Homozygous and heterozygous expression of a novel mutation of the acid-labile subunit

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Introduction

For both humans and rodents, it has been well established that insulin-like growth factor-I (IGF-I) plays a critical role in the regulation of intrauterine growth and development and postnatal growth and metabolism (1, 2). Total IGF-I deficiency leads to severe intrauterine and postnatal growth failure, microcephaly, mental retardation, and deafness (3, 4). Partial IGF-I resistance due to heterozygous mutations in the IGF-I receptor (IGF1R) gene results in moderate intrauterine and postnatal growth retardation and microcephaly (5–8).

A third defect that is associated with a reduced bioavailability of IGF-I is caused by a mutation in the acid-labile subunit gene (ALS). ALS can form, together with IGF-I (or IGF-II) and IGF-binding protein-3 (IGFBP-3), or IGFBP-5 for only 10%, a 150 kDa ternary complex. Under normal circumstances, 80–85% of circulating IGF-I is retained in this complex (9). The 150 kDa complexes cannot cross the capillary endothelial barrier, which prolongs the half-life of IGFs, IGFBP-3 and IGFBP-5, in the circulation (9–11). This seems to play an important role in the regulation of the bioavailability of IGFs to the tissue compartments.

ALS belongs to the superfamily of leucine-rich repeat (LRR) proteins, characterized by their ability to participate in protein–protein interactions. About 75% of the mature protein consists of the consensus motif for the LRR superfamily of proteins. These leucine-rich domains organize ALS into a doughnut-shaped structure (11, 12). Whereas ALS readily binds to binary complexes of IGFs and IGFBP-3, it does not interact directly with free IGFs and shows only a very low affinity for unliganded IGFBP-3 (13, 14).
So far, eight patients have been described with mutations in the ALS gene (10, 15–18). All cases described were characterized by moderate or mild growth failure, undetectable or very low circulating levels of ALS, and markedly reduced plasma concentrations of IGFBP-3 and IGF-I. In some cases, pubertal delay was noted, and a low bone mineral density (BMD) was found in one case (19).

In this report, we describe three brothers of Kurdish origin with short stature and microcephaly associated with a novel homozygous mutation in the ALS gene. In addition, we have evaluated the clinical and laboratory findings in ten relatives, five of whom were heterozygous for the mutation and five non-carriers.

Methods
All subjects described in this paper belong to a consanguineous family of Kurdish origin (Fig. 1). After mutation analysis, the subjects were divided into ‘patients’ (homozygous carriers), heterozygous carriers, and non-carriers. For each homozygous carrier, the medical history as well as the results of previous investigations was retrieved. In 2007, all the subjects were again thoroughly investigated. All the subjects provided written informed consent.

Molecular studies
Genomic DNA was isolated from whole blood or buffy coats according to the salting out procedure described by Miller et al. (20). All exons of ALS (GenBank accession number AF192554) were PCR amplified and sequenced as described previously (4). The various primer sequences employed are available upon request.

Clinical measurements and auxology
Physical examination was performed including measurements of height and sitting height with a Harpenden stadiometer and head circumference with a tape measure.

Results
Medical history of the ALS-deficient patients
Patient III-1 was born as the first of four children of consanguineous parents. In the first three years, he was...
short, frequently ill, had many episodes of diarrhea, and showed poor appetite and night sweating. At the age of 11.6 years, he moved with his family to the Netherlands. At the age of 12 and 14 years, he suffered fractures of the left wrist and arm, after minor trauma, and at radiographs, bone density appeared low. Reported pubertal onset was at about 14 years of age (mean age at Tanner stage 2 in Turkish adolescents living in the Netherlands is 12.2 years) (21). He had a somewhat dysmorphic face with mandibular hypoplasia and a prominent forehead. The growth curve is shown in Fig. 2A.

At the age of 16.1 years, dietary calcium and vitamin D intake appeared low, and serum vitamin D25 was slightly decreased, but serum calcium was in the upper normal range and parathyroid hormone (PTH) was normal. Vitamin D p.o. for 6 months resulted in normalization of D25 levels. A grade I mitral and tricuspid insufficiency was detected at cardiologic investigation. The growth hormone (GH) peak after exercise was high (63 mU/l), IGF-I was very low (−6.9 to −5.0 SDS), and IGFBP-3 extremely low (−12.0 SDS). There was a poor response to GH in an IGF-I generation test with two different doses (0.8 and 1.6 mg/m² per day for 4 days); only on the higher dose IGF-I increased from 16 to 60 ng/ml. IGFBP-3 did not change.

At the age of 17.8 years, his bone age was adult, and he had reached a final height of 149.7 cm (−4.2 SDS) (Fig. 2A), indicating a fast progression through puberty, which probably led to the poor pubertal height gain (13.3 cm). At the age of 16, 17, and 19 years, total BMD and z-scores were 0.81 g/cm² (−3.3 SDS), 0.89 g/cm² (−3.9 SDS), and 0.91 g/cm² (−2.5 SDS) respectively, and femoral neck BMD was 0.827 (−1.5 SDS) at 19 years of age.

Patient III-2 moved with his family to the Netherlands at the age of 5 years. At 7 years of age, he was seen by an ophthalmologist because of strabismus. His growth chart is shown in Fig. 2B. Data on reported pubertal onset and progression are inconsistent.

Patient III-4 had a reported birth weight of 2.0 kg at an unknown gestational age. He remained short during childhood and adolescence (Fig. 2C), but bone maturation was not retarded. During infancy, he experienced poor appetite, frequent episodes of diarrhea, and night sweating. His left leg was ~5 cm shorter than the right leg, probably due to congenital coxa vara by congenital femoral hypoplasia. After an osteotomy, the difference was reduced to 3 cm. He had a similar dysmorphic face as patient III-1. Puberty onset was reported to have occurred between 12 and 14 years. Total BMD at 6, 10, and 12 years of age was 0.68 (−1.3 SDS), 0.76 (−1.2 SDS), and 0.62 g/cm² (−2.0 SDS) respectively. The GH peak after exercise was high (118 mU/l), IGF-I was very low (−4.2 to −3.8 SDS), and IGFBP-3 extremely low (<−17 SDS). During IGF-I generation tests, employing GH doses 0.8 and 1.6 mg/m² per day for 4 days, the IGF-I levels increased only marginally, from 38 to 46 ng/ml and 44 to 52 ng/ml respectively. DNA analysis for GH and GHR was normal.
Molecular studies

Sequence analysis of the ALS gene of patients III-1, III-2, and III-4 revealed a homozygous duplication of a T nucleotide at position 1490 of the coding DNA, resulting in a frameshift and a premature stop codon (c.1490dupT, p.Leu497PhefsX40; further indicated as −/−) (Supplementary Fig. 1, which can be viewed online at http://www.eje-online.org-supplemental/). Five relatives were heterozygous carriers of the mutation (+/−) and the other five non-carriers (+/+) (Fig. 1).

Clinical, radiological, and biochemical features at recall

Clinical, radiological, and laboratory findings are summarized in Tables 1–3. Mean height and head circumference SDS of heterozygotes were 1 and 0.7 s.d. lower respectively than those of non-carriers, but the differences with non-carriers did not reach statistical significance ($P=0.25$ and 0.13 respectively). The sitting height/height ratio was in the upper normal range in most cases, without apparent association with the ALS defect. Two homozygous patients exhibited a low BMD and one of them also a low BMAD, but also in four out of nine relatives low values were observed. Tone audiometry did not show a clinically relevant perceptive hearing loss in the patients or their relatives (data not shown).

In the three patients, ALS was not detected by ELISA, which was confirmed by western blotting of patient’s sera (Supplemental Fig. 2, which can be viewed online at http://www.eje-online.org-supplemental/). No ALS bands with aberrant molecular weights were detected. Fasting glucose levels were normal (5.3, 4.5, and 4.6 mmol/l), but insulin was slightly elevated (12, 17, and 14 mU/l), indicating mild insulin resistance. Plasma levels of IGFBP-3 were extremely low, and of IGF-I, IGF-II, IGFBP-1, and IGFBP-2 markedly reduced. Plasma IGFBP-4 and IGFBP-6, and serum testosterone, follicle-stimulating hormone (FSH), luteinizing hormone (LH), and free thyroxine (FT$_4$) levels were normal. In heterozygous carriers, plasma ALS and IGFBP-3 were low. In non-carriers, all parameters were normal.

Neutral gel filtration

While in normal control serum, most IGF-I (90%) is associated with the 150 kDa complex (Fig. 3A); in the sera of the three ALS-deficient patients there was almost no 150 kDa complex present. The 40–50 kDa fraction (consisting of binary IGF–IGFBP complexes) was markedly elevated and the free $^{125}$I-IGF-I peak was reduced (Fig. 3B). Addition of purified hIGFBP-3 to these sera did not induce the 150 kDa complex formation, while a slightly increased 150 kDa peak was observed upon addition to normal serum (data not shown). Similar results were obtained with $^{125}$I-IGF-II (data not shown). In the sera of heterozygous carriers, the amount of the 150 kDa complexes was reduced and that of the 40–50 kDa complexes markedly increased (Fig. 3C). Column profiles of sera of the non-carriers were normal.

Table 1 Clinical features and biochemical characteristics of the homozygous carriers.

<table>
<thead>
<tr>
<th></th>
<th>III-1</th>
<th>III-2</th>
<th>III-4</th>
<th>Mean</th>
</tr>
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<tr>
<td>IGFALS gene</td>
<td>−/−</td>
<td>−/−</td>
<td>−/−</td>
<td>−/−</td>
</tr>
<tr>
<td>Gender</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>Age (year)</td>
<td>27.2</td>
<td>21.5</td>
<td>17.0</td>
<td>21.9</td>
</tr>
<tr>
<td>Height (cm) (SDS)</td>
<td>149.7 (−4.2)</td>
<td>153.2 (−3.6)</td>
<td>145.5 (−4.4)</td>
<td>149.5 (−4.1)</td>
</tr>
<tr>
<td>Head circumference (cm) (SDS)</td>
<td>52.5 (−3.0)</td>
<td>53.3 (−2.6)</td>
<td>51.2 (−3.2)</td>
<td>52.3 (−2.9)</td>
</tr>
<tr>
<td>BMI (kg/m$^2$)</td>
<td>21.2</td>
<td>23.4</td>
<td>21.6</td>
<td>22.1</td>
</tr>
<tr>
<td>Sitting height: Height (SDS)</td>
<td>0.54 (2.2)</td>
<td>0.54 (2.0)</td>
<td>0.53 (1.1)</td>
<td>0.54 (1.8)</td>
</tr>
<tr>
<td>Total BMD (g/cm$^2$) (z-score)</td>
<td>0.865</td>
<td>1.058</td>
<td>0.793 (−3.3)</td>
<td>0.905</td>
</tr>
<tr>
<td>BMD femoral neck (z-score)</td>
<td>0.591 (−2.4)</td>
<td>0.943 (0.1)</td>
<td>0.640 (−2.4)</td>
<td>0.725 (−1.6)</td>
</tr>
<tr>
<td>BMD L1–L4 (z-score)</td>
<td>0.625 (−4.2)</td>
<td>0.902 (−1.7)</td>
<td>0.590 (−3.1)</td>
<td>0.706 (−3.0)</td>
</tr>
<tr>
<td>BMAD L2–L4 (z-score)</td>
<td>0.209 (−2.8)</td>
<td>0.284 (−0.2)</td>
<td>0.230 (−1.7)</td>
<td>0.241 (−1.6)</td>
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<tr>
<td>IGF-I (ng/ml) (SDS)</td>
<td>&lt;12 (&lt;−7.2)</td>
<td>13 (−7.4)</td>
<td>29 (−5.6)</td>
<td>18 (−6.7)</td>
</tr>
<tr>
<td>IGF-II (ng/ml) (SDS)</td>
<td>66 (−6.6)</td>
<td>55 (−6.8)</td>
<td>49 (−6.7)</td>
<td>56.7 (−6.7)</td>
</tr>
<tr>
<td>IGFBP-1 (ng/ml) (SDS)</td>
<td>27</td>
<td>27</td>
<td>27</td>
<td>27</td>
</tr>
<tr>
<td>IGFBP-2 (ng/ml) (SDS)</td>
<td>67 (−2.5)</td>
<td>96 (−1.8)</td>
<td>77 (−2.4)</td>
<td>77 (−2.4)</td>
</tr>
<tr>
<td>IGFBP-3 (mg/l) (SDS)</td>
<td>0.09 (−18.2)</td>
<td>0.09 (−18.5)</td>
<td>0.10 (−17.6)</td>
<td>0.10 (−18.1)</td>
</tr>
<tr>
<td>IGFBP-4 (ng/ml) (SDS)</td>
<td>172 (−0.1)</td>
<td>136 (−0.7)</td>
<td>157 (−0.3)</td>
<td>155 (−0.4)</td>
</tr>
<tr>
<td>IGFBP-6 (ng/ml) (SDS)</td>
<td>180 (0.4)</td>
<td>138 (−0.3)</td>
<td>148 (0.2)</td>
<td>155.3 (0.1)</td>
</tr>
<tr>
<td>ALS (mg/l) (SDS)</td>
<td>&lt;0.07 (&lt;−7.1)</td>
<td>&lt;0.07 (&lt;−5.7)</td>
<td>&lt;0.07 (&lt;−4.5)</td>
<td>&lt;0.07 (&lt;−4.5)</td>
</tr>
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$^a$Normal range for non-fasting subjects: 24–57 ng IGFBP-1 per ml. After overnight fasting, there is an average fivefold rise in normal individuals. IGFBP-1 SDS values are not available for adults older than 24 years.

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suggest that other genetic factors may be involved. Biochemically, serum ALS was undetectable; IGFBP-3 extremely low: IGFs, IGFBP-2, and IGFBP-1 decreased; and the ternary 150 kDa complex was virtually absent. Most of the circulating IGFs were either sequestered by IGFBPs forming binary complexes or remained unbound. The western blotting experiments indicate that there is no truncated form of ALS present. Even if this were the case, a truncated ALS would not be able to form the stable 3D doughnut-shaped structure, which is essential for its function.

The growth pattern of all previously reported cases of ALS deficiency (10, 15, 16, 18) is characterized by moderate or mild short stature during childhood and adolescence and a normal or slightly decreased adult stature. Our ALS-deficient patients are much shorter in comparison with the reference population, but this may be due to other genetic factors in this highly consanguineous family. The difference between their mean (near) adult height SDS (−4.1 SDS) and that of the five heterozygous carriers (−2.3 SDS) is 1.7 s.d., comparable with earlier reports. Data on head circumference have not

<table>
<thead>
<tr>
<th>IGFBP-1 (ng/ml)</th>
<th>SDS</th>
<th>11</th>
<th>31</th>
<th>5</th>
<th>19</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMAD L2–L4 (SDS)</td>
<td>Head circumference (cm) (SDS)</td>
<td>53.8 (−0.9)</td>
<td>54.0 (−2.2)</td>
<td>55.0 (−1.6)</td>
<td>52.5 (−1.7)</td>
</tr>
</tbody>
</table>

| BMI (kg/m²) | Sitting height: height (SDS) | 0.53 (0.4) | 0.54 (2.1) | 0.53 (1.2) | 0.53 (0.6) | 0.53 (−0.4) | 0.53 (0.8) |
| Total BMD (g/cm³) (z-score) | 0.974 (−0.9) | 0.869 | 0.986 | 0.911 (−2.2) | 0.751 (0.4) | 0.898 (−0.9) |
| BMD femoral neck (z-score) | 0.721 (−0.7) | 0.648 (−1.3) | 0.855 (0.0) | 0.811 (−0.3) | 0.732 (1.4) | 0.753 (−0.2) |
| BMD L1–L4 (z-score) | 0.805 (−1.7) | 0.618 (−3.9) | 0.692 (−3.5) | 0.865 (−1.4) | 0.577 (0.7) | 0.711 (−0.20) |
| BMAD L2–L4 (z-score) | 0.307 (−0.4) | 0.164 (−4.4) | 0.184 (−3.7) | 0.308 (−0.4) | 0.169 | 0.226 (−2.2) |
| IGF-I (ng/ml) (SDS) | 0.99 (−11) | 73 (−1.5) | 86 (−1.6) | 141 (−1.8) | 73 (−1.4) | 94 (−1.5) |
| IGF-II (ng/ml) (SDS) | 352 (−1.0) | 305 (−1.8) | 305 (−1.7) | 308 (−1.5) | 296 (−1.1) | 313.2 (−1.4) |
| IGFBP-1 (ng/ml) (SDS)a | 11 | 31 | 5 | 19 | 164 (0.1) | 46 |
| IGFBP-2 (ng/ml) (SDS) | 184 (0.1) | 196 (0.3) | 255 (0.9) | 278 (1.5) | 360 (1.1) | 255 (0.8) |
| IGFBP-3 (mlg) (SDS) | 1.54 (−1.2) | 0.93 (−2.9) | 0.94 (−3.3) | 1.19 (−3.2) | 1.05 (−2.9) | 1.13 (−2.7) |
| IGFBP-4 (ng/ml) (SDS) | 196 (0.3) | 117 (−1.1) | 139 (−0.7) | 139 (−0.8) | 113 (−1.2) | 141 (−0.7) |
| IGFBP-6 (ng/ml) (SDS) | 185 (0.8) | 176 (−0.3) | 159 (−0.3) | 147 (0.1) | 112 (0.3) | 156 (0.1) |
| ALS (mg/l) (SDS) | 9.9 (−2.6) | 10.5 (−1.9) | 6.0 (−3.6) | 9.0 (−3.0) | 6.9 (−2.0) | 8.5 (−2.6) |

*Normal range for non-fasting subjects: 24–57 ng IGFBP-1 per ml. After overnight fasting, there is an average fivefold rise in normal individuals. IGFBP-1 SDS values are not available for adults older than 24 years.
been presented in earlier reports; thus it remains to be
established whether microcephaly represents a clinical
feature of homozygous ALS deficiency.

The growth pattern in humans with ALS deficiency is
comparable with the mild growth failure observed in
ALS gene knock-out (ALS-KO) mice, and is in contrast
with the severe growth failure as observed in patients
with a total IGF-I deficiency (2). This is in line with the
hypothesis that the contribution of locally produced
IGF-I to longitudinal growth is more important than
that of IGF-I derived from the circulation (10, 17).

As speculated previously, local production of IGF-I may
be even higher than normal due to increased GH
secretion, and might thereby compensate, at least in
part, for any deficiency of circulating IGF-I (10).

Another possible explanation of the near-normal
growth, in spite of reduced levels of circulating IGF-I,
may include an increase in the IGF-I flux toward the
peripheral tissue compartments at the expense of the
circulating IGF-I pool that cannot be maintained at
normal levels without ALS (17, 29). It is uncertain
whether ALS deficiency affects fetal growth.

At present, it is uncertain whether an ALS defect is
associated with pubertal delay. Three patients described
previously seem to have experienced late onset of puberty
(17, 18), but in the other cases puberty was normal. In
our index patient III-1, pubertal onset was late, in III-2
unknown, and in III-4 normal. The low BMD in two of
our patients and in the first published case (19),
in combination with the low BMD in ALS-KO mice
(30–32) and previous studies showing that circulating
IGF-I levels are related to sufficient bone growth and
acquisition of peak bone mass (33, 34), suggested that
ALS deficiency might be associated with a low BMD.
However, further observations in the patients’ relatives
did not support this.

Biochemically, in all cases reported so far, undetect-
able or very low levels of circulating ALS, extremely low
IGFBP-3, and low IGF-I and IGF-II have been reported.
The lack of the 150 kDa complex formation is expected
to shorten the half-life of IGF-I in the circulation (31),
and possibly also that of IGFBP-3 (10). The low IGFBP-1
and IGFBP-2 plasma levels observed in two earlier
papers (16, 18) and in our three patients suggest that
this is part of the syndrome. A consequence of the low
plasma IGFBP-1 (an inhibitor of IGF-I effects) may be
that growth is less compromised than would be
expected from the low circulating IGF-I levels alone.

In the cases tested, we found an increased GH
response to provocative stimuli, confirming earlier
observations (10, 16, 18). The increased GH secretion
can be explained by the reduction in free IGF-I as
observed in previous studies (10, 35, 36), but is in
contrast to the normal free IGF-I and GH levels in ALS-
KO mice (19, 31). An IGF generation test was performed
in three previous cases (10, 16, 18), and showed
virtually no response of IGF-I, similar to the two cases
tested. Our patients had a mild insulin resistance,
confirming earlier reports (16, 18).

Heterozygosity for an ALS defect may have some effect
on stature and head circumference compared with non-
carriers, but these differences did not reach statistical
significance. Within the heterozygous and wild-type
individuals, height and head circumference varied
considerably, which may be due to other genes that
negatively affect growth in this highly consanguineous
family. As in most previous reports, parents of affected
patients were relatively short (10, 18); a meta-analysis
of all cases may shed more light on this issue.

Biochemically, carriers had low plasma ALS and
IGFBP-3, and IGF-I in the low normal range, as reported
previously (10).

In conclusion, these three cases with a novel
frameshift mutation of ALS resulting in undetectable
circulating ALS levels show that besides short stature

Figure 3 S200 gel filtration column chromatography. (A) Representative column profile for normal adult serum. (B) Serum of
patient III-1 (solid line) and patient III-2 (dotted line), both exhibiting a homozygous mutation in the ALS gene. (C) Serum of the father
II-2 (solid line) and uncle (II-4) (dotted line) of the index cases, both being heterozygous for the mutation.

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and possible delayed onset of puberty. Microcephaly may also be part of the syndrome. Biochemically, the absence of circulating ALS leads to extremely low levels of IGFBP-3, very low levels of IGF-I and IGF-II, low plasma IGFBP-1 and IGFBP-2, and mild insulin resistance. Heterozygosity for an ALS defect may have a small effect on height and head circumference.

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