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

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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 \textit{MEN1}, protein kinase A regulatory subunit-1 alpha, \textit{GNAS1} and aryl hydrocarbon receptor interacting protein (\textit{AIP}) genes [5]. Feline acromegaly is clinically most similar to human \textit{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 \textit{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 \textit{AIP} gene variant human acromegalics compared to other causes of acromegaly [10]. \textit{AIP}-variant acromegaly has also been identified in patients with non-familial human acromegaly [11,12].

The human \textit{AIP} gene is located on chromosome 11q13 and containing six exons which encode for a 330 amino acid protein. The \textit{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 \textit{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 \textit{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].
This aim of the study was to sequence the feline $AIP$ gene, identify any genomic sequence variants 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 °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 anticoagulant 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 MgCl₂ (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 °C for 10 min followed by 94 °C for 40 s, annealing at 55 °C for 30 s, followed by elongation at 72 °C for 2 min. The final cycle was followed by a final elongation step at 72 °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. The PolyPhen-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


### Appendix

#### Table 1:

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

<table>
<thead>
<tr>
<th>HUMAN</th>
<th>CAT</th>
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</thead>
<tbody>
<tr>
<td>MADIIARLREDGIQKVRVIEGRELDPFDQDGTKATFHRYTLHSGDEGTVDLSRARGKPM</td>
<td>MADLIARLREDGIQKVRVIEGRELDPFDQDGTKATFHRYTLHSGDEGTVDLSRARGKPM</td>
</tr>
<tr>
<td>ELIIGKFLPVWETIVCTMRGEIAQFCDKTHVVLVPLVAKSLRNIAGDPLLEGQRH</td>
<td>ELIIGKFLPVWETIVCTMRGEIAQFCDKTHVVLVPLVAKSLRNIAGDPLLEGQRH</td>
</tr>
<tr>
<td>CCGVAMEMHSLGHADLDALQQNPQPLIFHMEMLVESPGTYQDPPAMTDEEKAQAVP</td>
<td>CCGIAQMMEHSLGHADLDALQQNPQPLIFDIEMLVESPGTYQDPPAMTDEEKAQAVP</td>
</tr>
<tr>
<td>LIHQEGRNLVREGHVKEAAKYYDAIAKNNQMKEQPGSPDWIQLDQITPLNLYQCQ</td>
<td>VIHQEGRNLVREGHVREAAKYYDAIAKNNQMKEQPGSPDWIQLDQITPLNLYQCQ</td>
</tr>
<tr>
<td>KLVEEYYEVLDHCSILNYDDNVKAYFKRGAAMWNAQFAQVDFAKVLDELPALAP</td>
<td>KLVAEYYYEVLDHCSILNYDDNVKAYFKRGAAMWNAQFAQVDFAKVLDELPALAP</td>
</tr>
<tr>
<td>VSRQLEARIQKDEEAKFRGIFSH</td>
<td>IVSRQLEARIQKDEEAKFRGIFSH</td>
</tr>
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</table>
Figure 2.

NCBI Gene ID: 101092293
Tables and Figures List:

Table 1: PCR primers and conditions for genomic \textit{AIP} gene amplification

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

Figure 2: Sanger sequencing chromatographs from three cats. The nucleotides shown represent the first 16 nucleotides of exon 1 of the feline \textit{AIP} gene. The top two chromatographs contain the \textit{AIP:c.9T>G} SNP (highlighted by red arrows) and the third chromatograph is the wild type (WT) feline \textit{AIP} sequence. The \textit{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.