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ORIGINAL ARTICLE

Novel polymorphisms and lack of mutations in the *ACD* gene in patients with ACTH resistance syndromes

Catherine E. Keegan*, Janna E. Hutz*, Andrea S. Krause*, Katrin Koehler†, Louise A. Metherell‡, Sosipatros Boikos§, Sotirios Stergiopoulos§, Adrian J. L. Clark‡, Constantine A. Stratakis§, Angela Huebner† and Gary D. Hammer¶**

**Department of Pediatrics, Division of Genetics, University of Michigan, Ann Arbor MI, USA, †Children's Hospital, Technical University Dresden, Dresden, Germany, ‡Department of Endocrinology, William Harvey Research Institute, Barts & the London School of Medicine and Dentistry, Queen Mary University of London, West Smithfield, London, UK, §Section on Endocrinology and Genetics, Developmental Endocrinology Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda MD, USA, ¶Department of Internal Medicine, Division of Metabolism, Endocrinology and Diabetes and **Department of Molecular and Integrative Physiology, University of Michigan, Ann Arbor MI, USA*

Summary

Objective ACTH resistance is a feature of several human syndromes with known genetic causes, including familial glucocorticoid deficiency (types 1 and 2) and triple A syndrome. However, many patients with ACTH resistance lack an identifiable genetic aetiology. The human homolog of the *Acd* gene, mutated in a mouse model of adrenal insufficiency, was sequenced in 25 patients with a clinical diagnosis of familial glucocorticoid deficiency or triple A syndrome.

Design A 3·4 kilobase genomic fragment containing the entire *ACD* gene was analysed for mutations in all 25 patients.

Setting Samples were obtained by three investigators from different institutions.

Patients The primary cohort consisted of 25 unrelated patients, primarily of European or Middle Eastern descent, with a clinical diagnosis of either familial glucocorticoid deficiency (FGD) or triple A syndrome. Patients lacked mutations in other genes known to cause ACTH resistance, including *AAAS* for patients diagnosed with triple A syndrome and *MC2R* and *MRAP* for patients diagnosed with familial glucocorticoid deficiency. Thirty-five additional patients with adrenal disease phenotypes were added to form an expanded cohort of 60 patients.

Measurements Identification of DNA sequence changes in the *ACD* gene in the primary cohort and analysis of putative *ACD* haplotypes in the expanded cohort.

Results No disease-causing mutations were found, but several novel single nucleotide polymorphisms (SNPs) and two putative haplotypes were identified. The overall frequency of SNPs in *ACD* is low compared to other gene families.

Conclusions No mutations were identified in *ACD* in this collection of patients with ACTH resistance phenotypes. However, the newly identified SNPs in *ACD* should be more closely examined for possible links to disease.

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Correspondence: Catherine E. Keegan, Department of Pediatrics, Division of Genetics, University of Michigan Medical School, 3520 MSRB I, Box 0652, 1150 W. Medical Center Dr., Ann Arbor, MI 48109–0652, USA. Tel.: +1 (734) 647 8237; Fax: + 1 (734) 763 9512; E-mail: keeganc@med.umich.edu

Introduction

The adrenocortical dysplasia (*acd*) mutation is a spontaneous autosomal recessive mouse mutant originally described in 1994 as a model for congenital adrenal insufficiency.[1] The phenotypic characteristics of *acd* mutant animals include growth deficiency, skin hyperpigmentation, adrenal hypofunction, and reduced survival. Adrenal glands from *acd* mutant mice are small relative to body weight, and they display unique histological characteristics that include enlarged cortical cells with enlarged nuclei, absence of the adrenocortical X-zone, and lack of a distinct boundary between cortical and medullary cells. Previous hormonal studies revealed low basal corticosterone levels in mutant female mice and elevated ACTH levels in both sexes, with a range of 52–2600 pg/ml in *acd* mutants compared to 15–98 pg/ml in wildtype littermates.[1] While *acd* mutant adrenals did respond to ACTH stimulation, adrenals from wildtype littermates secreted significantly more corticosterone in response to ACTH. Aldosterone levels at baseline and in response to ACTH stimulation did not differ significantly between *acd* mutants and wildtype littermates.[1] Furthermore, serum electrolytes were normal, suggesting normal mineralocorticoid synthesis.[1] Based on these findings, the adrenocortical defect in *acd* mutant animals was thought to resemble isolated glucocorticoid deficiency due to insufficient adrenocortical cell mass.[1] The recent identification of the mouse *acd* mutation has enabled further studies of its human ortholog, *ACD*, which encodes a novel telomeric regulator thought to tether the single-strand

DNA-binding protein POT-1 (protection of telomeres-1) to the TERF1 (telomere repeat binding factor 1) telomere protein complex.[2–4]

Primary adrenal insufficiency in humans can be caused by a defect in adrenocortical growth and/or function. Congenital defects in adrenocortical function are most often caused by deficiencies in steroidogenic enzymes such as 21-hydroxylase (*CYP21*, OMIM #201910), leading to glucocorticoid +/– mineralocorticoid deficiency and compensatory ACTH-driven hyperplasia of the adrenal glands. Another rare cause of adrenal insufficiency in male subjects is X-linked adrenoleucodystrophy (OMIM #300100), caused by mutations in an ATP-binding cassette transporter protein (*ABCD1*), leading to defects in peroxisomal function and accumulation of very long chain fatty acids, ultimately resulting in destruction of adrenocortical cells.

Defects in adrenal growth are collectively referred to as adrenal hypoplasia congenita (AHC, OMIM #300200). There are two major histological forms of AHC: the cytomegalic form and the miniature adult form.[5] The cytomegalic form typically presents in early infancy with adrenal insufficiency, skin hyperpigmentation, and ultimately delayed puberty due to hypogonadotropic hypogonadism. This form is characterized histologically by absence of the definitive cortex and persistence of the foetal zone, and is caused primarily by mutations in the *DAX-1* gene.[5] The miniature adult form is thought to be autosomal recessive and is characterized histologically by a thin rim of relatively normal definitive cortex, and an absent foetal zone.[5] While mutations in the orphan nuclear receptor SF-1 have been identified as a cause of adrenal hypoplasia and XY sex reversal in some patients,[6] the histological characteristics of the adrenal glands in these individuals have not been examined.

Isolated glucocorticoid deficiency, the presumed adrenocortical defect in *acd* mice, also causes adrenal insufficiency in several human syndromes. Patients with these disorders typically share some characteristics of the *acd* mutant phenotype, including low cortisol levels, significantly elevated ACTH levels, hyperpigmentation, and a poor response to exogenous ACTH, suggestive of ACTH resistance.[7] These individuals often present in the newborn period or during childhood with hypoglycaemia, frequently leading to seizures.[7] Salt wasting, typical of mineralocorticoid deficiency, is generally not observed in these patients but has been reported.[8] As predicted by this phenotype, homozygous mutations have been found in the ACTH receptor gene (*MC2R*) in some patients with these characteristics (FGD1, OMIM #202200).[7] However, mutations in *MC2R* only accounted for 25% of the patients with this phenotype, suggesting genetic heterogeneity. This group of patients has been referred to collectively as familial glucocorticoid deficiency type 2 (FGD2, OMIM #607398). Recently, homozygous mutations have been identified in the *MRAP* gene, which encodes a novel transmembrane protein that interacts with MC2R, in a subset of patients with FGD2.[9] However, many patients with isolated glucocorticoid deficiency have no identified mutation in either gene.

ACTH resistance is also a feature of triple A syndrome (OMIM #231550), a recessive disorder characterized by the triad of adrenal insufficiency, alacrima, and achalasia. Phenotypic heterogeneity is a well-described feature of triple A syndrome.[10,11] Adrenal insufficiency is not universally present; it can occur later in the course of the disease or not at all.[10,11] Furthermore, triple A syndrome patients with adrenal insufficiency can have isolated glucocorticoid deficiency or both glucocorticoid and mineralocorticoid deficiency.[11] Additional features that have been reported in triple A syndrome patients include abnormalities of the central, peripheral, and autonomic nervous system, as well as dermatological abnormalities.[7,11] The gene responsible for triple A syndrome (*AAAS*) has been identified to encode a novel WD repeat protein.[12,13] This protein product, named ALADIN, localizes to the nuclear pore complex, and it appears to be mislocalized in patients with triple A syndrome.[14,15] The exact mechanism by which deficiency in ALADIN causes adrenal insufficiency has not been determined. Nevertheless, triple A syndrome is considered an ACTH resistance syndrome because of the abnormal response to ACTH in these patients.[7]

To date, multiple inherited and sporadic cases have been described with features similar to FGD or triple A syndrome that lack mutations in *MC2R*, *MRAP* or *AAAS*, suggesting that mutations in additional genes may cause these phenotypes. Because of the phenotypic similarities between these patients and the hormonal disturbances observed in *acd* mice, we sequenced the *ACD* gene for mutations in 25 patients with a clinical diagnosis of FGD or triple A syndrome.

Materials and methods

Experimental subjects

For the primary cohort, 25 DNA samples from unrelated patients with clinical features of FGD or triple A syndrome were obtained from collaborators. Patients were referred by their paediatric endocrinologist, who had made the diagnosis of primary adrenal insufficiency, or another paediatric subspecialist, who had suspected triple A syndrome based on cardinal features of alacrima and/or achalasia. Thirteen were sporadic cases and 12 were familial. Clinical characteristics are reported in Table 1; genetic studies and family histories are reported in Table 2. For sporadic patients from non-consanguineous families, the diagnosis of FGD was made based on the presence of low cortisol levels, elevated ACTH levels, and lack of other identifiable causes of adrenal insufficiency, such as adrenal autoantibodies or adrenoleucodystrophy. Low aldosterone levels were present in two patients with a clinical diagnosis of FGD (FGD4, FGD11 in Table 1). In one patient (FGD4), aldosterone and renin levels normalized after initial treatment for hypoglycaemic shock. Patient FGD11 was considered to represent an atypical case of FGD.[8] Clinical diagnosis of triple A syndrome was based on the presence of one or more cardinal features (adrenal insufficiency, alacrima, or achalasia) in association with neurological symptoms such as peripheral neuropathy, muscle weakness, mental retardation, or dysautonomia. Two patients with a clinical diagnosis of triple A syndrome did not have evidence of adrenal insufficiency (TA2, TA3 in Table 1). For the expanded cohort, 35 additional DNA samples from patients with adrenal disease phenotypes were obtained from collaborators. These 35 individuals included the 15 patients with congenital adrenal hypoplasia and IMAGe association reported previously by our group;[16] 10 additional patients with FGD who were found to have *MRAP* mutations (Tables 1, 2), and seven additional patients with a clinical diagnosis of triple A syndrome in whom only the region of *ACD* containing the previously identified single nucleotide polymorphisms (SNPs) was sequenced; one patient

Table 1. Clinical phenotypes of patients in primary and expanded cohorts

				Hormone studies‡					Other symptoms	
Patient	Clinical diagnosis*	Gender	Age at diagnosis†	Cortisol	ACTH	Response to ACTH	Aldosterone	Renin	Hyper-pigmentation	Hypo-glycaemia
Primary cohort ($n = 25$)										
TA1	AAAS	M	–	Low	High	–	–	–	–	–
TA2	AAAS	M	5 years	–	–	–	–	–	–	–
TA3	AAAS	F	–	–	–	–	–	–	–	–
TA4	AAAS	F	–	Low	High	–	–	–	–	–
TA5	AAAS	M	–	44	686	No	Low	–	–	No
TA6	AAAS	F	5·2 years	204	High	No	Low	High	–	–
TA7	AAAS	F	4 years	66	> 275	No	nl	nl	–	–
FGD1	FGD	F	5 months	Low	High	–	–	–	–	–
FGD2	FGD	M	–	Low	High	–	–	–	–	–
FGD3	FGD	F	4·5 years	< 25	461	No	nl		Yes	Yes
FGD4	FGD	M	5 years	< 25	27·5	No	Low	High	Yes	Yes
FGD5	FGD	F	–	Low	High	–	–	–	–	–
FGD6	FGD	M	–	Low	High	–	–	–	–	–
FGD7	FGD	F	2 years	< 50	High	–	nl	nl	Yes	–
FGD8	FGD	F	5 years	Low	High	–	–	–	–	–
FGD9	FGD	M	3 years	ND	High	–	nl	–	–	–
FGD10	FGD	M	–	Low	High	–	–	–	–	–
FGD11	FGD	F	–	Low	High	–	Low	–	–	–
FGD12	FGD	M	4 years	ND	High	No	nl	–	–	–
FGD13	FGD	M	6 months	11·1	479	–	nl	–	Yes	–
FGD14	FGD	M	–	41	330	No	nl	nl	Yes	Yes
FGD15	FGD	M	1 year	ND	310	No	–	–	–	–
FGD16	FGD	F	3 years	< 33	High	–	nl	–	Yes	Yes
FGD17	FGD	F	12 years	< 10	1459	–	nl	–	–	–
FGD18	FGD	M	4 months	Low	High	–	nl	–	–	–
Expanded cohort ($n = 35$)§										
EXP1	FGD	F	Birth	ND	High	No	nl	–	Yes	Yes
EXP2	FGD	M	–	200	990	–	nl	nl	Yes	Yes
EXP3	FGD	M	10 weeks	ND	153	No	nl	nl	Yes	–
EXP4	FGD	F	–	Low	High	–	–	–	–	–
EXP5	FGD	F	Birth	71	110	No	–	–	–	–
EXP6	FGD	M	1·5 months	ND	High	No	nl	–	Yes	Yes
EXP7	FGD	M	–	Low	High	–	–	–	–	–
EXP8	FGD	M	3 months	Low	High	–	–	–	–	–
EXP9	FGD	F	Birth	Low	> 330	–	nl	–	Yes	Yes
EXP10	FGD	M	11 months	50	338	No	nl	nl	No	Yes
EXP11	FGD	M	Birth	< 33	> 48	No	High	High	Yes	Yes
EXP12	AI/HH	M	–	Low	High	–	Low	Low	–	–
EXP13	AI	F	–	Low	High	–	–	–	–	–

*Parameters for clinical diagnosis of AAAS and FGD as stated in Materials and Methods. AI, adrenal insufficiency; HH, hypogonadotropic hypogonadism. †Specific age given when known. All patients presented during infancy or childhood. ‡Hormone levels given where known. Cortisol levels presented in nmol/l (normal morning cortisol, 193–607), ACTH levels presented in pmol/l (normal = 1·1–11·4). For aldosterone and renin, normal (nl) is reported either based on laboratory value or on lack of symptoms in patients on glucocorticoid supplementation only. §Expanded cohort also includes 15 patients with congenital adrenal hypoplasia and IMAGe association previously reported,[16] and seven additional patients with a clinical diagnosis of triple A syndrome in whom only a portion of the *ACD* gene was sequenced and for whom limited clinical data are available. ND, not detectable; nl, normal; –, not available.

with adrenal insufficiency who was found to have a mutation in *AAAS* (Tables 1,2); one previously unreported patient with adrenal insufficiency and hypogonadotropic hypogonadism (Tables 1, 2); and one previously unreported patient with adrenal hypoplasia (Tables 1, 2). As a control population, SNP data from the 90 healthy individuals (180 chromosomes) comprising the Centre d'Etude Polymorphisme Humain (CEPH) sample from the HapMap project were used.[17] Informed consent was obtained from all patients who participated in this study in accordance with the established standards of the referring institutions.

Table 2. Genetic studies and family history of patients in primary and expanded cohorts

Patient	Genetic studies			Ethnicity/ country of origin	Familial or sporadic	Consanguinity
	AAAS	MRAP	ACTHR			
Primary cohort ($n = 25$)						
TA1	–	ns	ns	Tunisia	Familial	Yes
TA2	–	ns	ns	(Arab)	Familial	Yes
TA3	–	ns	ns	Germany	Familial	Yes
TA4	–	ns	ns	Turkey	Sporadic	No
TA5	–	ns	ns	Japan	Sporadic	No
TA6	–	ns	ns	Germany	Sporadic	No
TA7	–	ns	–	Israel (Arab)	Sporadic	No
FGD1	–	–	–	South Africa	Sporadic	No
FGD2	–	–	–	Netherlands	Sporadic	No
FGD3	–	–	–	Turkey	Familial	Yes
FGD4	–	–	–	Turkey	Familial	Yes
FGD5	–	–	–	Netherlands	Familial	No
FGD6	–	–	–	Netherlands	Sporadic	No
FGD7	–	–	–	UK	Familial	No
FGD8	–	–	–	USA/Arab	Familial	Yes
FGD9	–	–	–	Netherlands	Sporadic	Yes
FGD10	–	–	–	Germany/Turkish	Sporadic	No
FGD11	–	–	–	Germany/Turkish	Familial	No
FGD12	–	–	–	Germany	Sporadic	No
FGD13	–	–	–	Bulgarian (Gypsy)	Familial	No
FGD14	–	–	–	Turkey	Sporadic	Yes
FGD15	–	–	–	USA	Sporadic	No
FGD16	–	–	–	USA	Familial	No
FGD17	ns	–	–	UK/Pakistani	Familial	Yes
FGD18	ns	–	–	UK/Pakistani	Familial	Yes
Expanded cohort ($n = 35$)*						
EXP1	–	+	–	Netherlands	Sporadic	No
EXP2	–	+	–	UK	Sporadic	No
EXP3	–	+	–	Pakistani/UK	Sporadic	Yes
EXP4	–	+	–	UK	Sporadic	No
EXP5	–	+	–	India	Familial	No
EXP6	–	+	–	Belgium	Sporadic	No
EXP7	–	+	–	Netherlands	Sporadic	No
EXP8	–	+	–	Turkey	Familial	No
EXP9	–	+	–	Anglo-Saxon/US	Sporadic	No
EXP10	+	–	–	USA	Sporadic	No
EXP11	–	+	–	Israel (Bedouin)	Familial	Yes
EXP12	ns	ns	ns	Druze	Sporadic	Yes
EXP13	ns	ns	–	USA	Sporadic	No

*Expanded cohort also includes 15 patients with congenital adrenal hypoplasia and IMAGe association previously reported,[16] and seven additional patients with a clinical diagnosis of triple A syndrome in whom only a portion of the *ACD* gene was sequenced and for whom limited clinical data are available. –, no mutations found or ruled out by genotyping and linkage analysis (for consanguineous cases); +, disease-causing mutation identified; ns, not sequenced.

DNA sequencing

Genomic DNA was amplified by polymerase chain reaction (PCR) using six primer sets spanning 3442 basepairs (bp) and all 12 exons of the *ACD* gene.[16] PCR products were separated by electrophoresis and purified with the QIAGEN Gel Extraction Kit (Qiagen Inc., Valencia, CA). Samples were sequenced in both directions by the University of Michigan DNA Sequencing Core, and sequence data were analysed using Sequencher software, version 4·1·4 (Gene Codes Corp., Ann Arbor, MI). All novel SNPs were confirmed in at least two independent PCR reactions. Genotype data for rs6979 and rs14920 were obtained from http://www.hapmap.org and National Center for Biotechnology Information (NCBI)'s Single Nucleotide Polymorphism database (dbSNP).

Table 3. Characteristics of *ACD* polymorphisms identified in the primary and expanded patient cohorts

SNP	Novel or dbSNP* no.	Major allele	Minor allele	Minor allele frequency (no. of chromosomes)		Location	
				Primary cohort ($n = 50$)	Expanded cohort ($n = 120$)	Structural	Nucleotide†
1	+	G	A	1	1	Exon 1	88
2	+	C	T	1	1	Exon 10	1956
3	rs6979	T	C	24	55‡	Exon 11	2522
4	+	C	A	4	13	Intron 11	2545
5	rs14920	G	T	3	6	Exon 12	2675
6	+	T	A	4	10	3′ UTR§	2713
7	+	G	A	0	1	Upstream	–399
8	+	C	T	0	2‡	Exon 10	2112
9	+	G	A	0	1	Exon 10	2273
10	+	G	A	0	1	3′ UTR§	2685

*NCBI's dbSNP; †numbering of *ACD* genomic sequence relative to GenBank #AY971883; ‡includes one pair of siblings; §untranslated region.

Computational analysis

Haplotype analysis and calculations (r^2 and D') were performed using Haploview software, version 3·11 (http://www.broad.mit.edu/mpg/haploview/index.php). Exonic splicing enhancer (ESE) analysis was performed using RESCUE-ESE software (http://genes.mit.edu/burgelab/rescue-ese/).

Results

Sequence analysis of all 25 unrelated patients in the primary cohort revealed polymorphisms in six locations in the *ACD* gene (Table 3), two of which were previously represented in dbSNP (SNP3 and SNP5) and two of which were previously identified by our group (SNP4 and SNP6).[16] Of these six SNPs, two were identified in non-coding regions and four were identified in exons, consisting of two synonymous SNPs (SNP2 and SNP5) and two nonsynonymous coding SNPs. Of these, the following relatively conservative substitutions are produced: SNP1 Val→Ile and SNP3 Val→Ala. None of the non-synonymous SNPs was in *ACD*'s predicted POT-1 recruitment domain[3] but SNP3 was in *ACD*'s predicted TIN2 (TERF1-interacting nuclear protein 2)-interacting domain.[3] Four of the six identified SNPs were present on more than one chromosome; the remaining two were singletons (Table 3). Using only nonsingleton SNPs, the frequency of SNPs in *ACD* was approximately 1·16 SNPs per kilobase (kb). The distribution of SNPs appeared to be bimodal with respect to the structure of the *ACD* gene; there was an 1868 bp gap extending from exon 1 to exon 10 in which no polymorphisms were found.

SNP3 had a minor allele frequency (MAF) of 0·48 in our patient sample (Table 3), which is not significantly different from the MAF of 0·45 for this allele in the Centre d'Etude Polymorphisme Humain (CEPH) sample used by the HapMap project.[17] The MAFs of SNP4 and SNP6 were both 0·08 (Table 3), corresponding to their presence on four chromosomes in this sample. Interestingly, the same four patients who were heterozygous for SNP4 were also heterozygous for SNP6 ($r^2 = 1$) and also had at least one minor allele of SNP3. Additionally, SNP5 seemed to be linked with SNP3; all three patients with the minor allele for SNP5 also had at least one minor allele of SNP3. Genotypes from the CEPH population confirm this and also show that in CEPH families, SNP3 and SNP5 are in strong linkage disequilibrium (D' = 1). These putative haplotypes involving SNP4-SNP6-SNP3 and SNP5-SNP3 could not be verified in our sample because, in the absence of parental DNA, phase could not be determined. It is for this reason that we label these haplotypes as putative. Likewise, linkage disequilibrium could not be determined because the patients were from varied ethnic backgrounds. However, it is interesting to note that patients who had only one of the two putative haplotypes had only one minor allele of SNP3, while patients who had both were homozygous for SNP3's minor allele. The remaining SNPs identified in the original sample (SNP1, SNP2) were both previously unidentified polymorphisms.

To further explore these six polymorphisms an expanded cohort of 60 individuals, comprising the 25 original patients and 35 additional subjects with adrenal disease phenotypes, was studied. In an attempt to confirm the data regarding the putative haplotypes in the primary cohort, similar analyses were performed in the expanded cohort. Four additional SNPs were identified, of which three were novel singleton SNPs and one was previously identified by our group (SNP7-SNP10, Table 3). As SNP8 is only present in this sample in a sibling pair, it must also be considered a singleton. As a result, the number of nonsingleton SNPs per kilobase remained the same in the expanded cohort at 1·16 SNPs/kb. None of these was predicted to cause disease. The correlation between SNP4 and SNP6 was reduced in this sample ($r^2 = 0{\cdot}74$), but their individual correlations with SNP3 remained consistent. The SNP5-SNP3 relationship in the larger sample was also consistent with the primary cohort.

To investigate the possibility of a disease-causing effect, the major and minor alleles of all 10 SNPs and their surrounding sequences

were analysed for the presence of transcription factor binding sites. No significant transcription factor binding sites were abolished when any of the minor alleles were substituted. Additionally, none of the SNPs was in conserved or cryptic splice sites or known exonic splicing enhancer (ESE) sequences, nor were they predicted to create cryptic splice sites.

Discussion

The *acd* mutant mouse phenotype is characterized by adrenal hypoplasia, with striking changes in the histological characteristics of the adrenal cortex as well as hormonal evidence of adrenal insufficiency consisting of gluocorticoid, but not mineralocorticoid, deficiency. There is considerable overlap between the *acd* adrenal phenotype and several different human syndromes featuring adrenal insufficiency, making it difficult to immediately identify a corresponding human phenotype with mutations in *ACD*. We sought to examine for *ACD* mutations in patients with adrenal disorders because of the unique adrenal phenotype observed in *acd* mice. We previously reported an analysis of *ACD* mutations in patients with congenital adrenal hypoplasia and IMAGe association because this population of patients shares some features with *acd* mice, including adrenal hypoplasia with a cytomegalic histological appearance and the presence of skeletal defects.[16] Given the lack of mutations in this group of patients and the fact that the hormonal phenotype of *acd* mice also resembles aspects of FGD and triple A syndrome, we reasoned that these patients would be good candidates to examine for *ACD* mutations.

Twenty-five unrelated patients were sequenced for potential disease-causing mutations in a 3442 bp region spanning *ACD*, the human homolog of the gene that is mutated in the *acd* mouse model of adrenal insufficiency. Six polymorphisms were identified in this primary cohort, two of which are novel. Two SNPs were found on four chromosomes from unrelated individuals (Table 3), strongly suggesting that these polymorphisms are indeed SNPs, not rare isolated mutations. Three additional novel SNPs and one previously identified SNP were found when the cohort was expanded to include patients with other adrenal diseases.

Because it is the only nonsynonymous SNP identified in this sample and because it is in ACD's predicted TIN2 recruitment domain, SNP3 seems the most likely candidate for a disease-causing polymorphism. However, its high minor allele frequency in the 90 healthy CEPH samples does not support a disease-causing role for this SNP. It is unlikely that the other novel SNPs that we identified are responsible for causing adrenal insufficiency, given their location in noncoding, nonconserved regions. Although many diseases are caused by mutations in regulatory noncoding sequences, these particular polymorphisms are not predicted to disrupt known transcription factor binding sites or normal splicing patterns. Additionally, the fact that patients with adrenal disease from consanguineous families were never homozygous for the minor allele of any SNP but SNP3 indicates that these SNPs are not disease-causing.

The paucity of nonsingleton SNPs in the region of *ACD* suggests that this gene is under constraint, probably indicative of the importance of ACD's role in acting at the telomere. The *acd* mouse mutation, which may be a hypomorphic allele, is embryonic lethal on the DW/J and C57BL/6 genetic backgrounds.[2] It stands to reason then, that even minor mutations in *ACD* could result in early lethality, making them impossible to find in most patient samples. Strong support for the hypothesis that *ACD* is under evolutionary constraint comes from a similar study of genes encoding other telomeric proteins, including *POT1* and *TIN2*, two of ACD's interacting partners.[18] The SNP frequency in the seven genes sequenced in that study (*TERT*, *POT1*, *TERF1*, *TERF2*, *TNKS*, *TERF2IP*, *TINF2*) was found to be significantly lower than frequencies of SNPs in other gene families. *ACD*'s SNP frequency of 1·16 SNPs per kb is within the interval of SNP frequencies previously described for the other telomeric genes ($1{\cdot}4 \pm 0{\cdot}3$ SNPs per kb).[18]

The possible linkage disequilibrium between SNP4 and SNP6 is intriguing, given the fact that these are newly identified SNPs in unrelated families from varying ethnic backgrounds and with varying adrenal phenotypes. However, confirmation of linkage disequilibrium would require sequencing a cohort of unaffected individuals for SNP4 and SNP6 to determine whether this putative haplotype exists and whether it is associated with adrenal disease.

To date, there are no known human patients with *ACD* mutations. Based on the results of the current study and our previous work[16] it is unlikely that mutations in *ACD* are a major cause of congenital adrenal hypoplasia, IMAGe syndrome, FGD, and triple A syndrome, although we cannot exclude the possibility of an *ACD* mutation in rare patients with these phenotypes.

Although one hallmark of the *acd* mouse mutation is adrenal insufficiency, there is still much work to be done in order to elucidate the link between telomere biology and adrenal development and function. Once the molecular links between these areas are identified, the corresponding genes may be sequenced in patients with adrenal insufficiency. Any discovered mutations would greatly contribute to the understanding of adrenal function and the development of adrenal disease.

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