Three Novel IGSF1 Mutations in Four Japanese Patients With X-Linked Congenital Central Hypothyroidism

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Context: Congenital central hypothyroidism (C-CH) is a rare disease. We investigated the molecular basis of unexplained C-CH in 4 Japanese boys.

Patients and Methods: C-CH was diagnosed by low free T4 and/or T3 and low basal TSH concentrations. We used whole-exome sequencing of one patient with C-CH to identify potential disease-causing mutations. Thereafter, PCR direct sequencing was performed to identify genetic defects underlying C-CH in 3 more patients. We then assessed the effects of mutations identified in the Ig superfamily, member 1 (IGSF1), gene on protein expression and membrane trafficking.

Results: All patients had congenital hypothyroidism, and 2 had definitive prolactin deficiency. Two patients were detected by neonatal screening. The other patients were diagnosed by short stature and failure to thrive. We identified a novel nonsense variant in IGSF1 by whole-exome sequencing in patient 1, which was confirmed by PCR direct sequencing (p.R1189X). PCR direct sequencing identified the identical nonsense mutation in patient 2. Patients 3 and 4 harbored distinct missense (p.V1082E) or nonsense (p.Q645X) mutations in IGSF1. The mothers of patients 1, 3, and 4 were heterozygous for these mutations. The R1189X mutant, which lacks the transmembrane domain, failed to traffic to the plasma membrane. V1082E could be observed at the cell surface, but at greatly diminished levels relative to the wild-type form of the protein. The severely truncated Q645X mutant could not be detected by Western blot.

Conclusion: Our findings provide additional genetic evidence that loss-of-function mutations in IGSF1 cause an X-linked form of C-CH and variable prolactin deficiency. (J Clin Endocrinol Metab 98: E1682–E1691, 2013)
macro-orchidism is caused by loss-of-function mutations and deletions in the Ig superfamily, member 1 (IGSF1), gene. IGSF1 encodes a plasma membrane Ig superfamily glycoprotein (20–22), containing 12 C2-type Ig loops, a transmembrane domain, and a short intracellular C tail (22). The protein is cotranslationally cleaved such that only the C-terminal domain, containing 7 Ig loops, reaches the plasma membrane (22). Human IGSF1 and murine Igsf1 mRNAs are highly expressed in Rathke’s pouch and in adult pituitary gland and testis (19). IGSF1 protein is expressed in murine thyrotropes, somatotropes, and lactotropes (19).

In this study, we describe 3 novel IGSF1 mutations in 4 Japanese boys with C-CH from 4 unrelated families.

Patients and Methods

Neonatal screening for congenital hypothyroidism and serum thyroid hormone levels

Approximately 10 000 infants are born annually in Sapporo, Japan; almost all are subject to neonatal screening for congenital hypothyroidism (CH). The screening method and the frequency of C-CH in Sapporo have been described (3, 23).

Patients

We analyzed a Japanese boy with C-CH (patient 1) by whole-exome sequencing. After the identification of a novel IGSF1 variant, we recruited an additional 3 C-CH Japanese boys from unrelated families and analyzed IGSF1 by PCR direct sequencing. Two patients (patients 1 and 3) were detected by neonatal screening for CH. Their clinical characteristics and endocrine findings are summarized in Table 1.

Serum TSH and prolactin concentrations were determined in response to exogenous TRH (5 μg/kg). GH provocative tests were performed with insulin (0.1 IU/kg) or GHRH (1 μg/kg). Serum LH and FSH concentrations were determined in response to GnRH (2 μg/kg).

Our institutional ethics committee approved the study, and the patients’ parents provided written informed consent.

Target selection and sequencing

Exome sequencing was conducted on patient 1. Genomic DNA was extracted from whole blood using a NucleoSpin Blood Genomic DNA kit (Takara), sheared into approximately 150- to 200-bp fragments, and used to make a library for multiplexed paired-end sequencing (Illumina). The constructed library was hybridized to biotinylated cRNA oligonucleotide baits from the SureSelect Human All Exon 50Mb Kit (Agilent Technologies) for exome capture. Targets were sequenced on an Illumina HiSeq2000 platform in paired-end 101-bp configuration.

Mapping and single nucleotide variation (SNV)/Indel calling

Adapter sequences and low-quality bases were removed by Cutadapt (version 1.1). After quality control, reads were mapped to the reference human genome (hg19) using BWA (version 0.5.9). Mapping results were corrected using Picard (version 1.49) to remove duplicates and GATK (version 1.1–31) for local alignment and quality score recalibration. SNV and Indel calls were performed with multisample calling using GATK and filtered to coordinates with VQSR passed and genotype quality score ≥30. Annotations of SNVs and Indels were based on dbSNP135, CCDS (NCBI, November 2011), RefSeq (UCSC Genome Browser, November 2011), Encode (UCSC Genome Browser, version 7), and 1000 Genomes (October 2011). Variants were further filtered according to the following criteria: 1) genotype call existed in all of the 5 samples at the detected position; 2) predicted to cause framshift, nonsense, read-through, missense, deletion, insertion, or insertion-deletion; 3) located outside the Segmental Duplications region (UCSC Genome Browser, November 2011); and 4) located outside the Simple Tandem Repeats region (UCSC Genome Browser, November 2011).

Sequence analysis of IGSF1

Genomic DNA was extracted from patients 2 to 4 and the mothers of patients 1, 3, and 4 as described above for patient 1. Exon and exon-intron boundaries of IGSF1 were amplified from genomic DNA by PCR using primers reported previously (19). PCR products were sequenced directly with an ABI 373A automated fluorescent sequencer (PE Applied Biosystems).

In vitro analysis of mutant IGSF1

Constructs

The human Myc-IGSF1-3xHA expression construct was from Dr Peter Scheiffele (Biozentrum, University of Basel, Switzerland). The IGSF1-C-terminal domain (CTD) antibody epitope therein was modified using the QuikChange site-directed mutagenesis (Stratagene) to match that of the murine protein to facilitate detection with the antimurine IGSF1-CTD polyclonal antibody (22). The p.V1082E, p.R1189X, and p.Q645X mutations were introduced into the construct using the QuikChange method and the primers in Table 2. The constructs were verified by DNA sequencing (McGill University and Genome Québec Innovation Centre).

Cell culture and transfections

For Western blotting and cell surface biotinylation, HEK293 cells were seeded in 6-well plates, grown until 80% confluent, and transfected with 200 ng expression vector using Lipofectamine 2000 (Invitrogen) following the manufacturer’s instructions. For immunofluorescence, HEK293 cells were seeded on glass coverslips at 50,000 cells per well and transfected with 100 ng expression vector. For Northern blotting, HEK293 cells were seeded in 10-cm dishes and transfected when 70% confluent with 4 μg of the indicated expression constructs.

Deglycosylation and Western blotting

Whole-cell protein extracts were prepared from transfected HEK293 cells 24 hours after transfection using RIPA buffer (150 mM NaCl, 50 mM NaF, 10 mM NaPO4, 2 mM EDTA, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS)) with protease inhibitors (Roche). Protein lysates were deglycosylated using peptide-N-glycosidase F (PNGaseF) and endoglycosidase H (EndoH) (New England Biolabs) according to the manufacturer’s instructions (2 hours at 37°C). The
proteins were denatured at 100°C for 5 minutes in Laemmli buffer containing 2% 2-mercaptoethanol and resolved by SDS-PAGE on 8% Tris-glycine gels. The proteins were transferred to nitrocellulose membranes (25 V, 1.5 hours), the membranes blocked with 5% milk in 0.05% Tween 20 in Tris-buffered saline (TBST), and incubated overnight at 4°C in a 1:10 000 dilution of mouse antihuman influenza hemagglutinin (HA) antibody (H9658; Sigma) or a 1:1000 dilution of rabbit anti-IGSF1-CTD antibody in blocking buffer. The next day, membranes were washed 3 times for 15 minutes each in TBST, followed by incubation with goat antimouse or goat antirabbit horseradish peroxidase-conjugated secondary antibody (Bio-Rad; 1:3000 in blocking buffer) for 1 hour at room temperature. The membranes were washed 3 times for 15 minutes each in TBST, followed by incubation with goat antimouse or goat antirabbit horseradish peroxidase-conjugated secondary antibody (Bio-Rad; 1:3000 in blocking buffer) for 1 hour at room temperature. The membranes were washed 3 times for 15 minutes each in TBST, followed by incubation with goat antimouse or goat antirabbit horseradish peroxidase-conjugated secondary antibody (Bio-Rad; 1:3000 in blocking buffer) for 1 hour at room temperature.

### Table 1. Endocrinological Findings and IGSF1 Mutations in 4 Japanese Patients With C-CH

<table>
<thead>
<tr>
<th>Patient</th>
<th>1</th>
<th>1 (second test)</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at diagnosis</td>
<td>5 d (screening)</td>
<td>4 y</td>
<td>5 d (screening)</td>
<td>21 d</td>
<td></td>
</tr>
<tr>
<td>Symptoms</td>
<td>None</td>
<td>Short stature</td>
<td>None</td>
<td>Failure to thrive, constipation</td>
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<tr>
<td>Blood FT4 at MS, ng/dL&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.68</td>
<td>ND</td>
<td>0.79</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Blood TSH at MS, μU/mL</td>
<td>1.2</td>
<td>Normal</td>
<td>2.7</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>FT&lt;sub&gt;4&lt;/sub&gt; at referral, ng/dL&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.57&lt;sup&gt;d&lt;/sup&gt; (22 d)</td>
<td>0.76&lt;sup&gt;d&lt;/sup&gt; (4 y)</td>
<td>0.52&lt;sup&gt;c&lt;/sup&gt; (24 d)</td>
<td>0.78&lt;sup&gt;c&lt;/sup&gt; (21 d)</td>
<td></td>
</tr>
<tr>
<td>FT&lt;sub&gt;3&lt;/sub&gt; at referral, pg/mL</td>
<td>3.60&lt;sup&gt;d&lt;/sup&gt; (22 d)</td>
<td>2.35&lt;sup&gt;d&lt;/sup&gt; (4 y)</td>
<td>1.96&lt;sup&gt;c&lt;/sup&gt; (24 d)</td>
<td>3.82&lt;sup&gt;c&lt;/sup&gt; (21 d)</td>
<td></td>
</tr>
<tr>
<td>TSH at referral</td>
<td>2.7&lt;sup&gt;d&lt;/sup&gt; (22 d)</td>
<td>2.35&lt;sup&gt;d&lt;/sup&gt; (4 y)</td>
<td>3.5&lt;sup&gt;c&lt;/sup&gt; (24 d)</td>
<td>&lt;0.01&lt;sup&gt;c&lt;/sup&gt; (21 d)</td>
<td></td>
</tr>
<tr>
<td>PRL, ng/mL&lt;sup&gt;e&lt;/sup&gt; (age at test)</td>
<td>3.5 (6 y)</td>
<td>0.99 (4 y)</td>
<td>86.2 (24 d)</td>
<td>3.1 (7 mo)</td>
<td></td>
</tr>
<tr>
<td>TSH levels after TRH stimulation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(age at test)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>0.69</td>
<td>0.40</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Peak</td>
<td>0.82</td>
<td>0.38</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>FT&lt;sub&gt;4&lt;/sub&gt; levels after TRH test</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>(age at test)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>12.67</td>
<td>0.02</td>
<td>3.98</td>
<td>7.95</td>
<td></td>
</tr>
<tr>
<td>Peak</td>
<td>50.89</td>
<td>0.05 (11 y)</td>
<td>6.72 (4 y)</td>
<td>23.47</td>
<td></td>
</tr>
<tr>
<td>120 min</td>
<td>22.15 (6 y)</td>
<td></td>
<td>15.5 (24 d)</td>
<td></td>
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<tr>
<td>PRL levels after TRH stimulation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(age at test)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>3.5</td>
<td>1.7</td>
<td>0.99</td>
<td>86.2</td>
<td></td>
</tr>
<tr>
<td>Peak</td>
<td>21.5 (6 y)</td>
<td>1.52</td>
<td>131.8 (24 d)</td>
<td>ND</td>
<td></td>
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<tr>
<td>GH, ng/mL (stimulus)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>0.35</td>
<td>0.36</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Peak</td>
<td>20.1 (by GHRH)</td>
<td>0.82 (by ITT)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LH, mIU/mL&lt;sup&gt;f&lt;/sup&gt; (age at test)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>0.05</td>
<td>0.06</td>
<td>0.33</td>
<td>0.31 (4 mo)</td>
<td></td>
</tr>
<tr>
<td>Peak</td>
<td>2.6 (5 y)</td>
<td>3.1 (11 y)</td>
<td>8.48 (4 y)</td>
<td>0.43 (7 mo)</td>
<td></td>
</tr>
<tr>
<td>FSH, mIU/mL&lt;sup&gt;f&lt;/sup&gt; (age at test)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>1.6</td>
<td>2.0</td>
<td>2.42</td>
<td>4.29 (4 mo)</td>
<td></td>
</tr>
<tr>
<td>Peak</td>
<td>19.8 (5 y)</td>
<td>10.9 (4 y)</td>
<td>2.47 (7 mo)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mother</td>
<td>Carrier</td>
<td>Carrier</td>
<td>Carrier</td>
<td>Carrier</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: MS, mass screening; ITT, insulin tolerance test; ND, not determined; PRL, prolactin.

<sup>a</sup> The conversion factors to the SI unit are as follows: FT<sub>4</sub>, 12.87 (pmol/L); TSH, 1.0 (mIU/L); FT<sub>3</sub>, 1.536 (pmol/L); PRL, 1.0 (g/L); GH, 1.0 (g/L); LH, 1.0 (IU/L); and FSH, 1.0 (IU/L).
<sup>b</sup> Cutoff level of FT<sub>4</sub> at neonatal screening was 1.0 ng/dL.
<sup>c</sup> Normal range for this age, FT<sub>4</sub>, 1.40–1.74 ng/dL, FT<sub>3</sub>, 3.5–4.9 pg/mL, TSH, 0.30–3.50 μU/mL.
<sup>d</sup> Normal range for this age: FT<sub>4</sub>, 1.09–1.55 ng/dL; FT<sub>3</sub>, 3.9–5.1 pg/mL; TSH, 0.012–4.89 μU/mL.
<sup>e</sup> Normal range for 5-year-old, 1.6–14.8 ng/mL; normal range for 12-year-old, 1.3–10.8 ng/mL.
<sup>f</sup> Normal range for LH for 10-year-old, 0.02–0.15 (basal) and 1.70–3.77 (peak); for 10-year-old, 0.04–0.25 (basal) and 2.03–11.8 (peak) mIU/mL. Normal range for FSH for 10-year-old, 0.38–0.1.11 (basal) and 0.95–3.57 (peak); for 10-year-old, 4.38–9.48 (basal) and 5.69–16.6 (peak) mIU/mL.
bation in ECL Plus reagent (PerkinElmer) and then exposed to x-ray film (GE Healthcare).

**Cell immunofluorescence**

Transfected HEK293 cells grown on glass coverslips in 24-well plates were subjected to immunofluorescence as previously described (19).

**Cell surface biotinylation and immunoprecipitation**

Transfected HEK293 cells grown in 6-well plates were subjected to cell surface biotinylation as previously described (19).

**Northern blotting**

RNA was extracted from HEK293 cells approximately 24 hours after transfection using Trizol. Twenty micrograms of each RNA were resolved on formaldehyde-MOPS [3-N-morpholino]propanesulfonic acid] gels as previously described (24). RNA was transferred overnight with [32P]dCTP-labeled (3000 Ci/mmol, 10 mCi/mL; PerkinElmer) cDNA probes corresponding to exons 18 to 20 of murine *Igf1* or murine *Rpl19* (the same probes as in Sun et al [19]) in 50% formamide, 5 × SSC, 1 × Denhardt’s, 20M NaPO4 (pH 6.8), 1% SDS, 5% dextran sulfate, and 100 µg/mL denatured salmon sperm DNA. Membranes were washed twice for 30 minutes each in 2 × SSC/0.1% SDS at room temperature and at 65°C and then exposed to x-ray film.

**Patient reports**

**Patient 1**

Patient 1, now a 12-year-old Japanese boy, was born after 39 weeks gestation by normal vaginal delivery in Sapporo City and was the third child of nonconsanguineous, healthy parents. There was no family history of thyroid disease. His birth weight was 3330 g, and his length was 51.0 cm. Neonatal mass screening for CH showed blood TSH of 1.2 µU/mL and FT4 of 0.68 ng/dL at 5 days of age. On the basis of these results, a second blood filter paper sample was taken at 14 days, the results of which were as follows: TSH 1.5 µU/mL and FT4 0.47 ng/dL, indicating CH. He was referred to our hospital at 22 days. At that time, he showed no symptoms; however, bilateral femoral epiphyses were absent by x-ray examination. His serum thyroid function was: TSH 2.7 µU/mL, FT4 0.57 ng/dL, FT3 3.60 pg/mL (normal range for this age, TSH 0.30–3.50 µU/mL, FT4 1.40–1.74 ng/dL, and FT3 3.5–4.9 pg/mL, respectively). As he was confirmed to have CH, levothyroxine was initiated. At the age of 6 years, he underwent 123I scintigraphy after levothyroxine treatment was discontinued for 1 month, and his thyroid and pituitary function were evaluated. The scintigraph was normal, and uptake was 34% at 30 minutes each in 2 × SSC/0.1% SDS at room temperature and at 65°C and then exposed to x-ray film. Imaging (MRI) showed normal findings, including the hypothalamic-pituitary region. At 12 years of age, his height was 140.5 cm (~0.91 SD for a normal Japanese boy), his weight was 37.1 kg (~0.40 SD for a normal Japanese boy), and his body mass index was 18.9 (+0.34 SD for a normal Japanese boy) (25). His bilateral testicular sizes were 3 mL, and pubic hair was Tanner stage 1. As shown in Table 1, in response to GnRH provocation, LH remained low, which corresponds to the result expected at prepubertal age (basal LH 0.06 µIU/mL, peak LH 3.1 µIU/mL, basal FSH 2.0 µIU/mL, and peak FSH 17.4 µIU/mL). These findings indicated that he had delayed puberty compared with normal Japanese boys. The second TRH stimulation at this time showed a low response of serum TSH.

**Patient 2**

Patient 2 is now a 13-year-old Japanese boy born after 42 weeks gestation by normal vaginal delivery. His birth weight was 4000 g, and his birth height was 53.2 cm. CH screening in his area was based on TSH alone, and the result was normal. He was the first child of nonconsanguineous parents. At the age of 4 years, he was referred to the hospital because of short stature. At that time, his height was 85.5 cm (~3.9 SD for a normal Japanese boy) and his weight was 14.0 kg (~1.0 SD for a normal Japanese boy). His bone age was markedly delayed (1.6 years old by Japanese Tanner-Whitehouse 2). His serum IGF-1 was markedly low (0.64 ng/mL; normal range for this age, 29–173 ng/dL) and basal prolactin was also low (0.99 ng/mL) (Table 1). CH was evident (FT4 0.76 ng/dL and TSH 2.35 µU/mL [normal range for this age, FT4 1.09–1.55 ng/dL and TSH 0.12–4.89 µU/mL]). A TRH stimulation test showed low responses of serum TSH and prolactin (TSH, basal 3.98 µU/mL and peak 6.72 µU/mL; prolactin, basal 0.99 ng/mL and peak 1.52 ng/mL). An insulin tolerance test showed low responses of GH (0.36–0.82 ng/mL) (Table 1). A brain MRI showed a normal hypothalamic-pituitary region. Based on these findings, he was diagnosed as having GH deficiency and CH; GH and levothyroxine were initiated. At 12.9 years of age, his height was 147.1 cm (~1.4 SD for a normal Japanese boy), weight was 57.7 kg (~1.0 SD for a normal Japanese boy), and body mass index was 26.7 (~2.8 SD for a normal Japanese boy). His bilateral testicular size was 4 mL, and pubic hair was Tanner stage 1. Recent blood examination showed LH <0.2 mIU/mL, FSH 6.2 mIU/mL, and prolactin <1.0 ng/mL.

**Patient 3**

Patient 3 was born in Sapporo City by normal vaginal delivery after 36 weeks gestation. There was no family history of thyroid disease. His birth weight and height were 2924 g and 45.7 cm. Neonatal screening for CH at 5 days of age was positive (blood TSH 2.7 µU/mL, FT4 0.79 pg/dL). A second blood sample was obtained at 20 days, at which time TSH was <0.5 µU/mL, and FT4 was 0.52 ng/dL. At the age of 24 days, he was referred to us for endocrinological evaluation. FT4 and FT3 were 0.52 ng/dL and 1.96 pg/mL, respectively (normal range for this age, FT4 1.40–1.74 ng/dL and FT3 3.5–4.9 pg/mL, respectively). Brain MRI was normal. In response to TRH stimulation, serum TSH rose from 7.95 to 23.47 µU/mL at 30 minutes. At 120 minutes, serum TSH remained elevated (15.50 µU/mL). TRH also stimulated serum prolactin, from 86.2 to 131.8 ng/mL. Based on these findings, he was diagnosed with CH, and levothyroxine treatment was started. He is currently 1.3 years old, and his
developmental milestones are within the normal range. His bi-
lateral testicular size is 1 mL.

Patient 4

The patient was born by normal vaginal delivery after 40
weeks gestation. His birth weight and height were 4510 g and
53.6 cm. CH screening in his area was based on TSH alone, and
the result was normal. There was no family history of thyroid
disease. At 21 days, he showed failure to thrive and constipation.
At this time, his serum TSH was $<0.01 \mu U/mL$, FT₄ was 0.78
ng/dL, and FT₃ was 3.82 pg/mL. He was suspected of having CH,
and levothyroxine was started. MRI showed that the brain and

![Image of patient 1, mutation, and mother's heterozygosity]

**Figure 1.** *IGSF1* mutations in patients 1, 2, 3, and 4. A, Patient 1 had the mutation c.3565C>T (p.R1189X) in *IGSF1*. B, A chromatograph showing the corresponding sequence in a control sample. C, Patient 1’s mother was heterozygous for the c.3565C>T (p.R1189X) mutation. D, Patient 2 had an identical mutation to patient 1. E, Patient 3 had a missense mutation (c.3245T>A, p.V1082E) in *IGSF1*. The chromatograph presented is from the reverse (antisense) strand. F, Patient 3’s mother was heterozygous for this mutation. G, A chromatograph showing the corresponding sequence in a control sample. H, patient 4 harbored a nonsense mutation (c.1933 C>T, p.Q645X) in *IGSF1*. I, His mother was heterozygous for this mutation. J, A chromatograph showing the corresponding sequence in a control sample.
hypothalamic-pituitary regions were normal. Since initiation of levothyroxine, symptoms have disappeared and he has grown well. At the age of 7 months, he was referred to a pediatric endocrinologist for follow-up. At that time, endocrine examination demonstrated low normal serum prolactin (3.1 ng/mL; normal range for this age, 2.0–19.6 ng/mL) and normal LH and FSH levels (LH 0.43 mIU/mL and FSH 2.47 mIU/mL, respectively). He is currently 1.6 years old, and his growth and developmental milestones are within normal ranges. His bilateral testicular size is 1 mL.

Results

Whole-exome sequencing of DNA from patient 1 identified many nonsynonymous variants, but only 1 hemizygous mutation (c.3565C>T, p.R1189X) in the Xq25-linked IGSF1 gene. We suspected that this may explain his TSH concentrations, prolactin deficiency, and low responses of LH and FSH for his age, because the region of Xq25 in which IGSF1 is located was previously implicated in pituitary hormone deficiency (19, 26, 27). Furthermore, this base change was not identified in 200 normal Japanese control alleles. Sanger sequencing confirmed the presence of the mutation (Figure 1A). The corresponding sequence from a control subject is shown for reference (Figure 1B). The boy’s mother was heterozygous for the c.3565C>T mutation (Figure 1C), indicative of X-linked recessive inheritance. We therefore considered this nonsense mutation as a candidate cause of his C-CH.

We analyzed 3 more Japanese boys with C-CH by PCR direct sequencing. Patient 2, who has no known relationship to patient 1, nonetheless harbored the identical IGSF1 mutation (c.3565C>T, p.R1189X) (Figure 1D). His mother was heterozygous for the mutation (Figure 1F), whereas it was absent in 200 normal Japanese alleles (e.g., Figure 1G). Further sequence analysis in patient 4 identified yet another base change, c.1933C>T, in IGSF1 (Figure 1H), generating a premature stop codon (p.Q645X). In the course of our investigations, Sun et al (19) described 10 IGSF1 mutations or whole-gene deletions in males with C-CH, macro-orchidism, and variable prolactin deficiency. None of the 3 mutations we identified matched those described in Sun et al. However, similar to their observations, our mutations clustered in the 3’ part of the gene, which encodes the CTD of the IGSF1 protein (Figure 2).

We next examined whether the 3 mutations might be pathogenic. When introduced into a human IGSF1 expression vector, the V1082E mutation impaired protein maturation relative to wild-type in heterologous HEK293 cells (compare the upper bands in Figure 3A, lanes 2 and 5). Most of the mutant protein was EndoH-sensitive (compare lanes 4 and 7), indicating that it was retained in the endoplasmic reticulum (ER) during its biosynthesis. Although some V1082E mutant protein reached the cell surface in transfected cells (Figure 3B), it was greatly impaired in doing so compared with wild-type, as indicated by cell surface biotinylation (upper panel in Figure 3C).

A similar analysis was applied to the R1189X truncation mutant (Figure 4). Here, the mutant protein migrated as a single, EndoH-sensitive band on SDS-PAGE, again indicating ER retention (Figure 4A, compare lanes 5 and 7). Indeed, no R1189X protein could be detected at the cell membrane in intact (nonpermeabilized) cells (Figure 4B, top right panel). The Q645X mutant is predicted to generate a protein of only 63 amino acids that terminates within the first Ig loop of the CTD. IGSF1 is internally cleaved at G582-V583 by a signal peptidase-like protease.

Figure 2. Schematic representation of the IGSF1 protein domain structure and the relative locations of mutations identified in this study.
(22). Although the epitope recognized by our CTD antibody is retained within the 63-amino-acid peptide that would be generated by a Q645X expression vector, we failed to detect the predicted 6.9-kDa protein by Western blotting in lysates from transfected cells (data not shown). However, the mRNA transcribed from the Q645X vector was comparable to that of the wild-type vector (Supplemental Figure 1, published on The Endocrine Society’s Journals Online website at http://jcem.endojournals.org). Therefore, impairment in the expression of this mutant appears to be posttranscriptional.

**Discussion**

We identified 3 novel hemizygous IGSF1 mutations in 4 male patients with C-CH. In addition, X-recessive inheritance of this disease was confirmed in 3 cases. Although patients 1 and 2 had identical mutations, based on interviews, there was no apparent relation between the two families, although this was not ruled out experimentally. Consistent with our results, Sun et al (19) recently reported that loss-of-function mutations in IGSF1 caused X-linked C-CH and macro-orchidism in 11 unrelated European families. During the review of the present communication, we also reported an additional IGSF1 insertion mutation in a teenage male with C-CH, but normal-sized testes (28).

Four nonsense and 4 missense or in-frame deletion mutations were described in Sun et al (19). In vitro analyses to determine the functional consequences of these mutations indicated that the encoded proteins migrated predominantly as immature glycoforms on SDS-PAGE and were largely retained in the ER, resulting in decreased membrane expression levels. Application of these analyses to the 1 missense and 2 nonsense mutations identified here revealed similar results. V1082E was severely impaired in its posttranslational modification and membrane trafficking.

![Figure 3](https://example.com/figure3.png)

*Figure 3.* The V1082E mutation impairs protein trafficking to the plasma membrane. A, HEK293 cells were transfected with pcDNA3 (empty vector) or wild-type (WT) or V1082E IGSF1-1 expression vectors. Protein lysates were deglycosylated with either PNGaseF or EndoH, resolved by SDS-PAGE, and immunoblotted using an human influenza hemagglutinin (HA) antibody. B, HEK293 cells were transfected with the same constructs as in A. Expression of IGSF1-CTD was analyzed by immunofluorescence using the IGSF1-CTD antibody under nonpermeabilizing and permeabilizing conditions. Nuclei were stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (blue). C, HEK293 cells were transfected with the same constructs as in A. Membrane expression of IGSF1-CTD was analyzed by cell-surface biotinylation. Abbreviations: HRP, horseradish peroxidase; IB, immunoblot; IP, immunoprecipitation; ACTB, β-actin.
ing, whereas R1189X was completely absent at the cell surface. We were unable to detect Q645X protein in transfected cells. Because the mRNA is normally expressed from a Q645X expression vector, it is likely that the 63-amino-acid IGSF1-CTD peptide generated from this variant is highly unstable and is rapidly degraded after its translation. Thus, all of the pathogenic IGSF1 mutations associated with C-CH identified thus far cluster in the portion of the gene encoding the CTD (or are entire gene deletions) and are predicted to cause the loss of normal IGSF1 protein function and/or expression in the pituitary gland.

In Sun et al (19), 6 familial cases of C-CH in The Netherlands and 1 in Italy were identified in neonatal screening. Using a previously reported neonatal screening method in Sapporo City, based on simultaneous determination of TSH and FT$_4$ (3), we similarly identified 2 patients with CH and associated IGSF1 mutations. One patient (patient 2) showed growth retardation, and medical examination identified both GH deficiency and CH. It is possible that CH might impair GH secretion in this patient. Alternatively, because IGSF1 is expressed in somatotropes (at least in mice), we cannot rule out a direct effect of the gene defect in these cells. In Sun et al (19), 4 patients were diagnosed with GH deficiency along with CH; GH therapy was accordingly initiated. GH secretion was reevaluated in 3 patients in young adulthood, and GH deficiency was not detected (19). Reevaluation of GH secretion is therefore needed for our patient who was diagnosed with GH deficiency.

Three of our 4 patients had low prolactin levels. In Sun et al (19), 18 of 26 cases were similarly prolactin-deficient (19). Two of our patients showed delayed puberty (the other 2 are still too young to evaluate in this regard), and their serum LH and FSH levels remained at prepubertal levels even at 12 years of age. In Sun et al (19), 10 of 11 evaluated patients showed delayed testosterone production and pubertal growth. Delayed secondary sex characteristics may therefore be one of the clinical hallmarks in patients with pathogenic IGSF1 mutations. Moreover, Sun et al (19) reported testicular enlargement after adolescence as a defining feature of the syndrome associated with IGSF1 mutations. Careful follow-up for pubertal development and testicular size is therefore required in our current and future patients. However, it should be noted that we observed normal testicular size in another Japanese male with C-CH and an IGSF1 mutation (28).

A TRH stimulation test in our patient 1 at 5 years of age showed high basal and peak serum TSH, in addition to a delayed decrease at 120 minutes. In patient 3, an increased basal TSH level and delayed decrease of TSH after TRH stimulation were also observed. Sun et al demonstrated lower responses of serum TSH levels after TRH stimulation in patients with IGSF1 defects than in age-matched controls (19). Therefore, the results of our two studies appear to diverge with respect to the effects of TRH stimulation. Several reports show that an impaired TSH response is indicative of primary pituitary disease, whereas an excessive and delayed rise in serum TSH is indicative of primary hypothalamic disease (29, 30). However, there is still controversy surrounding this differentiation (31–33). Recent studies have demonstrated overlap between patients with pituitary disturbance and those with hypothalamic disturbance in terms of the serum TSH response to TRH (32, 33). Thus, it remains plausible that TSH deficiency is caused by a pituitary defect in our patients. That said, IGSF1 is expressed in murine hypothalamus (34); therefore, C-CH patients with inactivating IGSF1 mutations may possess both pituitary and hypothalamic defects.

A TRH test was performed twice in patient 1. Compared with the result of the first TRH test, a markedly low
response of serum TSH was observed in the second test. In Igsf1–knockout mice, pituitary TSH synthesis and secretion are reduced, and murine pituitary TRH receptor mRNA expression is decreased (19). It was speculated, therefore, that impaired TRH receptor signaling might be a mechanism of C-CH in IGSF1–deficient patients. TRH signaling through the TRH receptor is important for normal proliferation of thyrotropes (35, 36). A diminished TSH response to TRH stimulation with age in our patient 1 may be caused by failure of thyrotropes to proliferate normally during postnatal development. This may indicate that IGSF1, together with the TRH signaling system, is involved in thyrotrope proliferation and differentiation, in addition to TSH secretion. The physiological role of IGSF1 in TRH-mediated TSH secretion requires more investigation.

To date, the exact prevalence of C-CH caused by IGSF1 defects has not determined. We screened 83,232 neonates and identified 6 patients with C-CH from 2000 to 2004, as reported previously (3). Among these 6 patients, only 1 (patient 1 in the present study) had an IGSF1 mutation, and the remaining 5 patients were diagnosed as having congenital pituitary abnormalities or Prader-Willi syndrome (3). Therefore, the frequency of IGSF1 mutations may be rare, but more study is required to clarify the prevalence of C-CH caused by IGSF1 defects.

In conclusion, we identified 3 novel loss-of-function mutations in the IGSF1 gene as the likely cause of C-CH in 4 Japanese patients.

Acknowledgments

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