Clinical and biochemical characteristics and bone mineral density of homozygous, compound heterozygous and heterozygous carriers of three novel *IGFALS* mutations

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Abstract

Objective: Acid-labile subunit (ALS) deficiency (ACLSD), caused by homozygous or compound heterozygous *IGFALS* mutations, is associated with moderate short stature, delayed puberty, low serum IGF-I and ALS and extremely low serum IGFBP-3. Its effect on birth weight, head circumference, bone mineral density (BMD), serum IGF-II and IGFBP-2 is uncertain, as well as the phenotype of heterozygous carriers of *IGFALS* mutations (partial ACLSD).

Design: From all available members of five Turkish families, carrying three mutations in exon 2 of *IGFALS* (c.1462G > A, p.Asp488Asn (families A, B, E); c.251A > G, p.Asn84Ser (families C and E) and c.1477del, p.Arg493fs (family D)), clinical, laboratory and BMD data were collected.

Methods: Auxological and biochemical findings were expressed as SDS for age and gender. Ternary complex formation in serum was investigated by size-exclusion chromatography. BMD using DXA bone densitometry was adjusted for height and age (Ha-BMD z-score).

Results: In ACLSD ($n = 24$), mean ± s.d. height SDS (−2.7 ± 1.2), head circumference SDS (−2.3 ± 0.5) and body mass index (BMI) (−0.6 ± 1.0 SDS) were lower than those in partial ACLSD ($n = 26$, $P \leq 0.01$) and birth weight SDS ($n = 7$) tended to be lower (−2.2 ± 1.1 vs −0.6 ± 0.3 in partial ACLSD ($P = 0.07$)). Serum IGF-I was −3.7 ± 1.4 vs −1.0 ± 1.0, IGF-II: −5.6 ± 0.7 vs −1.3 ± 0.7, ALS: <−4.4 ± 1.2 vs −2.1 ± 0.9 and IGFBP-3: −9.0 ± 1.9 vs −1.6 ± 0.8 SDS respectively ($P < 0.001$). Ha-BMD z-score was similar and normal in both groups.

Conclusions: To the known phenotype of ACLSD (i.e. short stature, reduced serum levels of IGF-I and ALS, extremely low serum IGFBP-3 and disturbed ternary complex formation), we add reduced birth weight, head circumference and serum IGF-II.

Introduction

Insulin-like growth factors (IGFs) exert a broad range of biological actions including mitogenic and anti-apoptotic effects, which necessitates adequate regulation to ensure normal growth and development. The availability of biologically active IGFs to the tissue compartments is, at least in part, regulated by six different IGF-binding proteins (IGFBPs), designated as IGFBP-1 to IGFBP-6 that bind IGFs with high affinities. Normally, IGFBP-3 is the

most abundant IGFBP in the circulation. Another member of the IGF system is the acid-labile subunit (ALS). ALS is an 85 kDa glycoprotein that forms a relatively large ternary complex, 150 kDa, with IGFs and IGFBP-3 (or IGFBP-5) in the circulation and has a major role in its stabilization (1, 2, 3, 4). ALS has no affinity for free IGFs and only binds to binary complexes of IGFs/IGFBP-3 or -5 (3). Under normal circumstances, most of the IGFs are sequestered in these ternary complexes that cannot easily pass the capillary membranes. As a consequence, the half-life of IGFs in the circulation is extended to more than 12h. In contrast, unbound IGFs and binary IGF–IGFBP complexes are cleared much more rapidly from the circulation, i.e. with half-lives of only ~12 and ~25 min respectively (1, 4, 5). ALS is encoded by *IGFALS*, located on chromosome 16p13.3, and is secreted by the liver (2).

Acid-labile subunit deficiency (ACLSD) (OMIM #615961; #I.B.4d in the International Classification of Pediatric Endocrine Diagnoses (www.icped.org)), caused by homozygous or compound heterozygous mutations in *IGFALS*, leads to the failure of ternary complex formation and increased clearance of IGF-I, IGF-II and IGFBP-3 from the circulation (4). *IGFALS* consists of only 2 exons, with exon 2 encoding the mature ALS protein. Until November 2016, nineteen unique inactivating mutations in *IGFALS* have been described in 25 patients (all in exon 2) (4, 6, 7, 8, 9).

ACLSD is characterized by moderate short stature, low serum levels of IGF-1 and ALS and extremely low levels of IGFBP-3. In contrast, normal basal and stimulated growth hormone (GH) concentrations are found (4). GH treatment trials in ACLSD showed a poor response in terms of growth and serum IGF-1 and IGFBP-3 levels (2, 10, 11).

In our previous study on a Kurdish family, three siblings with an inactivating homozygous *IGFALS* mutation showed head circumferences (HCs) that were lower than those of heterozygous and wild-type (WT) relatives (12). A recent case report also reported microcephaly in a 14-year-old girl (8), and in 4 other cases, average HC was –2.2 SDS (range: 4.6–0.1) (9). Anecdotal reports have suggested that the average birth size in cases with ACLSD may be decreased (3, 6, 9, 13, 14, 15, 16), but studies on large families with sufficient heterozygous and WT controls are lacking. In approximately 50% of males carrying homozygous *IGFALS* mutations, delayed puberty has been noted (2, 3, 4, 6, 7, 10, 14, 17) and variable data on insulin insensitivity and reduced bone mineral density (BMD) have been reported (2, 3, 4, 6, 9, 10, 11, 12, 14, 18). The effects on body mass index (BMI)

and serum concentrations of IGF-II and IGFBP-2 have not been documented.

In a Kurdish family living in the Netherlands, we showed that heterozygous carriers of an *IGFALS* mutation are approximately 1 s.d. shorter than non-carriers (12), and this finding was confirmed in an international survey and other reports (19, 20, 21). However, because of the limited number of affected individuals within each family included in these studies, the statistical validity of the effect of partial ACLSD on the various clinical and laboratory parameters is still weak. Still, the available data indicate that two alleles of *IGFALS* are required for normal ALS levels, IGF-1 action and growth. We therefore propose that heterozygosity for an inactivating *IGFALS* mutation could be considered as a medical condition and can be labeled as partial ACLSD.

The present uncertainties regarding the clinical presentation of patients carrying *IGFALS* mutations, including the effect of complete and partial ACLSD on intrauterine growth, HC, BMI, serum IGF-II and IGFBP-2 levels and BMD, require additional documentation. Here, we report extensive clinical, laboratory and radiological features of a large group of homozygous and heterozygous carriers of three novel *IGFALS* mutations in reportedly five unrelated Turkish families.

Subjects and methods

Subjects

A total of 6 index cases from five consanguineous Turkish families (Fig. 1, panels A, B, C, D and E) were presented to pediatric endocrine clinics in Gaziantep (*n* = 5) and Diyarbakir (*n* = 1) because of short stature. Their medical history was unremarkable, and there were no dysmorphic features found on physical examination. Clinical and laboratory data are shown in Table 1 and further details are presented below. In all cases, the serum levels of IGF-I and ALS were reduced, associated with extremely low IGFBP-3 levels. Along with the pattern of inheritance these findings suggested ACLSD. After confirming the diagnosis by genetic testing, all available family members were invited for growth measurements, venipuncture (for genetic and biochemical testing) and assessment of BMD.

Family A

The index cases were two male cousins (A.III.5 and A.III.14) who were seen at the same visit for short stature (both –3.7 SDS), 1.5–1.6 s.d. shorter than target height (TH). In both
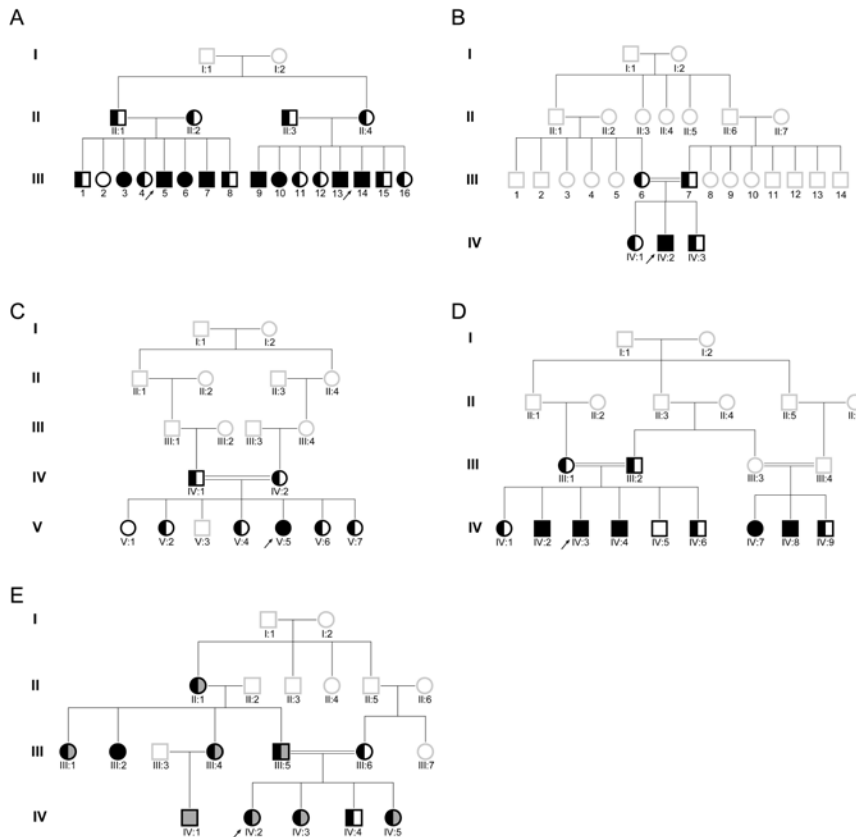
Figure 1

Pedigrees of families A, B, C, D and E and panels A, B, C, D and E prepared with PedigreeXP (PC PAL, Paris, France). In families A–D, heterozygous and homozygous carriers are indicated with half or completely filled squares and circles. In family E, the black color indicates the Asn84Ser mutation and the dark-gray color the Asp488Asn mutation. Light-gray symbols indicate family members who could not be investigated.

cases, serum GH in a random sample was considered too high for explaining the very low serum IGF-I (serum GH levels of 3.75 and 11.9 ng/mL respectively). Molecular genetics analysis for *IGFALS* showed a homozygous mutation in exon 2 (c.1462G > A and p.Asp488Asn), which was previously identified in heterozygous state in a South Asian population with an allelic frequency of 0.001 (EXAC – transcript ENST00000215539.3).

Family B

The index case (B.IV.2) was a 9.4-year-old boy with a height of −2.0 SDS, 0.8 s.d. shorter than target height. The peak GH level in a clonidine stimulation test was 26.4 ng/mL. *IGFALS* sequence analysis showed an identical (c.1462G > A and p.Asp488Asn) mutation, as detected in family A.

Family C

The index case (C.V.5) was a prepubertal 13.5-year-old girl with a height of −3.6 SDS, 2.8 s.d. shorter than TH. The GH response to a clonidine test was 8.2 ng/mL, and there was no response to an IGF generation test (data not shown).

Molecular genetic analysis showed a novel homozygous *IGFALS* mutation in exon 2 (c.251A > G and p.Asn84Ser).

Family D

The index case (D.IV.3) was an 11.4-year-old boy with a height of −2.3 SDS, 1.3 s.d. shorter than TH. An L-dopa stimulation test showed a normal peak GH level (10.3 ng/mL), and there was no response to an IGF generation test (data not shown). Genetic analysis showed a novel homozygous c.1477del and p.Arg493Alafs*176 mutation in exon 2.

Family E

The index case (IV.2) was a prepubertal 11.8-year-old girl with a height of −3.7 SDS, 2.3 s.d. shorter than TH. A clonidine stimulation test revealed a peak GH level of 8.6 ng/mL. In an IGF generation test, there was a small response of serum IGF-I (74.1–95.2 ng/mL; reference range 75–440), but no response of serum IGFBP-3 (0.87–0.74 mg/L, reference range 2.7–8.9). Genetic analysis showed a compound heterozygous (c.251A > G, Asn84Ser/c.1462G > A, p.Asp488Asn) mutation in exon 2 of *IGFALS*.

Table 1 Clinical features of the index cases of 5 families carrying *IGFALS* mutations.

Index case	A.III.5	A.III.14	B.IV.2	C.V.5	D.IV.3	E.IV.2
Mutation	D488N	D488N	D488N	N84S	R493fs	N84S/D488N
Sex	M	M	M	F	M	F
Age (years)	8.0	11.5	9.4	13.5	11.4	11.8
Birth weight (kg)	3.0		2.5	2.0		2.5
Birth weight SDS	−1.3		−2.6	−3.8		−2.4
Height (cm)	107.2	122.1	123.4	138.3	130.5	129
Height SDS	−3.7	−3.7	−2.0	−3.6	−2.3	−3.7
BA/CA (years)	5.0/7.4	10.0/10.4	6.0/9.0	12.0/13.5	9.0/11.4	12/11.8
Maternal Ht (cm)	145.5	157	161.1	158.3	159.5	159
Maternal Ht SDS	−3.0	−1.0	−0.3	−0.8	−0.5	−0.7
Paternal Ht (cm)	166	156.5	163.2	171.5	166.7	163
Paternal Ht SDS	−1.7	−3.2	−2.1	−0.8	−1.5	−2.1
TH (cm)	162.3	163.2	168.6	158.4	169.6	154.5
TH SDS	−2.2	−2.1	−1.2	−0.8	−1.0	−1.4
Ht SDS–TH SDS	−1.5	−1.6	−0.8	−2.8	−1.3	−2.3
Head circ SDS	−2.9	−2.8	−2.0	−2.1	−2.2	−1.9
BMI SDS	−0.3	−0.5	−1.2	−1.6	−1.6	−0.1
IGF-I (nmol/L)	2.2	4.7	4.8	8.3	4.2	8
IGF-I SDS	−4.5	−3.6	−3.3	−3.2	−3.8	−3.0
IGFBP-3 (nmol/L)	10	9	7.3	8	9.3	13.3
IGFBP-3 SDS	−9.1	−10.2	−11.2	−8.1	−10.0	−6.5
ALS (mU/mL)	<225	<225	225	<225	<225	<225
ALS SDS	<−2.9	<−3.3	<−3.1	<−5.5	<−3.3	<−5.4

ALS, acid labile subunit; BA, bone age; BMI, body mass index; CA, chronological age; circ, circumference; F, female; Ht, height; IGF-I, insulin-like growth factor I; IGFBP-3, insulin-like growth factor binding protein 3; M, male; SDS, standard deviation score; TH, target height.

Methods

We obtained approval for the study from the Ethics Committee of Gaziantep University (application 2016/139) and informed consent for genetic analysis and further studies from all family members and/or their legal guardians.

Height and weight were measured using standard anthropometric devices, and standard deviation scores (SDS) for height were calculated according to growth charts for the Turkish population (22). TH was calculated using the equation TH (centimeters) = (father's height (cm) + mother's height (cm))/2 −6.5 cm (girls) or +6.5 cm (boys) (23), and TH SDS was calculated using reference data for the Turkish population (22).

To evaluate the difference in height SDS between ACLSD and partial ACLSD, we took three approaches, the first two of which accounted for inter-family differences, to prevent confounding. First, for ACLSD patients in families A–D, we calculated the difference between their respective actual height SDS and TH SDS values. Secondly, for all ACLSD patients, we calculated the weighted average of the difference between height SDS of cases with p.ACLSD and partial ACLSD in the five families. Thirdly, we compared mean height SDS between all cases with ACLSD and partial ACLSD.

In the absence of Turkish reference data, HC SDS was calculated based on 1997 Dutch reference (24). Birth weight SDS was calculated based on the Swedish references (25), which proved similar to those of Turkish babies living in the Netherlands (26). In all cases, gestational age was reported as 'term', which we interpreted as 40 weeks. For children and adolescents, pubertal stage was assessed according to Tanner. Bone age was assessed using the Greulich and Pyle method (27). For all adolescent and adult females, the age at menarche was recorded. From adult males, we collected data on the age at onset of accelerated growth and shaving.

IGF-I in serum was determined by an immunometric technique on an IMMULITE 1000 Analyzer (Siemens Medical Solutions Diagnostics). Serum levels of IGFBP-3 and IGFBP-2 were determined by specific in-house RIAs. Serum total ALS levels were determined using the ELISA kit obtained from Mediagnost (Reutlingen, Germany). All plasma samples were diluted 150-fold before measurement in sample buffer, as recommended by the manufacturer. As the lowest standard in the ELISA was 1.5 mU/mL, measurements of less than 225 mU ALS/mL plasma may become inaccurate, although the duplicates showed less than 10% differences, which is in fact a quality requirement in the lab. We therefore decided to

report plasma levels <225 mU/mL as below the practical detection limit and calculated the SDS accordingly. Assay characteristics and smoothed normative ranges values, as constructed by the LMS method, of the various laboratory parameters mentioned previously have been published elsewhere (28, 29, 30).

Ternary complex formation was investigated by size-exclusion chromatography as described previously (12, 31). In the index cases in families C, D and E, IGF generation tests were performed (33 µg/kg/day for 3 days, venous samples taken before the first injection and on day 4 in index cases in family C and E and 50 µg/kg/day for 7 days, venous samples taken before the first injection and on day 8 in the index case in family D).

Genomic DNA was isolated from peripheral blood samples using PUREGENE nucleic acid purification chemistry on the AUTOPURE LS 98 Instrument (Qiagen). Sequence analysis of *IGFALS* (NC_000016.9/NM_004970.2) was performed as described previously (12).

BMD was measured by Dual-energy X-ray (Horizon DXA system, Hologic), and z-scores were calculated according to NHANES scores (32). To eliminate the impact of short stature on BMD z-score, sex and age-specific height-adjusted BMD (Ha-BMD), z-scores were calculated (33). As for subjects aged >20 years, no appropriate age references are available, we compared their Ha-BMD with 20-year-old adults.

Statistical analysis

Statistical analysis was carried out using IBM SPSS Statistics for Windows (version 22.0. Armonk, NY: IBM Corp. Released 2013). Data were expressed as mean ± s.d. (range). The Shapiro–Wilk test was used to test for a normal distribution of the data. Means were compared using Student's t test for normally distributed data, whereas for non-normally distributed data and for the groups with small number of cases, medians were compared using non-parametric Mann–Whitney U test. The Spearman–Rank test was performed for correlations. A P value <0.05 was considered statistically significant.

Results

Clinical, biochemical and radiological data per family

Clinical, biochemical and radiological data of all available individual members of the five families are shown in Supplementary Tables 1, 2, 3, 4 and 5 (see section on supplementary data given at the end of this article).

For two cases, medical disorders were reported. Case A.III.13 (homozygous for p.Asp488Asn) was born with anal atresia, had unilateral nephrectomy due to atrophic kidney at the age of 7 years and also had a history of frequent diarrhea and clavicle fracture after a minor trauma. Case E.IV.4 (heterozygous for p.Asn84Ser) reported gastroesophageal reflux disease complicated by bronchiectasis.

For families A and D (Supplementary Tables 1 and 4), the numbers of cases with ACLSD and partial ACLSD were sufficient to produce statistics with regard to the clinical, biochemical and radiological data. In family A, homozygous *IGFALS* mutations were encountered in 8 cases (6 < 18 years), heterozygotes in 11 family members (7 < 18 years) and one non-carrier (WT). In family D, 5 homozygous and 5 heterozygous carriers were found, and one WT. In both families, differences between homozygous and heterozygous carriers were statistically significant for SDS values of height, HC, IGF-I, IGFBP-3, ALS and IGF-II. Ternary complex formation in sera of the index cases was markedly diminished, whereas heterozygous
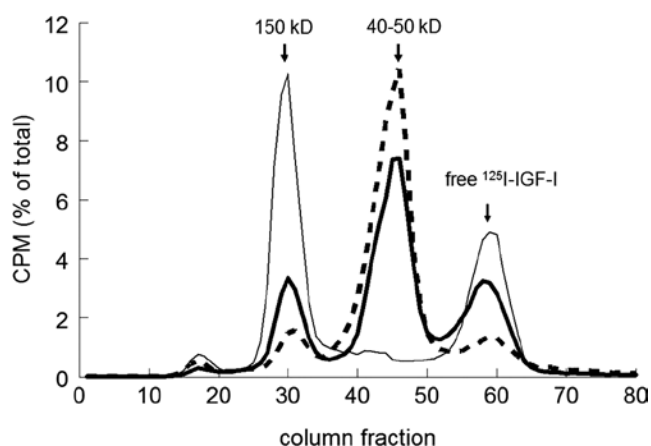

Figure 2

Distribution of radioactivity among the various MW size classes after S200 size-exclusion chromatography of pooled normal serum (thin black line), index patient III.14, homozygous for the c.1462G > A, p.Asp488Asn mutation in ALS (family A) (dashed line) and a heterozygous family member (II.2) (thick solid black line). Prior to column chromatography, 250 µL of each serum was incubated with 100 µL of ~100 000 cpm. ^{125}I-IGF-I for 18–20 h at 4°C. The ^{125}I content of each successive 1.2 mL fraction was counted in a γ-counter and expressed as percentage (II.2) radioactivity recovered from the column.

family members showed an intermediate pattern when compared to the normal situation. This is illustrated in Fig. 2 for one of the index cases of family A (III.14) and a carrier (II.2) of the *IGFALS* mutation. In these two families, the average height differences between ACLSD and partial ACLSD were 1.6 and 1.1 s.d.; the respective differences in HC were 1.2 and 0.6 s.d.

In families B and C (Supplementary Tables 2 and 3), the data of only one homozygous case could be compared with 4 and 6 heterozygotes respectively. Height SDS of the index cases vs the mean of the heterozygotes in these families were –2.0 vs –0.9 SDS (family B) and –3.6 vs –1.0 (family C), thus, differences of 1.1 and 2.6 s.d. respectively. In family C, HC SDS of the index case was 2.8 s.d. lower compared with the mean of the six heterozygous carriers (–2.1 vs +0.7 SDS).

In family E (Supplementary Table 5), father was compound heterozygous (p.Asn84Ser/p.Asp488Asn) and mother was heterozygous for the p.Asn84Ser mutation. One case, a cousin of the index patient, was homozygous for p.Asp488Asn mutation (IV.1), another case (III.2) was homozygous for the p.Asn84Ser mutation and seven were compound heterozygotes. There were only two heterozygous carriers. Mean height of the two heterozygotes (–1.9 SDS) was 0.3 s.d. above the combined

homozygous and compound heterozygous cases (–2.2 SDS). Ternary complex formation in sera from the index patients and heterozygous family members was affected in a similar way as for family A (data not shown).

Combined analysis

Tables 2 and 3 show the mean ± s.d. (range) of the clinical, biochemical and radiological data of 24 cases with ACLSD, 26 with partial ACLSD and 3 non-carriers (WT) in the five families combined. A graphical representation is shown in Fig. 3. In comparison with partial ACLSD, cases with ACLSD were shorter and leaner, had a smaller head circumference and lower SDS values of serum IGF-I, IGF-II, IGFBP-2, IGFBP-3 and ALS. The Spearman correlation analysis revealed that serum IGF-I SDS correlated stronger with HC SDS ($r = 0.653$, $P < 0.001$) than IGF-II ($r = 0.524$; $P < 0.001$). Serum IGFBP-2 levels also tended to be lower for ACLSD patients, but the difference did not reach statistical significance ($P = 0.06$). Five out of 7 cases with ACLSD had a birth weight < -2.0 SDS, vs none of the partial ACLSD cases ($n = 3$), but the difference between the means did not reach statistical significance ($P = 0.067$).

Table 2 Clinical features of 5 families carrying *IGFALS* mutations.

	Fam A	Fam B	Fam C	Fam D	Fam E	Mean ± s.d. (*n*)	Range	*P*-value*
Mutation	D488N	D488N	N84S	R493fs	N84S/D488N			
Sex								
–/–	5M 3F	1M	1F	4M 1F	2M 7F	12M 12 F		
–/+	5M 6F	2M 2F	5F 1M	3M 2F	1M 1F	12M 14F		
+/+	1F	–	1F	1M	–	2F 1M		
Age (years)								
–/–	11.7 ± 5.2 (8)	9.4 (1)	13.5 (1)	10.2 ± 2.5 (5)	28.4 ± 18.8 (9)	17.7 ± 14.4 (24)	4.8; 51.2	0.491
–/+	21.5 ± 16.0 (11)	19.7 ± 16.4 (4)	26.9 ± 18.8 (4)	8.4 ± 6.2 (3)	18.3 ± 15.0 (2)	20.7 ± 15.7 (26)	0.4; 53.9	
+/+	12.9 (1)	–	25 (1)	6.7 (1)	–	14.8 ± 9.3 (3)	6.7; 25	
BW SDS								
–/–	–1.3 (1)	–2.6 (1)	–3.8 (1)		–1.9 ± 1.1 (4)	–2.2 ± 1.1 (7)	–3.8; –0.3	0.067
–/+		–0.6 ± 0.4 (2)			–0.6 (1)	–0.6 ± 0.3 (3)	–0.8; –0.3	
Ht SDS								
–/–	–3.3 ± 0.6 (8)	–2.0 (1)	–3.6 (1)	–2.6 ± 0.7 (5)	–2.2 ± 1.7 (9)	–2.7 ± 1.2 (24)	–4.3; 0.6	<0.001
–/+	–1.7 ± 1.0 (11)	–0.9 ± 1.0 (4)	–1.0 ± 0.8 (5)	–1.5 ± 0.6 (5)	–1.9 ± 1.7 (2)	–1.4 ± 1.0 (26)	–3.2; 0.2	
+/+	–0.9 (1)	–	–1.3 (1)	–1.3 (1)	–	–1.2 ± 0.3 (3)	–1.3; –0.9	
HC SDS								
–/–	–2.7 ± 0.3 (8)	–2.0 (1)	–2.1 (1)	–2.3 ± 0.4 (5)	–1.7 ± 0.5 (4)	–2.3 ± 0.5 (19)	–3.2; –1.0	<0.001
–/+	–1.5 ± 1.0 (10)		0.7 ± 0.5 (4)	–1.6 ± 0.2 (3)	–1.1 (1)	–0.8 ± 1.1 (21)	–2.8; 1.0	
+/+	–1.5 (1)	NA	–	–	–	–		
BMI SDS								
–/–	–0.1 ± 1.2 (8)	–1.2 (1)	–1.6 (1)	–1.4 ± 0.5 (3)	–0.5 ± 0.8 (4)	–0.6 ± 1.0 (24)	–2.2; 2.2	0.013
–/+	0.8 ± 1.6 (11)	0.1 ± 1.0 (2)	0.2 ± 1.3 (4)	–1.1 ± 1.3 (3)	0.7 ± 1.2 (2)	0.3 ± 1.4 (26)	–2.4; 4.1	
+/+	–1.0 (1)	–	0.2 (1)	–1.0 (1)	–	–0.6 ± 0.7 (3)	–1.0; 0.2	

*P value represents comparision of biallelic (homozygous and compound heterozygous) cases vs monoallelic (heterozygous) ones.
BMI, body mass index; BW, birth weight; Ht, height; HC, head circumference; SDS, standard deviation score; –/–, biallelic; –/+, monoallelic; +/+ wild type.

Table 3 Biochemical features and height adjusted BMD z score of 5 families carrying *IGFALS* mutations.

	Fam A	Fam B	Fam C	Fam D	Fam E	Mean±s.d. (n)	Range	P-value
IGF-I SDS								
−/−	−3.5±1.0 (8)	−3.3 (1)	−3.2 (1)	−3.8±0.1 (5)	−4.0±2.2 (9)	−3.7±1.4 (24)	−7.9; −1.0	<0.001
−/+	−1.0±1.1 (11)	−0.4±1.1 (4)	−1.2±0.8 (6)	−0.9±0.5 (3)	−2.0±0.1 (2)	−1.0±1.0 (26)	−3.0; 0.8	
+/+	1.8 (1)			−0.6 (1)		0.6±1.7 (2)	−0.6; 1.8	
IGF-II SDS								
−/−	−5.4±0.7 (8)	−5.4 (1)	−6.4 (1)	−5.6±0.8 (5)	−5.7±0.8 (9)	−5.6±0.7 (24)	−6.8; −4.4	<0.001
−/+	−1.0±0.6 (11)	−1.3±0.8 (4)	−1.4±0.8 (6)	−1.8±0.9 (3)	−1.8±0.9 (2)	−1.3±0.7 (26)	−2.4; 0.1	
+/+	−0.2 (1)			−0.6 (1)		−0.4±0.3 (2)	−0.6; −0.2	
IGFBP-2 SDS								
−/−	−1.4±0.9 (8)	−4.6 (1)	0.5 (1)	−0.1±1.1 (5)	−0.4±2.0 (9)	−0.8±1.7 (24)	−4.6; 1.4	0.059
−/+	−0.7±1.4 (11)	−1.4±0.8 (4)	1.3±0.8 (6)	1.7±1.8 (3)	1.4±2.9 (2)	0.1±1.7 (26)	−2.6; 3.6	
+/+	−0.9 (1)			−0.3 (1)		−0.6±0.4 (2)	−0.9; −0.3	
IGFBP-3 SDS								
−/−	−8.8±1.3 (8)	−11.2 (1)	−8.1 (1)	−10.7±1.9 (5)	−8.1±2.0 (9)	−9.0±1.9 (24)	−12.6; −6.5	<0.001
−/+	−1.0±0.4 (11)	−1.7±0.4 (4)	−2.2±0.8 (6)	−2.4±0.8 (3)	−2.1±0.4 (2)	−1.6±0.8 (26)	−3.4; −0.2	
+/+	0.5 (1)			−0.4 (1)		0.0±0.6 (2)	−0.4; 0.5	
ALS SDS								
−/−	<−4.1±1.2 (8)	<−3.1 (1)	<−5.5 (1)	<−3.6±1.0 (5)	<−5.1±0.8 (9)	<−4.4±1.2 (24)	<−5.6; <−2.6	<0.001
−/+	−1.5±0.8 (11)	−1.8±0.6 (4)	−3.0±0.5 (6)	−2.0±0.4 (3)	−2.5±0.2 (2)	−2.1±0.9 (26)	−3.8; −0.2	
+/+	2.6 (1)			−0.7 (1)		0.9±2.3 (2)	−0.7; 2.6	
Ha-BMD z-score*								
−/−	0.0±1.1 (6)	−0.5 (1)	−0.3 (1)	0.9±0.5 (5)	0.1±1.0 (4)	0.1±0.9 (17)	−1.2; 1.5	0.666
−/+	0.2±0.8 (5)	−0.8 (1)	0.6 (1)	0.3 (1)	−1.5 (1)	−0.04±0.9 (9)	−1.6; 0.9	
+/+	–	–	–	–	–	–	–	

P value represents comparision of biallelic (homozygous and compound heterozygous) cases vs monoallelic (heterozygous) ones.
*Only calculated for children and adolescents.
ALS, acid labile subunit; Ha-BMD, bone mineral density z-score adjusted for height; IGF-I, insulin-like growth factor I; IGF-II, insulin-like growth factor II; IGFBP-2, insulin-like growth factor binding protein 2; IGFBP-3, insulin-like growth factor binding protein 3; SDS, standard deviation score.

Ha-BMD in adults could not be compared between groups because of the uneven distribution (no homozygous mutation carriers in families A, B and C, and only one heterozygous carrier in family E). If for adult cases reference data of 20-year-old adults were used, all Ha-BMD data were normal and not different between groups (data not shown). For children and adolescents, Ha-BMD SDS was 0.1±0.9 and −0.0±0.9 in ACLSD and partial ACLSD respectively (P=0.666).

Height difference between ACLSD and partial ACLSD

For all children with ACLSD in families A–D, we calculated the difference between height SDS and TH SDS, to assess the effect of homozygosity vs heterozygosity for *IGFALS* mutations. This difference in children with ACLSD (n=15) was −1.2±0.6 s.d. (95% confidence interval (CI) −1.6 to −0.9), whereas children with partial ACLSD (n=17) were predicted to become 0.3±0.9 s.d. (95% CI: −0.2 to +0.8) taller than their parents (P<0.001) (Fig. 4). Thus, using this approach, the effect of two defective alleles vs one could therefore be estimated as ~1.5 s.d. The weighted average of the difference between height SDS of cases with ACLSD and partial ACLSD in the five families was 1.3 s.d. When all cases with ACLSD and partial ACLSD were compared, the difference was also 1.3 s.d. (Table 2).

Due to the known increased occurrence of delayed puberty, we tested whether height SDS has a positive
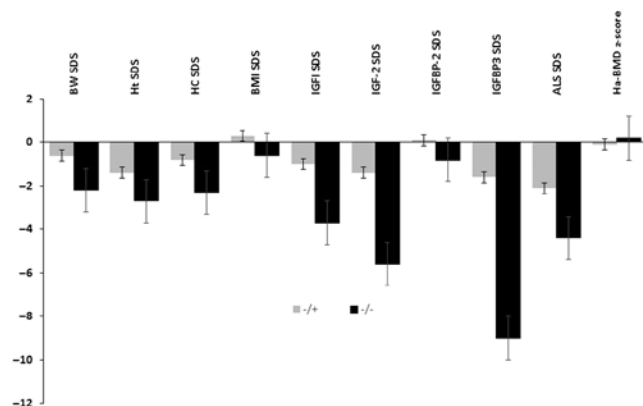
Figure 3

Graphical representation of mean±s.d. of SDS values of clinical and laboratory characteristics of cases with ACLSD (black bars) and partial ACLSD (gray bars). Ha-BMD z-score is only shown for cases ≤20 years.
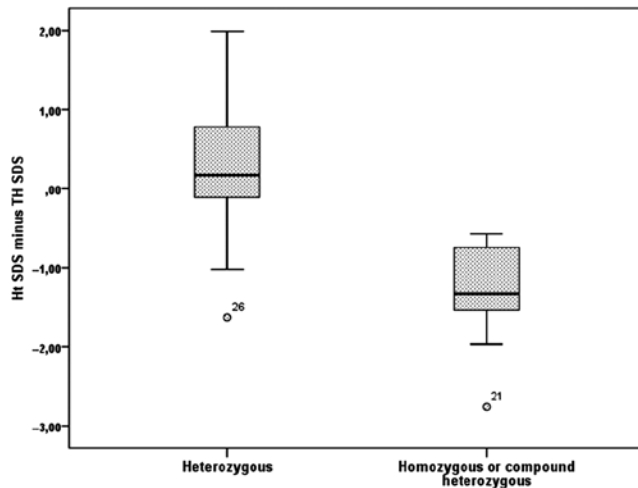
Figure 4

Boxplots of difference between height SDS and target height SDS for children with ACLSD and partial ACLSD.

correlation with age in cases with both groups. Regression analysis revealed no statistically significant correlation between age and height SDS in both ACLSD ($r=-0.116$; $P=0.589$) and partial ACLSD ($r=-0.136$; $P=0.507$).

Discussion

Fifty subjects belonging to five families carry one or two out of three *IGFALS* mutations in exon 2, which so far have not been associated with ACLSD. It is important that such mutations are registered in the literature and an international database (34) for future genetic diagnostic purposes. The c.1462G>A, p.Asp488Asn mutation (families A, B and E), previously only identified in heterozygous state in a South Asian population, introduces a potentially new N-glycosylation site (i.e. Asn-X-Thr/Ser), which will probably result in misfolding of the protein leading to its relatively rapid intracellular degradation (35). Several protein prediction algorithms conclude that this mutation, which is located in a highly conserved amino acid, is pathogenic (SIFT: deleterious (score 0; median: 3.74); PolyPhen: probably damaging (score of 1.000); MutationTaster: disease causing (P value: 0.787)). The p.Asn84Ser mutation (families C and E) replaces an asparagine by a serine. This amino acid is also highly conserved, and the mutation is predicted to be pathogenic by the algorithms (SIFT: Deleterious (score: 0, median: 3.64); PolyPhen: probably damaging (score of 1.000); MutationTaster: disease causing (P value: 1)). The c.1477del, p.Arg493Alafs*176 mutation is probably subject to nonsense-mediated decay and not expressed

at all. If expressed, the mutant ALS protein would loose two of its consensus motifs for leucine-rich repeats (LRRs), which would be replaced by 176 new amino acids. These LRRs are considered to be involved in the interaction between ALS and IGFBP-3 or-5 (35, 36, 37). Additional functional studies are needed to confirm these theoretical considerations, but the data on ternary complex formation, extremely low serum ALS and cosegregation of the mutations with the clinical and laboratory phenotype in these families make it extremely likely that these mutations are pathogenic. We speculate that the occurrence of the same mutations in more than one reportedly unrelated families is most likely caused by a founder effect because they all lived in the same region of Turkey.

Our findings confirm previous reports that the main clinical feature of children with ACLSD is moderate short stature (mean height –2.7 SDS, i.e. 1.3–1.5 s.d. shorter than heterozygous relatives); only 4 of 24 cases had a height above the cutoff of –2 SDS. We also confirm that a combination of a low serum IGF-I level with an extremely low IGFBP-3 concentration, low serum ALS and reduced ternary complex formation represent the main biochemical characteristics of ACLSD. We further show that heterozygous carriers are relatively short and that height-adjusted BMD is within the normal range in both groups. Novel findings include that most cases with ACLSD are born SGA, have a HC below –2 SDS (mean head circumference –2.3 SDS, i.e. 1.5 s.d. smaller than that in partial ACLSD) and have a low serum IGF-II, whereas serum IGFBP-2 levels are in the low-normal range. Our observation that BMI SDS in ACLSD is slightly lower (but reaching statistical significance) than that in partial ACLSD is novel, but not relevant from a clinical point of view.

Delayed puberty, as previously reported in 50% of males with ACLSD, is considered one of the most frequent clinical characteristics of this condition. As most of our cases with ACLSD were young, and information about pubertal onset of family members was of dubious accuracy, our data do not allow for any conclusion about pubertal timing. Assuming that the adults in the five families also had experienced a delayed puberty, we speculated that the effect on adult height might be less severe than that on height SDS during childhood. However, we could not confirm this in our study.

The difference in height SDS between ACLSD and partial ACLSD in our study (1.3–1.5 s.d.) is similar to what we previously reported in one family (12) and to results published by the International ALS Consortium (19). The

relatively low mean height SDS of partial ACLSD in our study is also consistent with previous reports indicating that heterozygosity for an *IGFALS* mutation leads to an average loss of approximately 1 S.D. (12, 19, 20), in line with a gene dosage effect (i.e. the expression of both *IGFALS* alleles is necessary for normal growth). However, the number of non-carriers in the five families in this study was too small to document this statistically.

In this study, we demonstrate that HC and birth size are significantly smaller in patients with ACLSD than in partial ACLSD, confirming previous anecdotal observations for head circumference (8, 9, 12) and birth weight (3, 6, 9, 12, 13, 14, 15, 16), although both in our study and the previous review (3) birth weight was only known for 7–8 cases, with unknown accuracy of the reported gestational age (in all except one case reported as 'term'). Previous studies have shown that IGF-I is necessary for normal fetal growth and development, and particularly for brain development, as illustrated by the severe microcephaly and low birth size of patients with *IGF1* or *IGF1R* defects (38, 39, 40, 41, 42, 43, 44). The effect of IGF-II deficiency on birth size and HC appears to be less than that of IGF-I (45), which is in line with our finding that the correlation between HC SDS with serum IGF-I was stronger than that with serum IGF-II. We speculate that the exposure of the developing fetus, including its brain, to IGFs is decreased in patients with ACLSD, resulting in a relatively low birth weight and HC, but apparently this has no detrimental effect on intellectual development. This speculation is also supported by a recent study showing that umbilical cord ALS levels are positively correlated with birth weight, head circumference and placental weight (46).

Several reports suggested that ACLSD may cause low BMD (3, 6, 12, 18), but it was found normal in other reports (9, 11, 14). The present results show that Ha-BMD in children and teenagers with ACLSD is normal and not different from partial ACLSD. Our observations in adults suggest that the same is true for that age range, although the unequal distribution of heterozygous and homozygous mutations in these families did not allow for a proper comparison. In addition, there was no history of bone pain or fracture, except for one homozygous patient (A.III.13) with serious coexisting medical disorders. Our findings are consistent with the most recent interpretation of data on bone biology i.e. ACLSD creates a phenotype of slender bones of normal density (9), similar to that observed in Als-deficient mice (47). Although there is no doubt that for a proper interpretation of BMD assessed by DXA an adjustment for body height is necessary (33, 48, 49), there is no consensus about the most appropriate

method. We compared apparent BMD (representing volumetric BMD instead of areal BMD) (50) and height-adjusted BMD (33) and chose the latter, because this method involves 4 comprehensive steps taking into account the bone mineral content, sex, age, race and height of the subjects in the calculation of BMD. Besides, it has been developed and tested in a large number of healthy children and adolescents with more than 10 000 DXA measurements.

With respect to biochemical findings, for the clinician the combination of a markedly low IGFBP-3, low IGF-I and a normal GH secretion in a moderately short child makes the diagnosis of ACLSD sufficiently likely to perform genetic testing. In the present paper, we show that serum IGF-II SDS is even lower than IGF-I SDS and that serum IGFBP-2 is usually in the low-normal range, but tends to be lower in ACLSD than that in partial ACLSD ($P = 0.059$). The ALS ELISA revealed some detectable ALS immunoreactivity in sera from all patients with ACLSD, albeit clearly below the lowest standard in the assay. It is unclear whether the (very) small amount of ALS detected (which may also represent fragments, cross reactive material, or a matrix effect) is of any biological importance. On the other hand, the column gel filtration experiments showed the presence of a (very) small amount of ternary complex in ACLSD patients, as shown in Fig. 2.

In conclusion, our results expand the characteristics of the clinical phenotype of ACLSD by an increased percentage of low birth weight (SGA), low HC (microcephaly) and leanness, and show that besides decreased serum IGFBP-3, IGF-I and ALS, also IGF-II is decreased, whereas BMD appears normal for height.

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