueplo AP, Cameron FJ, Borson-Chazot F, Holdaway I, Toledo SP, Stalla GK, Spada A, Zacharieva S, Bertherat J, Brue T, Bours V, Chanson P, Aaltonen LA, Beckers A. Clinical characteristics and therapeutic responses in patients with germ-line AIP mutations and pituitary adenomas: an international collaborative study. J. Clin. Endocrinol. Metab. 2010;95(11):E373-83.

[11] Preda V, Korbonits M, Cudlip S, Karavitaki N, Grossman AB. Low rate of germline AIP mutations in patients with apparently sporadic pituitary adenomas before the age of 40: a single-centre adult cohort. Eur. J. Endocrinol. 2014;171(5):659–666.

[12] Karaca Z, Taheri S, Tanriverdi F, Unluhizarci K, Kelestimur F. Prevalence of AIP mutations in a series of Turkish acromegalic patients: are synonymous AIP mutations relevant? Pituitary 2015;18(6):831–837.

[13] Trivellin G, Korbonits M. AIP and its interacting partners. J. Endocrinol. 2011;210(2):137–55.

[14] Georgitsi M, Karhu A, Winqvist R, Visakorpi T, Waltering K, Vahteristo P, Launonen V, Aaltonen LA. Mutation analysis of aryl hydrocarbon receptor interacting protein (AIP) gene in colorectal, breast, and prostate cancers. Br. J. Cancer 2007;96(2):352–6.

[15] Beischlag TV., Luis Morales J, Hollingshead BD, Perdew GH. The aryl hydrocarbon receptor complex and the control of gene expression. Crit. Rev. Eukaryot Gene Expr. 2008;18(3):207–250.

[16] Dirtu AC, Niessen SJ, Jorens PG, Covaci A. Organohalogenated contaminants in domestic cats' plasma in relation to spontaneous acromegaly and type 2 diabetes mellitus: a clue for endocrine disruption in humans? Environ. Int. 2013;57–58:60–7.

[17] Morgan RM, Hernández-Ramírez LC, Trivellin G, Zhou L, Roe SM, Korbonits M, Prodromou C. Structure of the TPR domain of AIP: lack of client protein interaction with the C-terminal α-7 helix of the TPR domain of AIP is sufficient for pituitary adenoma predisposition. PLoS One 2012;7(12):e53339.

[18] Kelley LA, Mezulis S, Yates CM, Wass MN, Sternberg MJ. The Phyre2 web portal for protein modeling, prediction and analysis. Nat. Protoc. 2015;10(6):845–858.

[19] Ozfirat Z, Korbonits M. AIP gene and familial isolated pituitary adenomas. Mol. Cell. Endocrinol. 2010;326(1–2):71–9.

[20] Bell DR, Poland A. Binding of aryl hydrocarbon receptor (AhR) to AhR-interacting protein. The role of hsp90. J. Biol. Chem. 2000;275(46):36407–14.

[21] Jonson PH, Petersen SB. A critical view on conservative mutations. Protein Eng. 2001;14(6):397–402.

[22] Lee DY, Kim KA, Yu YG, Kim KS. Substitution of aspartic acid with glutamic acid increases the unfolding transition temperature of a protein. Biochem. Biophys. Res. Commun. 2004;320(3):900–6.

[23] Georgitsi M, Raitila A, Karhu A, Tuppurainen K, Mäkinen MJ, Vierimaa O, Paschke R, Saeger W, van der Luijt RB, Sane T, Robledo M, De Menis E, Weil RJ, Wasik A, Zielinski G, Lucewicz O, Lubinski J, Launonen V, Vahteristo P, Aaltonen LA. Molecular diagnosis of pituitary adenoma predisposition caused by aryl hydrocarbon receptor-interacting protein gene mutations. Proc. Natl. Acad. Sci. U.S.A. 2007;104(10):4101–5.

[24] Sudmant PH, Rausch T, Gardner EJ, Handsaker RE, Abyzov A, Huddleston J, Zhang Y, Ye K, Jun G, Hsi-Yang Fritz M, Konkel MK, Malhotra A, Stütz AM, Shi X, Paolo Casale F, Chen J, Hormozdiari F, Dayama G, Chen K, Malig M, Chaisson MJP, Walter K, Meiers S, Kashin S, Garrison E, Auton A, Lam HYK, Jasmine Mu X, Alkan C, Antaki D, Bae T, Cerveira E, Chines P, Chong Z, Clarke L, Dal E, Ding L, Emery S, Fan X, Gujral M, Kahveci F, Kidd JM, Kong Y, Lameijer E-W, McCarthy S, Flicek P, Gibbs RA, Marth G, Mason CE, Menelaou A, Muzny DM, Nelson BJ, Noor A, Parrish NF, Pendleton M, Quitadamo A, Raeder B, Schadt EE, Romanovitch M, Schlattl A, Sebra R, Shabalin AA, Untergasser A, Walker JA, Wang M, Yu F, Zhang C, Zhang J, Zheng-Bradley X, Zhou W, Zichner T, Sebat J, Batzer MA, McCarroll SA, Mills RE, Gerstein MB, Bashir A, Stegle O, Devine SE, Lee C, Eichler EE, Korbel JO. An integrated map of structural variation in 2,504 human genomes. Nature 2015;526(7571):75–81.

[25] Sachidanandam R, Weissman D, Schmidt SC, Kakol JM, Stein LD, Marth G, Sherry S, Mullikin JC, Mortimore BJ, Willey DL, Hunt SE, Cole CG, Coggill PC, Rice CM, Ning Z, Rogers J, Bentley DR, Kwok PY, Mardis ER, Yeh RT, Schultz B, Cook L, Davenport R, Dante M, Fulton L, Hillier L, Waterston RH, McPherson JD, Gilman B, Schaffner S, Van Etten WJ, Reich D, Higgins J, Daly MJ, Blumenstiel B, Baldwin J, Stange-Thomann N, Zody MC, Linton L, Lander ES, Altshuler D, International SNP Map Working Group. A map of human genome sequence variation containing 1.42 million single nucleotide polymorphisms. Nature 2001;409(6822):928–933.

[26] 1000 Genomes Project Consortium, Abecasis GR, Auton A, Brooks LD, DePristo MA, Durbin RM, Handsaker RE, Kang HM, Marth GT, McVean GA. An integrated map of genetic variation from 1,092 human genomes. Nature 2012;491(7422):56–65.

[27] Mullikin JC, Hansen NF, Shen L, Ebling H, Donahue WF, Tao W, Saranga DJ, Brand A, Rubenfield MJ, Young AC, Cruz P, Driscoll C, David V, Al-Murrani SWK, Locniskar MF, Abrahamsen MS, O'Brien SJ, Smith DR, Brockman J a. Light whole genome sequence for SNP discovery across domestic cat breeds. BMC Genomics 2010;11:406.

[28] Ng PC, Levy S, Huang J, Stockwell TB, Walenz BP, Li K, Axelrod N, Busam DA, Strausberg RL, Venter JC. Genetic variation in an individual human exome. PLoS Genet. 2008;4(8):e1000160.

[29] Chasman D, Adams RM. Predicting the functional consequences of non-synonymous single nucleotide polymorphisms: structure-based assessment of amino acid variation. J. Mol. Biol. 2001;307(2):683–706.

[30] Ng PC, Henikoff S. Accounting for human polymorphisms predicted to affect protein function. Genome Res. 2002;12(3):436–446.

[31] Lamb CR, Ciasca TC, Mantis P, Forcada Y, Potter M, Church DB, Niessen SJ. Computed tomographic signs of acromegaly in 68 diabetic cats with hypersomatotropism. J. Feline Med. Surg. 2014;16(2):99–108.

[32] Tichomirowa MA, Barlier A, Daly AF, Jaffrain-Rea ML, Ronchi C, Yaneva M, Urban JD, Petrossians P, Elenkova A, Tabarin A, Desailloud R, Maiter D, Schürmeyer T, Cozzi R, Theodoropoulou M, Sievers C, Bernabeu I, Naves LA, Chabre O, Montañana CF, Hana V,

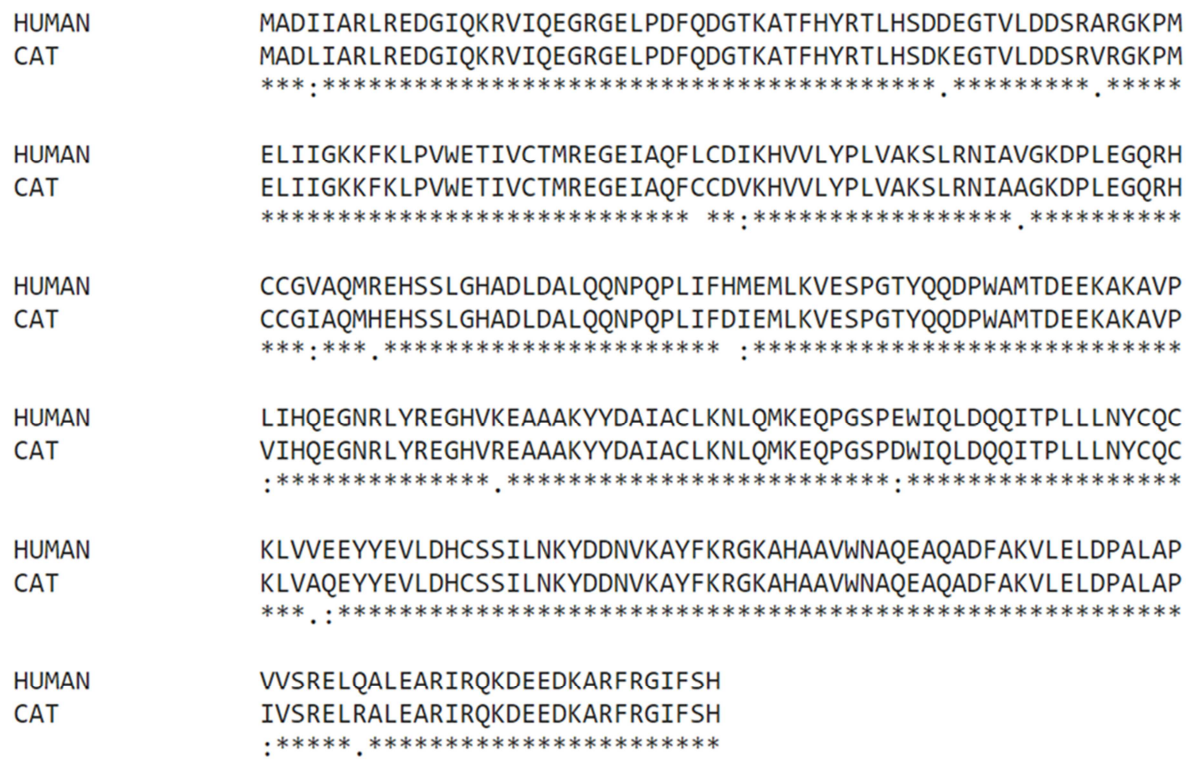
345 Halaby G, Delemer B, Aizpún JI, Sonnet E, Longás AF, Hagelstein MT, Caron P, Stalla GK,
346 Bours V, Zacharieva S, Spada A, Brue T, Beckers A. High prevalence of AIP gene mutations
347 following focused screening in young patients with sporadic pituitary macroadenomas. Eur.
348 J. Endocrinol. 2011;165(4):509–15.

Appendix

Table 1:

Exon	Primers	Amplicon	Denaturation		Annealing 35 cycles	Elongation
1	For 5’-3’ TAG AAG TTG CCG AAG CAG GT	431 bp	95 °C	94 °C	55 °C	72 °C
	Rev 5’-3’ CCC TGC AAC GTT CTT ACG AT		10 min	40 s	2 min	2 min
2	For 5’-3’ GGG TAA AGG TCA GGT GGT GA	369 bp	95 °C	94 °C	64 °C	72 °C
	Rev 5’-3’ GAT GGG GAA TAG GGG ATG AC		10 min	40 s	2 min	2 min
3	For 5’-3’ GAG GAC TCC TGA GGG AAA GG	400 bp	95 °C	94 °C	64 °C	72 °C
	Rev 5’-3’ GGT TTG GTG AGG CAC CTG		10 min	40 s	2 min	2 min
4	For 5’-3’ CAG GGG TGT TGG TAG GAG AA	1348 bp	95 °C	94 °C	64 °C	72 °C
			10 min	40 s	2 min	2 min
5	For 5’-3’ CAG CTC TCA GCG TCT CCT G	220 bp	95 °C	94 °C	64 °C	72 °C
	Rev 5’-3’ GGT CAG AGG CCC AGT TGT G		10 min	40 s	2 min	2 min
6	Rev 5’-3’ GGG AGA GAT AAA TAC GGC CTT T	1348 bp	95 °C	94 °C	64 °C	72 °C
			10 min	40 s	2 min	2 min

352 **Figure 1:**

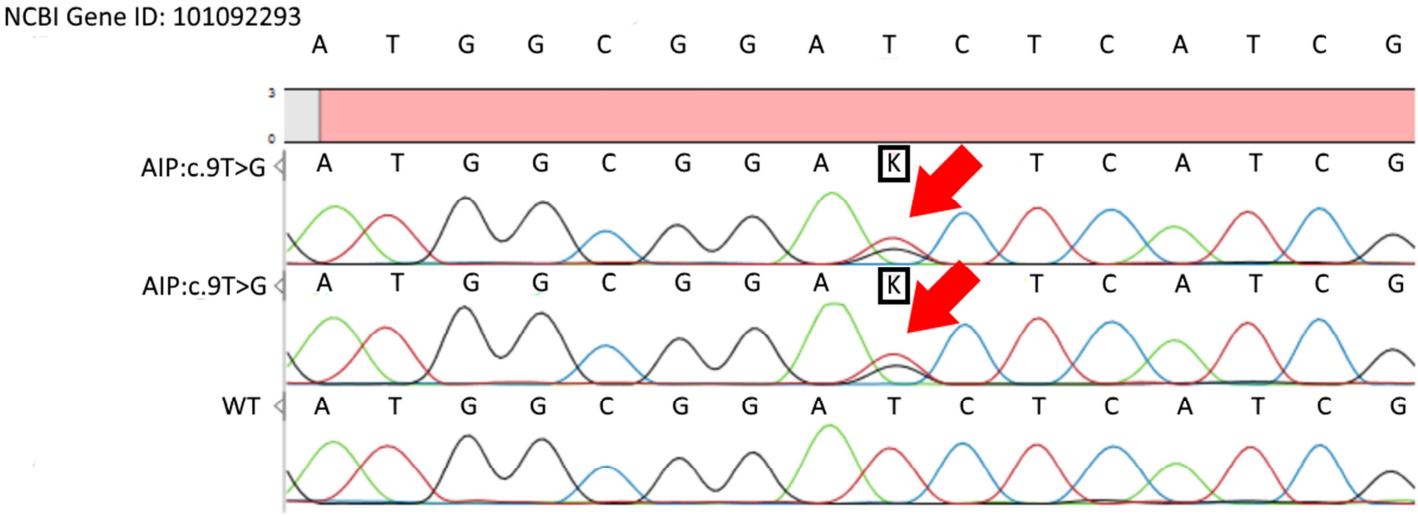


353

354

355 **Figure 2.**

356



357

Tables and Figures List:

Table 1: PCR primers and conditions for genomic *AIP* gene amplification

Figure 1: Comparison of the homology of the human and feline AIP amino acid sequence using CLUSTAL multiple sequence alignment by MUSCLE (3.8) (http://www.ebi.ac.uk/Tools/msa/muscle). The feline AIP protein was 96% homologous to the human AIP protein.

Figure 2: Sanger sequencing chromatographs from three cats. The nucleotides shown represent the first 16 nucleotides of exon 1 of the feline *AIP* gene. The top two chromatographs contain the AIP:c.9T>G SNP (highlighted by red arrows) and the third chromatograph is the wild type (WT) feline AIP sequence. The AIP:c.9T>G SNP is heterozygous at nucleotide 9 and labelled K as denoted by the IUPAC nucleotide ambiguity code nomenclature.

Feline hypersomatotropism and acromegaly tumorigenesis: A potential role for the AIP gene

- A non-synonymous heterozygous germline variant of the AIP gene (AIP:c.9T>G) was only found acromegalic cats
- The AIP:c.9T>G variant encodes for an amino acid change from aspartic acid to glutamic acid in a region of the AIP protein considered to be important for its tumour suppressor activity
- The AIP:c.9T>G variant may predispose to pituitary macroadenomas. Three of the four cats having this variant had pituitary tumours in the upper quartile of reported pituitary heights in acromegalic cats, as measured using contrast-enhanced computed tomography.