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

4

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17

18 **Abstract**

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

34

35 Keywords: feline acromegaly hypersomatotropism genetic SNP *AIP*

36

37 1. Introduction

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

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

52 The human *AIP* gene is located on chromosome 11q13 and containing six exons which encode for a
53 330 amino acid protein. The *AIP* protein is thought to act as a tumour suppressor by mediating gene
54 transcription via interaction with the aryl-hydrocarbon receptor (AhR), and modulates oestrogen and
55 androgen receptors and response to xenobiotics[13–15]. The latter is of extra interest since cats
56 with acromegaly demonstrate increased circulating concentrations of organohalogenated
57 contaminants [16]. The tertiary structure of the C-terminal region of the *AIP* protein is a
58 tetratricopeptide double helix motif and a terminal seven amino acid helix known as the TPR domain
59 [17]. Sequence variants within the TPR domain may affect the binding properties of the *AIP* protein
60 and the importance of this region is highlighted by the finding that 70% of clinically relevant genomic
61 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.

ACCEPTED MANUSCRIPT

64 2. Materials and methods

65 2.1 Animals

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

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

89

90 *2.2 Identification of the feline AIP gene sequence*

91 The feline genome was searched for nucleotide similarity to the coding sequence of the human *AIP*
92 gene using a BLAST search and *Felis catus* (domestic cat) nucleotide database

93 (<https://blast.ncbi.nlm.nih.gov>). This revealed a six exon, 1250 base pair sequence located on

94 chromosome D1 (NCBI Reference Sequence: NW_004065058.1, Assembly *Felis_catus_6.2*). Primers

95 for cDNA were designed using Primer3Plus (<http://www.primer3plus.com>) and NCBI PrimerBLAST

96 (<http://www.ncbi.nlm.nih.gov/tools/primer-blast>) for DNA polymerase. The nucleotide sequence of

97 the sense primer was 5'-3' TAG AAG TTG CCG AAG CAG GT and anti-sense primer was 5'-3' GGG AGA

98 GAT AAA TAC GGC CTT T. Polymerase chain reactions (PCRs) were performed using 1 µL of cDNA

99 derived from the pituitary of an acromegalic cat (tissue obtained during necropsy), 13 µL of water, 5

100 µL of 5xHispec (Bioline, London, UK), 2.5 µL of PCR buffer (Roche, Welwyn Garden City, UK), 1.25 µL

101 of MgCl₂ (5 nM) (Bioline, London, UK), 0.25 µL of 250 µM dNTPs (Bioline, London, UK), 1 µL of each

102 sense and anti-sense primer (each at 200 pmol/µL) and 0.1 µL of Immolase (Bioline, London, UK).

103 PCR amplification cycles (n = 35) were performed using PCR thermal cycler (G-Storm GS1 thermal

104 cycler, Somerton, UK) according to the following protocol: denaturation at 95 °C for 10 min followed

105 by 94 °C for 40 s, annealing at 55 °C for 30 s, followed by elongation at 72 °C for 2 min. The final

106 cycle was followed by a final elongation step at 72 °C for 10 min. Agarose gel electrophoresis was

107 performed for 30 min followed by visualisation using 590 nm UV light then DNA purification using a

108 commercially available kit (GenElute Gel Extraction Kit, Sigma-Aldrich, Dorset, UK). The extracted

109 DNA was submitted for standard Sanger sequencing (Source BioScience LifeSciences, Nottingham,

110 UK) and compared to the reference feline sequence using sequence analysis software (CLC Main

111 Workbench 7, Qiagen Aarhus, Waltham, MA, USA).

112

113 2.3 SNP discovery and assessment of siblings

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

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

125

126 2.4 Structural effect assessment

127 The structural and functional effect of the identified non-synonymous SNP was estimated using
128 protein modelling software (Pyhre2 version 2.0 [18], PyMOL Molecular Graphics System Version
129 1.7.4.4 Schrödinger LLC, Sorting Tolerant from Intolerant [SIFT]
130 [http://sift.jcvi.org/www/SIFT_seq_submit2.html] and Polyphen-2
131 [<http://genetics.bwh.harvard.edu/pph2/>] programmes).

132

133 2.5 Statistics

134 Statistical analysis was performed using Windows Excel 2010 and SPSS (IBM Statistics SPSS 21).

135 Statistical significance was established using $P < 0.05$. Normality testing was performed visually using

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

138

139 **3 Results**

140 *3.1 Animals*

141 The mean age of control cats (19.2 ± 2.4 yr) was greater than acromegalic cats (10.7 ± 2.7 yr; $P <$

142 0.001). There were eight domestic short hair (DSH) cats, one British short hair and one Maine Coon

143 cat in the acromegalic group and nine DSH and one domestic long hair cat in the control group.

144 There were six male and four female cats in the acromegalic group and three male and seven female

145 cats in the control group.

146 Genomic DNA of three pairs of sibling cats (all with a diagnosis of acromegaly) was acquired; all were

147 DSH with a median age of 11 yr (range 9 to 12), four were male and two were female, all were

148 neutered. These siblings had a mean serum IGF-1 of 1640 ng/mL (range 1460 to 2000).

149

150 *3.2 Feline AIP coding sequence identification*

151 A single amplicon was identified using the pituitary cDNA template and primers designed for sense

152 and anti-sense AIP primers. Sanger sequencing of an acromegalic case revealed an 1181 base pair

153 amplicon, coding for a 330 amino acid protein. The coding sequence identified from the amplicon

154 shared 100 % homology to the predicted mRNA transcript variant X1 of feline *AIP* gene

155 (XM_003993700.2, Assembly: GCF_000181335.2). The feline *AIP* nucleotide and predicted amino

156 acid sequences were compared to the human AIP nucleotide and amino acid sequences and were

157 found to be 91 % and 96 % homologous, respectively (Figure 1).

158

159 *3.3 SNP discovery and assessment of acromegalic siblings*

160 Two female DSH cats in the acromegalic group had a heterozygote non-synonymous SNP in exon 1,
161 position 9 of the coding sequence (AIP:c.9T>G) changing the third amino acid from aspartic acid to
162 glutamic acid (Figure 2). Two additional heterozygote synonymous SNPs were identified;
163 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
164 one male DSH cat in the acromegalic and one female DSH cat in the control group. The AIP:c.481C>T
165 SNP has previously been reported in cats (rs783758897, <http://www.ncbi.nlm.nih.gov/snp>).

166 The results of 3-D protein modelling predicted that the AIP:c.9T>G SNP resulted in a minor effect on
167 the tertiary structure of the protein at the N-terminal. The SIFT score was 0.00, indicating the amino
168 acid change could affect the protein function (scores > 0.05 are not predicted to have deleterious
169 effects). Nevertheless, the prediction was deemed to be of low confidence. The PolyPhen-2 report
170 described the predicted mutation to be benign with a score of 0.003 (sensitivity 0.98 and specificity
171 0.44).

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

175

176 **4 Discussion**

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

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

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

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

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

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

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

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

230

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ACCEPTED MANUSCRIPT

349 **Appendix**

350 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	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		
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		
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		
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

351

352 **Figure 1:**

HUMAN MADIIARLREDGIQKRVIQEGRGELPDFQDGTKATFHVRTLHSDDEGTVLDDSRARGKPM
 CAT MADLIARLREDGIQKRVIQEGRGELPDFQDGTKATFHVRTLHSDKEGTVLDDSRVRGKPM
 :**.*****.*****

HUMAN ELIIGKKFKLPVWETIVCTMREGEIAQFLCDIKHVLYPLVAKSLRNIAVGGKDPLEGQRH
 CAT ELIIGKKFKLPVWETIVCTMREGEIAQFCCDVKHVLYPLVAKSLRNIAAGKDPLEGQRH
 ***** **:******.*****

HUMAN CCGVQMREHSSLGHADLDALQQNPQPLIFHMEMLKVESPGTYQQDPWAMTDEEKAKAVP
 CAT CCGI AQMHEHSSLGHADLDALQQNPQPLIFDI EMLKVESPGTYQQDPWAMTDEEKAKAVP
 :.*****:*****

HUMAN LIHQEGNRLYREGHVKEAAAKYYDAIACLKNLQMKEQPGSPEWIQLDQQITPLLLNYCQC
 CAT VIHQEGNRLYREGHVREAAAKYYDAIACLKNLQMKEQPGSPDWIQLDQQITPLLLNYCQC
 :*****.*****.*****

HUMAN KLVVEEYEVLDHCSSILNKYDDNVKAYFKRGKAHAAVWNAQEAQADF AKVLELDPALAP
 CAT KLVAQEYEVLDHCSSILNKYDDNVKAYFKRGKAHAAVWNAQEAQADF AKVLELDPALAP
 .:**

HUMAN VVSRELQALEARIRQKDEEDKARFRGIFSH
 CAT IVSRELRALEARIRQKDEEDKARFRGIFSH
 :*****.*****

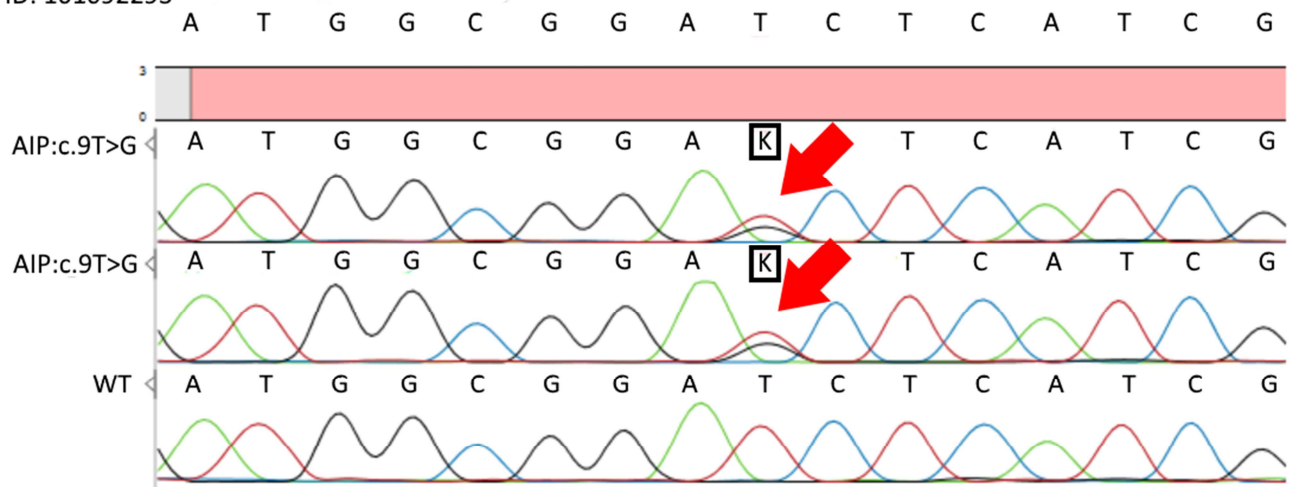
353

354

355 **Figure 2.**

356

NCBI Gene ID: 101092293



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358 **Tables and Figures List:**359 Table 1: PCR primers and conditions for genomic *AIP* gene amplification

360

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

364

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

370

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 in 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.