Genome-wide scan identifies novel modifier loci of acromegalic phenotypes for isolated familial somatotropinoma

S K Khoo¹, R Pendek², R Nickolov³, D C Luccio-Camelo^{4,5}, T L Newton¹, A Massie¹, D Petillo¹, J Menon⁶, D Cameron⁷, B T Teh^{1,8} and S-P Chan²

¹Laboratory of Cancer Genetics, Van Andel Research Institute, Grand Rapids, Michigan 49503, USA

²Department of Medicine, University of Malaya Medical Centre, 50603 Kuala Lumpur, Malaysia

³Department of Mathematics and Computer Science, Fayetteville State University, Fayetteville, North Carolina 28301, USA

⁴Serviço de Endocrinologia, Hospital Universitário Clementino Fraga Filho, UFRJ, Rio de Janeiro 21949-590, Brazil

⁵Department of Urology, University of Illinois at Chicago, Chicago, Illinois 60612, USA

⁶Department of Medicine, Queen Elizabeth Hospital, 88586 Kota Kinabalu, Sabah, Malaysia

⁷Department of Diabetes and Endocrinology, Princess Alexandra Hospital, Brisbane, Queensland 4102, Australia

⁸NCCS-VARI Translational Cancer Research Laboratory, National Cancer Centre, Singapore, Republic of Singapore 169610

(Correspondence should be addressed to S K Khoo; Email: sok-kean.khoo@vai.org; S-P Chan; Email: spchan88@hotmail.com)

Abstract

Isolated familial somatotropinoma (IFS) accounts for 18% of familial isolated pituitary adenoma (FIPA) cases. Recently, germline mutations of the aryl hydrocarbon receptor-interacting protein gene (*AIP*) have been found in families with pituitary adenoma predisposition, FIPA, and IFS. In this study, we investigate the *AIP* mutation status and perform a genome-wide scan to search for the modifier regions of acromegalic phenotypes in an IFS family of 31 aborigines from Borneo. Complete endocrine diagnosis and data could not be collected due to logistical and cultural reasons. *AIP* mutation screening was carried out by direct sequencing and the genome-wide scan was performed using 400 microsatellites. Non-parametric linkage analysis was performed to obtain the logarithm of odds (LOD) scores. A novel *AIP* frameshift mutation in exon 4 (c.500delC) (p.P167HfsX3) was identified in all members with acromegalic features, as well as in 15 members without acromegalic features, revealing incomplete penetrance of *AIP*. The data showed that patients with the same mutation may express acromegalic features of differing severity, suggesting the existence of modifier genes. The highest LOD score of 2.2 was obtained near D19S571 (19q13.41). We also found weak linkages on chromosomes 3q28, 8q12.1, and 21q22.13, with LOD scores of 1.1, 1.8, and 1.4 respectively. Our results show the first genome-wide scan that identifies novel modifier loci for acromegalic phenotypes in an IFS family. Identification of modifier loci may provide further insight into the disease mechanism and explain the clinical variability observed in its patients.

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Introduction

The prevalence of GH-secreting pituitary adenomas is ~1 in 1000 (Daly *et al.* 2006b), although biochemical evidence of acromegaly in previously undiagnosed subjects can occur even more frequently (Schneider *et al.* 2008). Although these adenomas are not classified as malignant, their excessive production of hormones can cause severe clinical conditions such as obesity and disfigurement, hypertension, diabetes mellitus, and accelerated heart disease. Thus, patients

are often faced with an array of debilitating symptoms and difficult treatments.

Familial GH-secreting pituitary adenoma (also known as familial somatotropinoma or familial acromegaly) is a rare clinical entity. Its occurrence is associated with three separate clinical syndromes: multiple endocrine neoplasia type 1 (MEN1); Carney complex; and isolated familial somatotropinoma (IFS). The *MEN1* gene and the protein kinase A regulatory subunit 1 (*PRKARIA*) gene have been identified as

genes related to MEN1 and Carney complex respectively (Chandrasekharappa *et al.* 1997, Kirschner *et al.* 2000). IFS is defined as the occurrence of at least two cases of acromegaly or gigantism in a family not exhibiting MEN1 or Carney complex. Recently, IFS has been included in the clinical syndrome of familial isolated pituitary adenomas (FIPA) and accounts for ~18% of FIPA cases (Daly *et al.* 2006a). Till date, there are ~50 families with 120 affected members reported with IFS (Beckers & Daly 2007) and 99 families with 223 affected members reported with FIPA (Daly *et al.* 2007, Leontiou *et al.* 2008).

Recently, germline mutations in the aryl hydrocarbon receptor-interacting protein (*AIP*) gene have been identified in individuals with pituitary adenoma predisposition (PAP; Vierimaa *et al.* 2006). Since then, a few reports of *AIP* mutations in both familial and sporadic acromegaly have been published (Barlier *et al.* 2007, Daly *et al.* 2007, Iwata *et al.* 2007, Toledo *et al.* 2007, Leontiou *et al.* 2008). Low penetrance of *AIP* in reported PAP and FIPA families suggests the existence of additional modifier genes. Patients with *AIP* mutations may or may not present with pituitary adenomas, besides presenting with acromegalic features of differing severity. Since the development of acromegalic phenotypes is insidious, it is important to find the associated genes so that patients can be treated promptly. Here, we performed direct sequencing on all 31 individuals in a large IFS family from Borneo to screen for *AIP* mutations. Subsequently, to identify the modifier regions based on acromegalic features in IFS, we performed a genome-wide scan using 400 microsatellite markers on the same family.

Materials and methods

Subjects

One previously unreported IFS family with 31 members was located in Borneo, Malaysia. The pedigree is shown in Fig. 1. All participating subjects provided informed consent and this study was approved by the Institutional Review Board of the Van Andel Research Institute and the University of Malaya. The studied subjects are the aborigines who live in the mountainous area of Borneo with minimal access to civilization. Our medical group traveled to the village in order to collect the samples. Owing to logistical reasons, the subjects refused to travel to the regional hospital for magnetic resonance imaging (MRI). Blood samples were collected, and the GH levels and insulin-like growth factor-1 (IGF-1) levels were measured from serum. Genomic DNA was obtained from blood using a QIAamp DNA Blood Maxi Kit (Qiagen).

AIP mutation screening

All six coding exons of *AIP* were sequenced. The exon-specific primer sequences were designed according to Vierimaa *et al.* (2006), except those of exon 1 (*AIP*-SK-1 – forward primer: 5'-AAGCAAGTCCGG-AAGCTA-3'; reverse primer: 5'-GTCGAGTTCTG-CATGTGAG-3'). PCR condition for exon 1 was performed with 0.4% dimethyl sulfoxide (DMSO), 0.4 µM of each primer, 1.5 mM MgCl₂, 0.04 mM dNTPs, and 0.2 U *Taq* DNA polymerase (Invitrogen) with cycling conditions as follows: 95 °C for 10 min, followed by 35 cycles of 95 °C for 45 s; 57 °C for 45 s; 72 °C for 45 s; and a final extension at 72 °C for 7 min.

Figure 1 Pedigree of a large Borneo family with IFS, showing incomplete penetrance of *AIP*. Affected and unaffected individuals are shown by filled and open symbols respectively. Suspected carriers are indicated by a dot in the square or circle and deceased individuals are identified by a slash (/). Subjects with *AIP* mutation are marked by +.

PCR conditions for exons 2–6 were 1 μ M of each primer, 1.5 mM MgCl₂, 0.2 mM dNTPs, and 0.25 U FastStart *Taq* DNA polymerase (Roche Applied Science). The cycling conditions for exons 2–6 were as follows: 95 °C for 10 min, followed by 30 cycles of 95 °C for 30 s; 68 °C for 20 s; 72 °C for 30 s; and a final extension at 72 °C for 7 min. All amplifications were carried out with 25 ng of genomic DNA in a total volume of 25 μ l, and PCR was performed using a MJ Research Tetrad Thermal Cycler (Bio-Rad Laboratories). Amplicons were analyzed on 1.5% agarose gels before purification using Multiscreen PCR cleanup plates (Millipore, Billerica, MA, USA). Sequencing was performed using Big Dye Terminator (Applied Biosystems, Foster City, CA, USA) and run on an ABI 3700 genetic analyzer (Applied Biosystems). The *AIP* sequence was obtained from Ensembl (<http://www.ensembl.org/index.html>) and was aligned with Emboss pairwise alignment algorithms (<http://www.ebi.ac.uk/emboss/align/index.html>), as well as being manually verified. All sequence alterations were verified by reamplification of the corresponding *AIP* exon and repeating the sequencing procedure with both forward and reverse primers.

Genome-wide scanning

The genomic DNA of 31 individuals from the IFS family was included in a 10 cM density genome-wide scan by using 400 fluorescence-labeled microsatellite markers from the ABI PRISM Human Linkage Mapping Set v. 2 (Applied Biosystems). PCR was performed in a 7.5 μ l reaction, and its volume containing 0.17 μ M each of fluorescence-labeled forward and unlabeled reverse primer, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 4 mM MgCl₂, 0.3 U AmpliTaq Gold polymerase (Applied Biosystems), 0.25 mM dNTPs (Invitrogen), and 15 ng genomic DNA. PCR was performed using a DNA Engine Tetrad (Bio-Rad Laboratories) with an initial set of 10 cycles (15 s at 94 °C, 15 s at 55 °C, 30 s at 72 °C) followed by 20 cycles (15 s at 89 °C, 15 s at 55 °C, 30 s at 72 °C). All PCR-amplified products were then denatured and run on an ABI 3700 genetic analyzer (Applied Biosystems). Allele sizes were assigned using Genescan v. 3.1 and Genotyper v. 2.5 software (Applied Biosystems). Genotypes inconsistent with Mendelian laws were detected using the PedCheck program (O'Connell & Weeks 1998). Erroneous genotypes were re-examined manually and corrected, or the samples were rerun to confirm the allele calls. Pedigrees and haplotypes were drawn using Cyrillic v. 2.1 (Exeter Software, Setauket, NY, USA).

Linkage analysis

The biochemical data did not show any clear-cut clinical sign of acromegaly in the subjects (same phenomenon reported by Schneider *et al*). Therefore, we could not use this set of biochemical data to define the affected subjects for linkage study. On the other hand, since IFS has a much younger age at onset compared with sporadic acromegaly (Soares & Frohman 2004, Beckers & Daly 2007). We can assume that subjects >20 years old should have their acromegalic features defined. Thus, without MRI data or clearly defined biochemical data, to identify the affected subjects, and until a better diagnosis of acromegaly is defined, it is rational to use the presence or absence of acromegalic features in the *AIP* mutants and perform linkage analysis to identify the modifier genes related to this phenotype. *AIP* mutants with acromegalic features are considered as affected (II:3, III:6, III:9, IV:7, IV:8, and IV:9) and *AIP* mutants without acromegalic features or asymptomatic carriers are non-affected (III:2, III:3, III:7, III:10, III:12, IV:1, IV:3, IV:5, IV:10, IV:13, and IV:14). Teenaged *AIP* mutants (IV:11, IV:12, IV:17, and IV:18) are considered as unknown. The rest are considered as non-informative. We used a non-parametric approach based on identical-by-descent allele sharing among affected individuals. Calculation of multipoint non-parametric linkage statistics (NPL scores) was carried out using GENEHUNTER beta v. 2.1_R2 (Kruglyak *et al.* 1996, Kruglyak & Lander 1998). NPL scores were then converted to LOD scores (Kong & Cox 1997). Owing to the large number of family members, the data was divided into three separated branches for linkage analysis. The LOD plot was created with the R software package (www.R-project.org).

Results

The clinical features of the studied subjects are summarized in Table 1. Since the subjects are aborigines from a remote area, they do not have accurate birth records and/or do not know their exact age. Therefore, their ages are given as estimates in decades. Three individuals have prominent acromegalic features (protruding lower jaws and brows, and enlarged hands and feet; IV:7, IV:8, and IV:9), while another three individuals have mild acromegalic features (enlarged hands and feet only; II:3, III:6, and III:9). The rest of the members do not exhibit any acromegalic features. There has not been any new incidence of acromegaly reported in this family since the last collection of data, which was 6 years ago.

Table 1 Clinical data of members in the Borneo family with isolated familial somatotropinomas

Subjects	Age at diagnosis ^a	Sex	Height (cm)	Acromegalic features	AIP mutation	Laboratory results	
						GH (mIU/l)	IGF-1 (μg/l)
II:3	90s	M	160	Yes	Yes	<0.5	143.3
III:2	50s	M	163	No	Yes	<0.5	130.0
III:3	50s	M	162	No	Yes	<0.5	205.0
III:4	50s	M	N/D	No	No	N/D	N/D
III:5	60s	M	N/D	No	No	<0.5	174.2
III:6	60s	F	170	Yes	Yes	<0.5	102.6
III:7	60s	F	167	No	Yes	<0.5	163.3
III:9	50s	F	168	Yes	Yes	<0.5	216.2
III:10	50s	F	165	No	Yes	4.1	191.3
III:11	50s	F	162	No	No	<0.5	200.0
III:12	50s	F	163	No	Yes	4.8	113.4
III:13	50s	M	162	No	No	<0.5	224.6
IV:1	20s	F	N/D	No	Yes	<0.5	312.9
IV:2	20s	F	165	No	No	<0.5	84.7
IV:3	20s	F	168	No	Yes	<0.5	151.3
IV:4	20s	F	166	No	No	<0.5	219.0
IV:5	20s	M	171	No	Yes	<0.5	236.1
IV:6	20s	M	N/D	No	No	<0.5	457.8
IV:7	30s	M	175	Yes	Yes	N/D	N/D
IV:8	30s	F	170	Yes	Yes	<0.5	59.8
IV:9	20s	F	182	Yes	Yes	124.2	577.5
IV:10	20s	F	169	No	Yes	1.3	411.1
IV:11	Teens	F	N/D	No	Yes	<0.5	235.4
IV:12	Teens	F	N/D	No	Yes	<0.5	257.0
IV:13	20s	M	165	No	Yes	<0.5	268.7
IV:14	20s	M	167	No	Yes	<0.5	264.0
IV:15	20s	M	N/D	No	No	<0.5	178.4
IV:16	20s	M	N/D	No	No	<0.5	315.1
IV:17	Teens	M	N/D	No	Yes	6.0	328.0
IV:18	Teens	M	N/D	No	Yes	<0.5	515.6

IGF-1, insulin-like growth factor-1; M, male; F, female; N/D, not determined.

^aAge is estimated in decades.

We used sex- and age-dependent reference ranges of IGF-1, which were established in a large group of patients (Brabant *et al.* 2003) and considered IGF-1 levels above +2 SDS as elevated (Schneider *et al.* 2008). With these criteria, all subjects have non-elevated levels of IGF-1, except IV:9. Besides having pronounced acromegalic features and elevated IGF-1 level, IV:9 also had an extremely high level of GH. She refused further diagnostics or treatment and died of hypopituitarism 2 years after her blood was collected.

We found a novel *AIP* frameshift mutation in exon 4 (c.500delC) (p.P167HfsX3) in all members with acromegalic features (II:3, III:6, III:9, IV:7, IV:8, and IV:9) as well as in 15 members without acromegalic phenotypes (III:2, III:3, III:7, III:10, III:12, IV:1, IV:3, IV:5, IV:10, IV:11, IV:12, IV:13, IV:14, IV:17, and IV:18), revealing incomplete penetrance of *AIP* in this IFS family (Fig. 1). The penetrance of *AIP* in this family was 28.6% (6/21). The frameshift mutation

resulted from deletion of a cytosine, which changed from proline to histidine and caused an early stop codon at amino acid 170 (Fig. 2). This germline mutation may prevent the translation of the tetra-tricopeptide repeat (TPR) regions downstream and possibly creates a malfunctioning *AIP* protein.

It is known that somatotrope axis over-activity causes acromegalic features. However, in this study, we could not find strong or significant evidence of GH and/or IGF-1 levels associated with the features. This could be a mild form of acromegaly in this studied pedigree. Since the symptoms of acromegaly could not be clearly defined using the biochemical data in our study, we did not perform the linkage analysis based on biochemical data. Instead, linkage analysis was performed using the acromegalic features data, which could define the disease status according to the age of on-set (> 20 years old). The genome-wide scan results showed the highest LOD score, 2.2, located on

Figure 2 Sequence electropherogram showing the frameshift mutation c.500delC (P167fsX170) in exon 4 of the *AIP* gene of the Borneo IFS family.

19q13.41 (D19S571), which indicated suggestive evidence of linkage. In addition, we detected three weak linkages on chromosomes 3q28 (D3S1580), 8q12.1 (D8S285), and 21q22.13 (D21S1252) with LOD scores of 1.1, 1.8, and 1.4 respectively (Table 2). The whole-genome LOD plots are shown in Fig. 3.

Discussion

Germline mutations in the *AIP* gene were first discovered in PAP patients. The PAP phenotype, with a very low-penetrance susceptibility to somatotropinoma and prolactinoma, did not fit well in any of the known familial pituitary adenoma syndromes (Vierimaa *et al.* 2006). In FIPA, *AIP* mutations occur only in 50% of the IFS families and 15–30% of the entire FIPA cohort (Daly *et al.* 2007, Leontiou *et al.* 2008). In addition, *AIP* mutations are extremely rare in sporadic pituitary adenomas from patients of various countries (Barlier *et al.* 2007, Iwata *et al.* 2007, Raitila *et al.* 2007, Leontiou *et al.* 2008).

In our present study, we identified a novel *AIP* germline mutation, p.P167HfsX3, in a large IFS family from Borneo ($n=31$). The newly identified *AIP*

germline mutation has a deletion of cytosine at nucleotide position 500, resulting in a premature stop codon at amino acid 170. It is similar to many known frameshift germline mutations of *AIP*, which are predicted to result in loss of translation of the TPR domains, which may disrupt the protein–protein interactions, resulting in malfunction of the *AIP* protein (Daly *et al.* 2007, Naves *et al.* 2007).

All members with acromegalic features ($n=6$) have the germline mutation. Interestingly, a large fraction of members ($n=15$) also carry the same mutation without showing any of the acromegalic features. Therefore, these asymptomatic carriers showed evidence of incomplete penetrance of *AIP* in IFS. Penetrance is time-dependent and in this particular family, the penetrance was 28.6% at the time of sample collection. The low penetrance of *AIP* in PAP and FIPA families also suggests the existence of additional genes (Vierimaa *et al.* 2006, Daly *et al.* 2007, Naves *et al.* 2007). This indicates the mutation alone may not lead to the development of the symptoms of the disease. There is a high possibility that additional factors, either genetic or environmental, are assisting in or preventing the onset of IFS.

By using the established sex- and age-dependent reference ranges of IGF-1, it is known that healthy subjects can also have hormone levels outside of the normal range. Elevated IGF-1 levels are therefore not necessarily indicative of pathological acromegaly. Even with elevated IGF-1, it is not clear whether these patients will develop the clinical symptoms of acromegaly (Schneider *et al.* 2008). Therefore, IGF-1 values do not give a clear-cut clinical phenotype and we did not use it for linkage analysis. To identify the modifier genes/loci, the clinical phenotype has to be clearly defined and in some cases, it can be difficult to do so (Genin *et al.* 2008). In our study, subjects > 20 years old should have their acromegalic features defined since IFS has a much younger age at onset compared with sporadic acromegaly (Soares & Frohman 2004, Beckers & Daly 2007). Thus, *AIP* mutants with the presence or absence of acromegaly feature were used in the linkage analysis to identify the modifier genes related to this phenotype. Although no

Table 2 Modifier regions of acromegaly features for isolated familial somatotropinoma

Chromosomal region	Region size (cM)	Flanking microsatellites	Microsatellite ^a	LOD
3q26.31–q28	18.2	D3S1565–D3S1601	D3S1580	1.1
8q12–q21.3	58.5	D8S505–D8S270	D8S285	1.8
19q12–q13.43	25.1	D19S414–D19S210	D19S571	2.2
21q22.11–q22.3	10.5	D21S263–D21S266	D21S1252	1.4

^aMicrosatellite with the highest LOD score in each modifier region.

Figure 3 Multipoint nonparametric scores of the genome-wide scan for the Borneo IFS family. The chromosome number is plotted on the upper X-axis and the LOD score on the Y-axis. Suggestive linkage (LOD=2.2) is shown in 19q13.41.

significant evidence of linkage was found (LOD > 3) in the genome-wide linkage analysis, one suggestive linkage at chromosome 19q13.41 with a LOD score of 2.2 was detected. The definition of suggestive linkage (LOD > 1.9) is based on [Lander & Kruglyak \(1995\)](#). We also detected three chromosomes, 3q28, 8q12.1, and 21q22.13, with weak linkage to the phenotype. These regions are different from previously known putative loci for somatotropinoma susceptibility found on chromosomes 2p16–12 and 13q14 ([Gadelha et al. 2000](#), [Donangelo et al. 2005](#)). However, the genome-wide scan results in PAP family members reported by [Vierimaa et al. \(2006\)](#) detected a significant LOD score of 3.08 at chromosome 8q. It is the same chromosomal region where our data showed a weak evidence of linkage (LOD = 1.8 at 8q12.1) and may merit further investigation. At present, we are fine mapping all candidate regions in order to identify modifier genes. Since the development of acromegalic features is insidious, this hormonal disorder is often not recognized immediately. Therefore, it is important to identify genes related to acromegaly, so that patients can be treated promptly to reduce the risk of complications. Moreover, identifying the modifier genes may lead to better understanding of the variety of clinical phenotypes of *AIP* mutants.

In summary, we have identified a novel *AIP* germline mutation in a large IFS family, although complete endocrine diagnosis and data could not be collected due to logistical and cultural reasons. We also confirmed that *AIP* is an incomplete-penetrance hereditary gene conferring a predisposition to IFS. More importantly, this is the first genome-wide linkage

analysis to detect modifier loci for acromegalic features in IFS. A suggestive linkage on chromosome 19q13.41 and three weak linkages on chromosomes 3q28, 8q12.1, and 21q22.13 were identified. We conclude that genes located at those regions, especially near 19q13.41, may modify the severity of acromegalic features in IFS patients.

Declaration of interest

The authors declare no conflicts of interest that could perceive as prejudicing the impartiality of the research reported.

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Author contribution statement

S K Khoo and B T Teh designed research; R Pendek, J Menon and S-P Chan contributed samples; S K Khoo, D C Luccio-Camelo, T L Newton and A Massie performed research; S K Khoo, R Nickolov, D Cameron, and D Petillo analyzed data; and S K Khoo wrote the paper.

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