CLINICAL STUDY

High allele frequency of the p.Q258X mutation and identification of a novel mis-splicing mutation in the *STAR* gene in Korean patients with congenital lipoid adrenal hyperplasia

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Abstract

Objective: Steroidogenic acute regulatory (*STAR*) protein plays a crucial role in steroidogenesis, and mutations in the *STAR* gene cause congenital lipoid adrenal hyperplasia (CLAH). This study investigated the *STAR* mutation spectrum and functionally analyzed a novel *STAR* mutation in Korean patients with CLAH.

Methods: Mutation analysis of *STAR* was carried out in 25 unrelated Korean CLAH patients. A region of *STAR* comprising exons 4–7 was cloned from human genomic DNA into an expression vector, followed by site-directed mutagenesis and transient expression in COS7 cells. The splicing pattern was analyzed by *in vitro* transcription, and each transcript was functionally characterized by measuring pregnenolone production in COS7 cells cotransfected with the cholesterol side chain cleavage system.

Results: Mutation p.Q258X was identified in 46 of 50 alleles (92%); mutation c.653C>T was detected in two alleles (4%); and mutations p.R182H and c.745–6_810del were found in one allele (2%). Reverse transcriptase-PCR products amplified from a patient heterozygous for compound c.653C>T and c.745–6_810del mutation revealed multiple alternatively spliced mRNAs. *In vitro* expression analysis of a minigene consisting of exons 4–7 containing the c.653C>T yielded two transcripts in which exon 6 or exons 5 and 6 were skipped. The encoded proteins exhibited defective pregnenolone-producing ability. The c.745–6_810del mutation led to full and partial intron retention.

Conclusions: p.Q258X is the most common *STAR* mutation in Korea. A previously reported c.653C>T variant was found to cause aberrant splicing at the mRNA level, resulting in perturbation of *STAR* function. The c.745–6_810del mutation also resulted in aberrant splicing.

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Introduction

Congenital lipoid adrenal hyperplasia (CLAH) is an autosomal recessive disorder characterized by impaired synthesis of all adrenal and gonadal steroid hormones. CLAH is the most severe form of congenital adrenal hyperplasia and generally manifests as hyperpigmentation and adrenal insufficiency in the neonatal period. CLAH is either caused by mutations of the steroidogenic

acute regulatory (*STAR*) gene or the gene encoding the cholesterol side chain cleavage enzyme, *P450scc* (1). Defects in the *STAR* gene account for a majority of cases; very few reported cases of CLAH are caused by a defect in *P450scc* (2–4). Molecular analyses of the *STAR* gene in patients with CLAH have provided insight into the structure and function of the *STAR* protein. To date, 48 different mutations in the *STAR* gene have been identified in various ethnic groups

(<http://www.hgmd.org/>). The incidence of certain mutations is very high in specific ethnic groups. Genetic clusters consistently identified to date include the p.Q258X mutation in the Japanese and Korean populations (5, 6), the p.R182L mutation among Palestinian Arabs (7), the p.R182H mutation in eastern Saudi Arabia (8), and the p.L260P mutation in the Swiss population (9). However, in most Palestinian cases, a founder c.201_202delCT mutation in *STAR* is the most common (10); the p.R182L mutation has also been described in a Japanese patient (11).

This study was carried out to investigate the clinical and endocrinological characteristics and mutation spectrum of the *STAR* gene in 25 unrelated Korean patients with CLAH. The significance of the molecular analysis of the *STAR* gene in Korean patients with CLAH and the relationships between genetic alterations in the *STAR* gene and clinical features of CLAH are discussed.

Subjects and methods

Subjects

Twenty-five unrelated Korean patients were diagnosed with CLAH based on endocrine profiles and mutation analysis. Clinical features (skin hyperpigmentation, external genitalia, and salt-wasting) and endocrine data (serum electrolytes, plasma ACTH, renin, and 17-OHP levels) were analyzed. This study protocol was approved by the Institutional Review Board of Asan Medical Center, and written informed consent was obtained from all subjects or their parents.

Mutation analysis of the *STAR* gene

Genomic DNA was extracted from peripheral blood leukocytes using a Puregene DNA isolation kit (Gentra, Minneapolis, MN, USA). Seven exons and associated intronic flanking regions of the *STAR* gene were amplified by PCR and directly sequenced in both orientations using a BigDye Terminator v3.1 Cycle Sequencing Kit and an ABI3130xl Genetic analyzer (Applied Biosystems, Foster City, CA, USA).

Reverse transcriptase-PCR

Total RNA was isolated from testis fibroblast cells of subject 25 using the RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's instructions and cDNA was synthesized. Reverse transcriptase-PCR (RT-PCR) of exon 4 through 7 of the *STAR* gene was performed using the primers 4S (forward: 5'-GAC_AAT_GGG_GAC_AAA_GTG-3') and 7AS (reverse: 5'-TCA_ACA_CCT_GGC_TTC_AGA_GG-3'). The amplified products were separated on a 1.2% agarose gel and directly sequenced.

Computational prediction of splicing alterations

We used publicly available *in silico* tools to predict the impact of c.653C>T mutation. Splicing efficiencies of wild-type (WT) and mutant sequences were calculated using three splice prediction programs: the Berkeley *Drosophila* Genome Project (BDGP, available at http://www.fruitfly.org/seq_tools/splice.html), Net-gene2 (available at <http://www.cbs.dtu.dk/services/NetGene2/>), and EX-SKIP (available at <http://ex-skip.img.cas.cz/>). The first two programs provide a similar type of data output, namely, quantitative scores for WT and mutant splice site sequences that reflect splice site strength. The third program simply compares the exonic splice enhancer (ESE) and exonic splice silencer (ESS) profiles of a WT and a mutated allele to rapidly determine which exonic variant has the highest likelihood of skipping a given exon. It calculates the total number of ESSs, ESEs, and their ratio.

In vitro expression of the *STAR* minigene

A 2175 bp region of the *STAR* gene comprising exons 4 through 7, including introns 4, 5, and 6, was amplified by PCR from human genomic DNA using the primers of 4S and 7AS and then cloned into the expression vector, pcDNA3.1/NT-GFP (Invitrogen). Mutants were generated by site-directed mutagenesis and verified by direct sequencing. Three micrograms of the mutant (MT) or WT constructs were transfected into COS7 cells using JetPEI (Polyplus-Transfection, Illkirch, France). After 48 h, total RNA was extracted from cells using the RNeasy Plus Mini Kit (Qiagen) and the cDNA was synthesized. The resulting cDNA was amplified by PCR using the primers of 4S and 7AS. The products were directly sequenced and separated on a 1.2% agarose gel and visualized with ethidium bromide. Gels containing different RT-PCR fragments were examined on an u.v. imaging system, and relative band intensities were evaluated using LabWorks 4.6 Software (UVP Products, Upland, CA, USA).

Functional expression study of mutant *STAR* proteins in COS7 cells

The full-length *STAR* cDNA was cloned into pCMV-Flag vector and a fusion protein (F2) plasmid that consists of the cholesterol side chain cleavage system (NH₂-cholesterol side chain cleavage enzyme (P450_{sc})-adrenodoxin reductase-adrenodoxin-COOH) (12). The pCMV-Flag-*STAR* plasmid and F2 were provided by Dr Walter L Miller (Department of Pediatrics, University of California San Francisco, San Francisco, CA, USA). Mutant *STAR* (MT-*STAR*) cDNAs were prepared from patient's testis RNA after RT-PCR and were generated by site-directed mutagenesis of cloned WT-*STAR* cDNA. All mutants were confirmed by direct sequencing.

COS7 cells were cotransfected with the F2 plasmid (1.5 µg) and 1.5 µg pCMV-Flag-WT-*STAR*, pCMV-Flag-MT-*STAR*, or empty pCMV-Flag. Forty-eight hours after transfection, the medium was collected for assay of pregnenolone. Pregnenolone secreted into the culture medium was determined by ELISA according to the manufacturer's instructions (Pregnenolone ELISA Kit, Alpco Diagnostics, Windham, NH, USA) and was analyzed in at least three separate transfections.

Results

Clinical and hormonal characteristics

Subjects ranged in age from 0.4 to 16.0 years. None had a history of consanguinity, but the first phenotypically female offspring of subject 2 had died of unknown etiology at the age of 1 month (Tables 1 and 2). All patients manifested adrenal insufficiency in the neonatal period. Their presenting symptoms varied and included recurrent vomiting, fever, lethargy, seizure, and skin hyperpigmentation. Two subjects (subjects 14 and 15) presented with persistent cholestasis. Basal ACTH and renin levels were extremely elevated in all patients, with minimal concentrations of most adrenal and gonadal steroids. Adrenal gland imaging was performed in 15 patients, revealing an enlarged adrenal gland in 13 patients. Fourteen of 25 patients were 46,XY karyotype males with female

external genitalia and 11 had a 46,XX karyotype. Seven of 14 46,XY patients underwent bilateral gonadectomy at the age of 12 months to 8 years. All subjects were reared as female. All affected individuals responded to hydrocortisone and 9 α -fludrocortisone replacement therapy. Two subjects (subjects 9 and 21) had neurodevelopmental deficits. Two genetically female patients (subjects 1 and 9) experienced spontaneous puberty that manifested as breast development at the age of 10.3 and 13.7 years respectively. Spontaneous menarche occurred in these patients at the age of 12.9 and 15.8 years respectively. Ovarian cyst torsion occurred at the age of 14 years in subject 1, who received a laparoscopic left salpingo-oophorectomy at the age of 14 years (13). Subject 14 died of adrenal crisis at the age of 2 months due to poor adherence to medication.

Mutation analysis of the *STAR* gene

A molecular analysis of the *STAR* gene was performed in 25 unrelated Korean patients with CLAH using genomic DNA from peripheral blood leukocytes. Four different mutations, including the novel splicing mutation c.745–6_810del, were identified (Table 2). Of these mutations, the c.772C>T (p.Q258X) mutant allele was the most common and was identified in 46 of the 50 alleles (92%). The c.653C>T mutation was detected in two of the 50 alleles (4%). The c.545G>A (p.R182H) and c.745–6_810del mutations were found

Table 1 Clinical characteristics of patients with congenital lipid adrenal hyperplasia.

Subject	Age at diagnosis	Current age	Major presenting symptoms and signs	Adrenal gland enlargement	Pubertal development
1	15 days	14.5	Lethargy, vomiting, and hyperpigmentation	+	+
2	7 days	6.2	Poor feeding and hyperpigmentation	–	–
3	75 days	4.3	Vomiting, fever, and hyperpigmentation	+	–
4	20 days	11.0	Vomiting and hyperpigmentation	NA	–
5	18 days	2.4	Lethargy, hyperpigmentation, vomiting, and fever	NA	–
6	7 days	3.6	Hyperpigmentation and poor feeding	NA	–
7	16 days	7.1	Hyperpigmentation	NA	–
8	24 days	6.4	Lethargy and seizure	NA	–
9	30 days	16.0	Seizure, vomiting, and hyperpigmentation	+	+
10	70 days	2.5	Hyperpigmentation and vomiting	NA	–
11	5 days	0.5	Lethargy and fever	+	–
12	9 days	1.7	Hypoglycemia	+	–
13	30 days	1.5	Lethargy and vomiting	NA	–
14	30 days	Expired	Hyperpigmentation and cholestasis	+	–
15	5 days	1.0	Hyperpigmentation and cholestasis	NA	–
16	14 days	7.0	Apnea and hyperpigmentation	+	–
17	13 days	2.8	Lethargy and hyperpigmentation	+	–
18	39 days	0.4	Lethargy, fever, and hyperpigmentation	+	–
19	20 days	1.0	Lethargy, anuria, and hyperpigmentation	+	–
20	3 months	0.4	Lethargy, vomiting, failure to thrive, and hyperpigmentation	+	–
21	20 days	11.4	Apnea and hyperpigmentation	–	–
22	17 days	0.5	Hyperpigmentation and poor feeding	NA	–
23	30 days	2.9	Lethargy, hyperpigmentation, and vomiting	NA	–
24	21 days	5.1	Hyperpigmentation and hypoglycemia	+	–
25	10 days	0.4	Hyperpigmentation and lethargy	+	–

NA, not assessed.

Table 2 Endocrinological data at diagnosis and mutation analysis of patients with congenital lipoid adrenal hyperplasia.

Subject	ACTH (pg/ml; 0–60)	17-HP (ng/ml; <12)	PRA (ng/ml per h; 0.68–1.36)	Na/K/Cl (mmol/l)	Karyotype	DNA analysis
1	>2000	<1.0	34.2	117/9.2/98	46,XX	p.Q258X/p.Q258X
2	1059	0.47	19.2	117/4.0/87	46,XX	p.Q258X/p.Q258X
3	>2000	ND	37.4	124/4.2/ND	46,XX	p.Q258X/p.Q258X
4	1081	0.05	65.4	118/5.4/81	46,XX	p.Q258X/p.Q258X
5	383	15.1	18.3	120/8.1/109	46,XX	p.Q258X/p.Q258X
6	1394	4.1	>20.0	119/6.4/92	46,XX	p.Q258X/p.Q258X
7	1389	1.3	28.0	127/6.2/95	46,XX	p.Q258X/p.Q258X
8	1655	0.1	21.4	127/5.3/102	46,XX	p.Q258X/p.Q258X
9	>2000	0.1	96.5	114/8.5/94	46,XX	p.Q258X/p.Q258X
10	345	0.1	26.9	125/6.6/96	46,XX	p.Q258X/p.Q258X
11	1855	3.1	ND	125/4.7/77	46,XX	p.Q258X/p.Q258X
12	>2000	0.5	ND	126/6.6/97	46,XY	p.Q258X/p.Q258X
13	>2000	1.1	20.9	140/5.1/111	46,XY	p.Q258X/p.Q258X
14	1250	1.6	74.0	122/5.8/97	46,XY	p.Q258X/p.Q258X
15	1152	0.4	14.1	129/5.8/100	46,XY	p.Q258X/p.Q258X
16	>2100	0.2	4.0	132/6.0/109	46,XY	p.Q258X/p.Q258X
17	1086	1.1	29.5	128/7.8/94	46,XY	p.Q258X/p.Q258X
18	>2000	0.1	87.6	124/5.4/104	46,XY	p.Q258X/p.Q258X
19	>2000	0.1	50.0	120/5.9/ND	46,XY	p.Q258X/p.Q258X
20	>2000	0.1	112.7	109/8.6/ND	46,XY	p.Q258X/p.Q258X
21	>2000	0.1	39.7	119/7.8/85	46,XY	p.Q258X/p.Q258X
22	>2000	0.5	90.8	131/6.9/97	46,XY	p.Q258X/p.Q258X
23	1120	0.1	ND	127/6.4/94	46,XY	p.Q258X/p.R182H
24	>2000	0.1	80.2	135/4.8/102	46,XY	p.Q258X/c.653C>T
25	>2000	1.0	20.0	128/5.4/99	46,XY	c.653C>T/c.745–6_810del

ND, not determined. 17-HP, 17-hydroxy progesterone; PRA, plasma renin activity.

in one of the 50 alleles (2%). Twenty-two patients (88%) were homozygous for the p.Q258X mutation, and three patients (12%) were compound heterozygous for c.772C>T/c.545G>A, c.772C>T/c.653C>T, and c.653C>T/c.745–6_810del. The *STAR* gene analysis identified subject 25 as compound heterozygous for c.653C>T and c.745–6_810del. Sequencing analysis of her parents demonstrated that the father was heterozygous for the c.745–6_810del mutation in the *STAR* gene, whereas the mother was heterozygous for the c.653C>T mutation.

Identification of multiple transcripts of c.653C>T and c.745–6_810del

To assess the transcript patterns of the compound heterozygous c.653C>T and c.745–6_810del mutation found in subject 25, we performed RT-PCR analysis using this subject's testes fibroblast cells. RT-PCR products demonstrated the presence of multiple alternatively spliced mRNAs (Fig. 1).

Computational prediction of splicing alterations

Two web-based tools were used to determine whether the c.653C>T mutation affected the strength of the natural splice site. According to the splice prediction programs BDGP and Netgene2, the 3' splice scores of

c.653T were reduced from 0.92 to 0.87 and 0.26 to 0.24 respectively. In addition, the Netgene2 program predicted an increase in the 5' splice score of c.653T from 0.72 to 0.80. The ESS/ESE ratio obtained from the EX-SKIP program was greater in c.653T (0.17) than in c.653C (0.00); that is, the EX-SKIP program predicted that c.653T has a higher likelihood of exon skipping than c.653C.

In vitro expression of the *STAR* minigene

To more accurately analyze the transcriptional effects of the c.653C>T mutation, we created a minigene construct of *STAR* and used it to assess *in vitro* transcription following transfection in COS7 cells. This construct consisted of the expression vector pcDNA3.1/NT-GFP and a region of the c.653C>T mutant or WT *STAR* gene spanning exons 4 through 7, including introns 4, 5, and 6. Two amplicons were transcribed from the WT construct: a 552 bp amplicon, which accounted for 97% of all transcripts, and a 367 bp amplicon, which accounted for the remaining 3%. The amplification products of WT *STAR* cDNA represent exons 4 through 7 and the normally spliced exon 5. The c.653C>T mutant construct also yielded two amplicons, but of different sizes – 458 and 273 bp; the larger and smaller transcripts accounted for 72 and 28% of the total respectively. For further confirmation, the 458 and 273 bp fragments were retrieved and subjected to

Figure 1 RT-PCR analysis of *STAR* mRNA from testis fibroblast cell of subject 25. Electrophoretic patterns of the RT-PCR products of wild-type and mutant mRNAs yielded two transcripts reflecting skipping of exon 6, or both exons 5 and 6.

sequencing analyses. The results indicated that exon 6 skipping in the 458 bp transcript, and skipping of both exons 5 and 6 in the 273 bp transcript (Fig. 2A). For the 458 bp transcript, exon skipping created a frameshift that extended the open reading frame and introduced a stop codon at codon 289. For the 273 bp transcript, the frameshift caused by skipping of exons 5 and 6 led to an in-frame deletion of 93 amino acids (Fig. 2A).

The *STAR* minigene construct containing the c.745–6_810del mutation yielded two transcripts of 1317 and 1077 bp that accounted for 94 and 6% of the total transcript mRNA respectively. The 1317 bp of RT-PCR product was generated by retention of intron 6 and encoded a novel peptide consisting of 47 amino acids after codon 248 and containing a premature termination codon (TGA) at codon 295. Using cryptic splice sites in intron 6, the 1077 bp product retained partial intronic sequences, resulting in the +595 bp variant. This transcript is predicted to encode a novel five-amino-acid peptide after codon 248 and contains a premature termination codon (TGA) at codon 253. A single transcript of ~552 bp was present in normal testis cDNA, whereas amplicons of ~1317, 1077, 458, and 273 bp were detected in the cDNA of subject 25. Sequencing analyses revealed that these abnormal RT-PCR products arose due to intron 6 retention, partial intron 6 retention, exon 6 skipping, and exons 5 and 6 skipping (Fig. 2B).

Functional expression of MT-*STAR* proteins in COS7 cells

To evaluate possible functional defects of proteins encoded by *STAR* c.653C>T mutation splicing variants, we investigated pregnenolone-producing

activity in non-steroidogenic COS7 cells cotransfected with either WT or MT-*STAR* expression constructs and a construct expressing the fused cholesterol side chain cleavage system. WT *STAR* efficiently facilitated the conversion of cholesterol to pregnenolone (261.3 ± 118.5 ng/dish of pregnenolone), whereas transient *in vitro* expression of the exon 6-skipping and exons 5 and 6-skipping mutations showed markedly decreased pregnenolone-producing activity (16.50 ± 5.33 and 15.03 ± 5.35 ng/dish respectively), indicating complete inactivation of *STAR* function ($P < 0.05$; two-tailed Student's *t*-test; Fig. 3).

Discussion

This study analyzed the *STAR* gene in 25 unrelated Korean patients with CLAH and identified four different mutations of the *STAR* gene, including one novel splicing mutation. Previously, the p.Q258X mutation was shown to account for about 70% of affected alleles in most patients of Japanese and Korean ancestry (14). However, we found that the p.Q258X mutation is more prevalent (92.3%) in the Korean population. These results suggest that the genetic defect in the *STAR* gene in Korean patients with CLAH is highly homogeneous, probably reflecting a founder effect. The majority of CLAH patients carrying the p.Q258X mutation typically exhibit severe adrenal insufficiency within the first 2 months of life (1, 5, 6, 15). It has been demonstrated that p.Q258X is a null mutation, resulting in

Figure 2 (A) RT-PCR analysis of *STAR* mRNA obtained in minigene assays. The size standards and their corresponding lengths are indicated at left. Alternatively spliced transcripts of c.653C>T and c.745–6_810del mutant variants are designated at right. Note that exon 5 is followed by exon 7 because of the skipping of exon 6 in the mutant transcript, and exon 4 is followed by exon 7 because of the skipping of exons 5 and 6. (B) Schematic representation of splicing in the c.653C>T and c.745–6_810del mutant variants. The proportions (%) of mis-spliced transcripts are described at the right.

Figure 3 Functional analysis of mutant and wild-type STAR proteins in COS7 cells. The ability to produce pregnenolone from cholesterol was tested for wild-type and mutant STAR *in vitro*. COS7 cells were cotransfected with the wild-type (pCMV-Flag-STAR-WT), exon 6-skipping, or exons 5 and 6-skipping STAR constructs and a plasmid expressing the human cholesterol side chain cleavage enzyme as a fusion protein. An empty vector was used as a negative control. Cotransfection of expression vectors for mutant STAR proteins (exon 6 skipping and exons 5 and 6 skipping) resulted in decreased induction of pregnenolone production. Data represent the mean $\pm$ s.d. from three independent experiments (* $P < 0.05$ versus wild-type STAR; two-tailed Student's *t*-test).

elimination of STAR activity (1, 7). The authors found that the gene frequency for the p.Q258X mutation in the Korean population can be presumed to be $\sim 1/500$, with $1/250$ the carrier frequency. The confidence limits of the gene frequency for the mutant allele are 0.5–8.0 among 1000 alleles. Therefore, the carrier frequency could be lower ($1/1000$) or higher ($16/1000$). However, the estimated incidence could be inaccurate due to the insufficient sample size (6).

Most patients with CLAH harboring the c.653C>T mutation, which has been found in several Japanese and Caucasian patients (5, 7, 16, 17), also exhibit adrenal insufficiency during the neonatal period. The c.653C>T variant was previously considered a missense mutation that encoded the mutant protein p.A218V; the results of expression studies indicated that this mutant possessed little or no ability to enhance pregnenolone production, although it could enter mitochondria and be processed (5, 16). However, the current study clearly demonstrated that the c.653C>T variant gives rise to aberrant splicing at the mRNA level, leading to premature termination of the protein (p.R217fsX48). Moreover, the results of our *in vitro* transcription analyses using a minigene construct containing exons 4–7 and the intervening introns of the STAR gene demonstrate that the C-terminal region of the STAR gene can yield two alternative transcripts; a full-length transcript and a shorter transcript that skips exon 5, even in the WT. In the WT, the full-length transcript is predominant, accounting for 97% of total transcripts. On the other hand, the c.653C>T mutation not only leads to exon 6 skipping but it also increases the proportion of the short transcript in which both exons 5 and 6 are skipped. This finding was predicted by

the computer program, EX-SKIP, and was demonstrated by analyzing both patients' testicular tissue and *in vitro* transcripts. The c.653C is part of the trinucleotide, GCG, which is the second codon in exon 6 and encodes alanine. Located on the exon–intron boundary, the c.653C>T mutation is expected to alter the splicing pattern. A similar phenomenon was demonstrated previously (15). The p.R217T mutation, located at the terminal position in exon 5, was shown to result in aberrantly spliced transcripts rather than a single amino acid substitution. In the case of the c.653C>T mutation, the resulting altered transcript encoded a nonfunctional protein, as demonstrated by the absence of pregnenolone-producing activity in our *in vitro* transient expression study. Therefore, our findings suggest that splicing pattern should be investigated by examining tissue mRNA or through *in vitro* transcription analysis if a STAR mutation is located near an exon–intron boundary, even when the mutation is a suspected missense mutation. Because STAR protein is primarily expressed in gonadal or adrenal tissues, *in vivo* transcription assays using peripheral blood are not feasible. Therefore, *in vitro* transcription analysis is recommended as a simple, effective method for validating the mutation.

The elevated ESS/ESE ratio of c.653T (0.17) predicted a higher risk of exon skipping compared with the c.653C. Interestingly, a semiquantitative assay of alternatively spliced transcripts showed that exon 6-skipping transcripts are more abundant than those that skip both exons 5 and 6. The former are predicted to cause a frameshift and the latter an in-frame deletion, which might explain the severe phenotype of the patient carrying this mutation. However, defective conversion of cholesterol to pregnenolone was observed in *in vitro* cotransfection assays using both transcripts. Although assays of the c.653C>T missense mutant construct showed that this mutant also exhibited defective conversion of cholesterol to pregnenolone, it was not the underlying molecular defect in the patient.

The novel mutation identified in our study, c.745–6_810del, also resulted in aberrant splicing. This mutation encompasses the exon 7–intron 6 boundary and is thus expected to affect normal splicing patterns. As was the case for WT STAR gene transcripts, this mutation also yielded two alternative transcripts, one with exon 5 and the other without exon 5. The proportions of these two transcripts were similar to those in the WT, indicating that this mutation does not alter the splicing patterns of exon 5. These two transcripts led to transcripts that were prematurely terminated in the C-terminal region. This unique genomic deletion mutation resulted in the production of an alternatively spliced transcript that retained the entire intron 6, generating a novel 47-amino acid peptide after codon 248 with a premature termination codon (TGA) at codon 295. Moreover, this alternatively spliced transcript accounted for $\sim 94\%$ of all mRNA

transcripts. Subject 25, who was compound heterozygous for the c.653C>T and c.745–6_810del mutations, manifested severe adrenal insufficiency in the neonatal period, suggesting that these mutations completely abolish STAR function.

The C-terminal 200–210 residue motif of STAR known as the STAR-related lipid transfer (START) domain is found in many proteins involved in diverse cell functions (18). Most STAR missense mutations that eliminate STAR activity are found in the C-terminal 40% of the 285-amino acid STAR protein (14), particularly in the region between exons 5 through 7, and thus affect the critical START domain (7, 11). Mutations in the START domain drastically affect the activity of STAR; indeed, deletion of just 28 C-terminal residues suppresses all STAR activity (1, 19). *In vitro* studies have shown that STAR protein lacking the N-terminal targeting sequence retain the ability to stimulate steroidogenesis in transfected COS1 cells, whereas mutations in the C-terminal region lead to severely diminished or absent function (19–21).

The p.R182H mutation has been previously reported, and many of the patients bearing this mutation have clinically milder and later onset CLAH symptoms (8). This mutation changes the strong basic arginine residue to the weak basic histidine. Functional studies have shown that p.R182H is devoid of activity (8). In the seven patients who carried the same p.R182H mutation, the onset of symptoms ranged from 1 to 14 months (8). This is the most surprising finding for which there is no clear explanation. In our CLAH cohort, only one allele of p.R182H (allele frequency, 1%) was found; it was present as a compound heterozygote with p.Q258X in a patient presenting with severe adrenal crisis in the newborn period.

At the time of puberty, 46,XX females with CLAH may develop spontaneous puberty, feminization, and even regular menstruation, observations that can be explained by the STAR-independent steroidogenic capacity of the ovary (5, 7, 22–24). In this study, two pubertal-aged patients exhibited spontaneous breast development. Subject 1 showed spontaneous menarche at age 12.9 years after thelarche at age 10.6 years and experienced ovarian cyst torsion at age 14. Subject 9, after menarche at 10.3 years of age, is undergoing regular menstruation without ovarian cyst or failure. The remaining 46,XX patients are of prepubertal age and thus should be followed up until puberty for the evaluation of ovarian function. The bilateral inguinal gonads were removed in seven genetic males, and histological examination of the gonads confirmed the presence of normal testicular tissue. There was no adrenal enlargement on adrenal ultrasonography in subjects 2 and 21. These findings suggest that adrenal glands are not always enlarged in CLAH and the accuracy of adrenal ultrasonography is doubtful for the diagnosis of CLAH.

In conclusion, we have identified four different mutations in the STAR gene, including a novel mutation, in 25 unrelated Korean patients with CLAH. The p.Q258X mutation is the most commonly found STAR gene mutation in Korean patients with CLAH. Thus, STAR gene mutation analysis in suspected patients is helpful for early diagnosis of CLAH as well as for genetic counseling in families at risk. The alternative splicing patterns in the C-terminal region of the STAR gene, demonstrated here, indicate that the aberrant splicing should be suspected for mutations located near exon–intron boundaries, even if the mutation is expected to result in a single amino acid substitution.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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