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# Simple and rapid genetic testing for citrin deficiency by screening 11 prevalent mutations in *SLC25A13*

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ABSTRACT

Citrin deficiency is an autosomal recessive disorder caused by mutations in the *SLC25A13* gene and has two disease outcomes: adult-onset type II citrullinemia and neonatal intrahepatic cholestasis caused by citrin deficiency. The clinical appearance of these diseases is variable, ranging from almost no symptoms to coma, brain edema, and severe liver failure. Genetic testing for *SLC25A13* mutations is essential for the diagnosis of citrin deficiency because chemical diagnoses are prohibitively difficult. Eleven *SLC25A13* mutations account for 95% of the mutant alleles in Japanese patients with citrin deficiency. Therefore, a simple test for these mutations is desirable. We established a 1-hour, closed-tube assay for the 11 *SLC25A13* mutations using real-time PCR. Each mutation site was amplified by PCR followed by a melting-curve analysis with adjacent hybridization probes (HybProbe, Roche). The 11 prevalent mutations were detected in seven PCR reactions. Six reactions were used to detect a single mutation each, and one reaction was used to detect five mutations that are clustered in a 21-bp region in exon 17. To test the reliability, we used this method to genotype blind DNA samples from 50 patients with citrin deficiency. Our results were in complete agreement those obtained using previously established methods. Furthermore, the mutations could be detected without difficulty using dried blood samples collected on filter paper. Therefore, this assay could be used for newborn screening and for facilitating the genetic diagnosis of citrin deficiency, especially in East Asian populations.



## 1. Introduction

Citrin deficiency is an autosomal recessive disorder that results from mutations in the *SLC25A13* gene [1] and causes two diseases: adult-onset type II citrullinemia (CTLN2; OMIM #603471) and neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD; OMIM#605814) [1–4]. The clinical appearance of these diseases is variable and ranges from almost no symptoms to coma, brain edema, and severe liver failure requiring transplantation [5–8]. In a study of patients with NICCD, only 40% of individuals were identified by newborn screenings to have abnormalities, such as hypergalactosemia, hypermethioninemia, and hyperphenylalaninemia [9]. Other patients were referred to hospitals with suspected neonatal hepatitis or biliary atresia, due to jaundice or discolored stool [9]. Hypercitrullinemia was not observed in all patients [9]. Mutation analysis of *SLC25A13* is indispensable because of the difficulties associated with the chemical diagnosis of citrin deficiency. The *SLC25A13* mutation spectrum in citrin deficiency is heterogeneous, and more than 31 mutations of *SLC25A13* have been identified to date [1,10–18]. However, there are several predominant mutations in patients from East Asia. As shown in Table 1, 6 prevalent mutations account for 91% of the mutant alleles in the Japanese population [12,19]. Five additional mutations also occur within a 21-bp cluster in exon 17 (Table 1 and Fig. 1D). The six prevalent mutations, together with the five mutations in exon 17, account for 95% of the mutant alleles in Japan [12,19].

Several different methods, such as direct sequencing, PCR restriction fragment length polymorphism (PCR-RFLP), and denaturing high performance liquid chromatography (DHPLC), are currently used for the detection of mutations in *SLC25A13* [1,10–14,19]. However, these methods are too complex for clinical use. Direct sequencing is a standard but cumbersome method. The PCR-RFLP method is

*Abbreviations:* CTLN2, adult-onset type II citrullinemia; FRET, fluorescence resonance energy transfer; HRM, high resolution melting; NICCD, neonatal intrahepatic cholestasis caused by citrin deficiency; Tm, melting temperature.

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**Table 1**
Seven primer/probe sets and 11 targeted mutations of *SLC25A13*.

| Primer/probe set | Mutation | | Location | Nucleotide change | Effects of mutations | Allele frequency*[19] | References |
|---|---|---|---|---|---|---|---|
| A | Mutation [I] | :851del4 | exon 9 | c.851_854delGTAT | p.R284fs(286X) | 33.2% | [1] |
| B | Mutation [II] | :g.IVS11+1G>A | intron 11 | c.1019_1177del | p.340_392del | 37.6% | [1] |
| C | Mutation [III] | :1638ins23 | exon 16 | c.1638_1660dup | p.A554fs(570X) | 3.4% | [1] |
| D | Mutation [IV] | :S225X | exon 7 | c.675C>A | p.S225X | 5.3% | [1] |
| E | Mutation [V] | :g.IVS13+1G>A | intron 13 | c.1231_1311del | p.411_437del | 8.2% | [1] |
| F | Mutation [XIX] | :IVS16ins3kb | intron 16 | c. aberrant RNA | p.A584fs(585X) | 4.6% | [19] |
| G | Mutation [VI] | :1800ins1 | exon 17 | c.1799_1800insA | p.Y600X | 1.3% | [10] |
| | Mutation [VII] | :R605X | exon 17 | c.1813C>T | p.R605X | 0.90% | [10] |
| | Mutation [VIII] | :E601X | exon 17 | c.1801G>T | p.E601X | 1.2% | [11] |
| | Mutation [IX] | :E601K | exon 17 | c.1801G>A | p.E601K | 0.30% | [11] |
| | Mutation [XXI] | :L598R | exon 17 | c.1793T>G | p.L598R | 0% | [15] |
| | | | | | | Total 95.1% | |

* The frequency of each mutant allele among Japanese patients with citrin deficiency.

complicated and can lead to genotyping errors, due to incomplete digestion by the restriction enzymes. DHPLC is time-consuming and requires expensive equipment. Thus, there is a strong need for the development of a simple test for these mutations.

The goal of this study was to establish a rapid and simple test for the detection of the 11 most common *SLC25A13* mutations. We adopted the HybProbe format (Roche) for the detection of the mutations using real-time PCR followed by a melting-curve analysis with adjacent hybridization probes [20,21]. This assay can be completed in less than 1 h and has the advantage of being a closed-tube assay. The fundamental process for detecting point mutations using the HybProbe assay is presented in Fig. 1A. The 11 prevalent mutations contain not only point mutations but also include a 4-bp deletion and insertions of 1-bp, 23-bp and 3-kb genomic fragments (Table 1 and Fig. 1). Careful design of the PCR primers and HybProbes enabled us to test for these various *SLC25A13* mutations.

## 2. Methods

### 2.1. Subjects

CTLN2 and NICCD were diagnosed, as previously described [9,10,19,22–24]. Genomic DNA of the patients was obtained from peripheral blood leukocytes using the DNeasy blood kit (Qiagen Inc., Valencia, CA, USA). Genomic DNA was purified from filter paper blood samples using the ReadyAmp Genomic DNA Purification System (Promega, Madison, WI, USA). Mutations in these DNA samples were analyzed at Kagoshima University using a combination of PCR with or without restriction enzyme digestion or by direct sequencing, as previously described [1,10–14,19]. Another set of samples was obtained from 420 healthy volunteers (mainly from Miyagi prefecture in the northeastern region of Japan) at Tohoku University. Genomic DNA from leukocytes was extracted, as described above.

### 2.2. Detection of seven prevalent mutations in SLC25A13 using the HybProbe assay

HybProbe probes comprise a pair of donor and acceptor oligonucleotide probes designed to hybridize adjacent to their target sites in an amplified DNA fragment [20,21]. The donor probes are labeled at their 3′ end with fluorescein isothiocyanate (FITC), whereas the acceptor probes are labeled at their 5′ end with LC Red640; these acceptor probes are phosphorylated at their 3′ end to prevent extension by the DNA polymerase. When two probes hybridize to the amplicon, the fluorescent dyes are located within 5 bases of each other, which allows fluorescence resonance energy transfer (FRET) between the excited FITC and the LC Red640; this process emits light that can be quantified by real-time PCR. Following PCR amplification, a melting-peak analysis is performed. The melting peak is produced by the reporter probe, which has a lower melting temperature (Tm) than the other probe, called the anchor probe. As the reporter melts from the target, the fluorophores are separated, and the FRET ceases. The Tm of the reporter probe determines the reaction

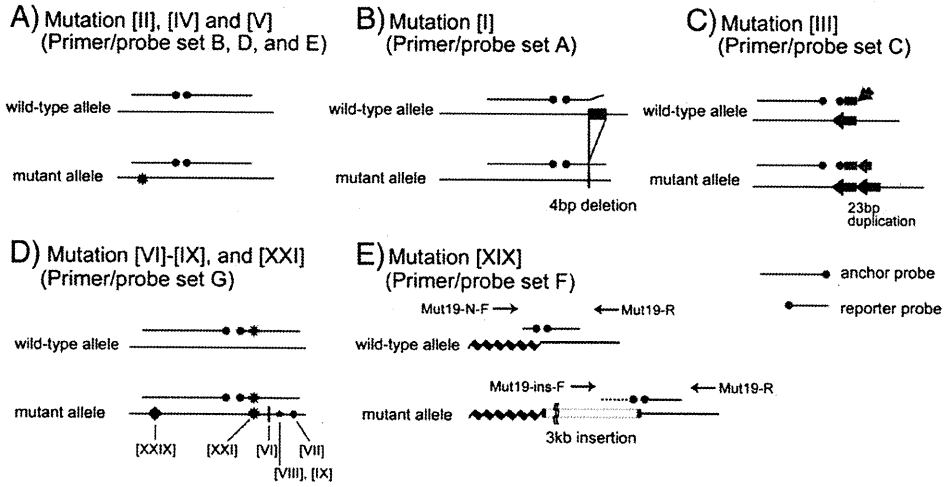


**Fig. 1.** Principle of *SLC25A13* mutation detection by melting-curve analysis with the HybProbe assay. In primer/probe sets A–E, and G, PCR was performed with a pair of primers, whereas in primer/probe set F, two forward primers and one common reverse primer were used for the amplification of both wild-type and mutant alleles. Note that mutation [XXIX], located on the anchor probe of primer/probe set G, is a non-target mutation.

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specificity (i.e., binding of the probe to a perfectly matched sequence rather than to regions with sequence mismatches).

Seven primer/probe sets were designed for this study. Fig. 1 shows a schematic diagram of the strategy for mutation detection using these primer/probe sets. Tables 1 and 2 list the primer/probe sets and corresponding sequences and primer concentrations that were used to target the 11 mutations. Primer/probe sets A, B, C, D, E, and F were designed to detect mutations [I], [II], [III], [IV], [V], and [XIX], respectively. Primer/probe set G was designed to detect the five mutations clustered on exon 17: mutations [VI], [VII], [VIII], [IX], and [XXI] (Fig. 1D). All primers and probes were synthesized based on the NCBI reference SLC25A13 gene sequence (GenBank accession no. **NM_014251**) with the exception of mutation [XIX]:IVS16ins3kb, which was designed according to [19].

Real-time PCR and subsequent melting curve analyses were performed in a closed tube using a 20-μL mixture on a LightCycler 1.5 (Roche Diagnostics, Tokyo, Japan). The PCR mixture contained 2.0 μL of genomic DNA (10–50 ng), 0.5 μM of forward primer, 0.5 or 0.1 μM of reverse primer, 0.2 μM of each sensor and anchor probe, and 10 μL of Premix ExTaq™ (Perfect Real Time) reagent (TaKaRa Bio Inc., Otsu, Japan).

The thermal profile conditions were identical for all seven assays and consisted of an initial denaturation step (30 s at 95 °C), followed by 45 amplification cycles with the following conditions: denaturation for 5 s at 95 °C and annealing and extension for 20 s at 60 °C. The transition rate between all steps was 20 °C/s. After amplification, the samples were held at 37 °C for 1 min, followed by the melting curve acquisition at a ramp rate of 0.15 °C/s extending to 80 °C with continuous fluorescence acquisition.

**Table 2**
Primers, probes and target amplicon sequences, target mutation sites, and primer concentrations.

| Primer/probe set | Name | Sequences of PCR products, primer locations, probe sequences, and mutation sites (5′ to 3′) | Concentration (μmol/L) |
|---|---|---|---|
| A | | GGCTATACTGAAATATGAGAAatgaaaaaagggatgtttttaaatttttataatgtaaattgtaataaattggtatatttgttgcttgtgtttgtttttcccctacagac | |
| | | **gtat**gaccttagcagacattgaacggattgctcctctggaagagggaactctgccCTTTAACTTGGCTGAGG (181 bp) | |
| | Mut1-F | GGCTATACTGAAATATGAGAA | 0.5 |
| | Mut1-R | CCTCAGCCAAGTTAAAG | 0.5 |
| | Mut1-UP | ATGTAAATTGTAATAAATTGGTATATTTGTTGCTTGTGTT-FITC | |
| | Mut1-DW | LC Red640-GTTTTTCCCCTACAGAC**GACC**-P | |
| B | | GAATGCAGAACCAACGAtcaactggctcttttgtgggagaactcatgtataaaaacagctttgactgttttaagaaagtgctacgctatgaaggcttctt | |
| | | tggactgtatagaggttagtgccacatgctcaatacctgttaggtgaaataacactcaaaggtttggtttctcatcttagtgcctGACATGAATTAGCAAGACTG (205 bp) | |
| | Mut2-F | GAATGCAGAACCAACGA | 0.5 |
| | Mut2-R | CAGTCTTGCTAATTCATGTC | 0.1 |
| | Mut2-UP | ACCTAACAGGTATTGAGCATGTG-FITC | |
| | MUt2-DW | LC Red640-CACTAA**C**CTCTATACAGTCCA-P | |
| C | | GCAGTTCAAAGCACAGTTATTtttatatagtgagaatgtgaccagactgagatggtgttgtgtctctcctgcaggtatgcctgcagcatctttagtg | |
| | | accccctgctgatgttatcaagacgagattacaggtg | |
| | | gctgcccggg(**gagatta**caggtggctgcccggg)ctggccaaaccaCTTACAGCGGAGTGATAGAC (175 bp) | |
| | Mut3-F | GCAGTTCAAAGCACAGTTATT | 0.5 |
| | Mut3-R | GTCTATCACTCCGCTGTAAG | 0.5 |
| | Mut3-UP | ACCCCTGCTGATGTTATCAAGACGAGATTACAGGT-FITC | |
| | Mut3-DW | LC Red640-GCTGCCCGGG**GAGATTA**-P | |
| D | | TCAATTTATTTGAGGCTGCtggaggtaccacatcccatcaagttagtttctcctattttaatggatttaattcgctccttaacaac | |
| | | atggaactcattagaaagatctatagcactc | |
| | | tggctggcaccaggaaagatgttgaagtGACTAAGGGTGAGTGAGAA (164 bp) | |
| | Mut4-F | TCAATTTATTTGAGGCTGC | 0.5 |
| | Mut4-R | TTCTCACTCACCCTTAGTC | 0.5 |
| | Mut4-UP | AATGGATTTAATT**C**GCTCCTTAACA-FITC | |
| | Mut4-DW | LC Red640-ATGGAACTCATTAGAAAGATCTATAGCACTC-P | |
| E | | TGCACAAAGATGGTTCGgtcccacttgcagcagaaattcttgctggaggctgc**g**taagtacctttgaagctctcttcattgaaaagacttgtttcac | |
| | | atatatatcactaccatggtcaacaggtgtggactaaggcttctgttTAACCACAGATCCTGCA (162 bp) | |
| | Mut5-F | TGCACAAAGATGGTTCG | 0.5 |
| | Mut5-R | TGCAGGATCTGTGGTTA | 0.5 |
| | Mut5-UP | GTGAAACAAGTCTTTTCAATGAAGAGAGCTTC-FITC | |
| | Mut5-DW | LC Red640-AAGGTACTTA**C**GCAGCCTC-P | |
| F | normal allele | GGAGCTGGTGGTATGGAAataatgtgttcttaactaactctttggtatcaggtaaatttttaaaatatctaattatatctgtgatttctc | |
| | | catttttttaaagctcgtgtatttcgatcctcaccccagtttggt | |
| | | gtaactttgctgacttacgaattgctacagcgatggttctacattgattttggaggagtgtaagtatcatgctaaatctgctgctaaatttt | |
| | | GGCTGCTGCTAATGCTC (244 bp) | |
| | insertion allele | CCATCTTCCTCCTCCCTTggcagccccgccccccgatttctccatttttttaaagctcgtgtatttcgatcctcaccccagtttggt | |
| | | gtaactttgctgacttacgaattgctacagcgatggttctacattgatttt | |
| | | ggaggagtgtaagtatcatgctaaatctgctgctaaattttGGCTGCTGCTAATGCTC (196 bp) | |
| | Mut19-N-F | GGAGCTGGTGGTATGGAA | 0.5 |
| | Mut19-ins-F | CCATCTTCCTCCTCCCTT | 0.5 |
| | Mut19-R | GAGCATTAGCAGCAGCC | 0.5 |
| | Mut19-UP | ACCAAACTGGGGTGAGGATCGAAATACACGAGCTTTAAAAAAATG-FITC | |
| | Mut19-N-DW | LC Red640-AGAAATCACAGATATAATTAGATATTT-P | |
| | Mut19-ins-DW | LC Red640-AGAAATCGGGGGGCGGGG-P | |
| G | | TCTTAACTAACTCTTTGGTATCAGGTaaatttttaaaatatctaattatatctgtgatttctccatttttttaaagctcg | |
| | | tgtatttcgatcctcaccccagtttggtgtaactttgc**t**gactt**a(a)cg**aattgctacag**c**ga | |
| | | tggttctacattgattttggaggagtgtaagtatcatgctaaatctgctgctaaattttGGCTGCTGCTAATGCTC (217 bp) | |
| | Mut6-9, 21-F | TCTTAACTAACTCTTTGGTATCAGGT | 0.5 |
| | Mut6-9, 21-R | GAGCATTAGCAGCAGCC | 0.5 |
| | Mut6-9, 21-UP | TGTATTTCGATCCTCACCCCAGTTTGGTGTAACTT-FITC | |
| | Mut6-9, 21-DW | LC Red640-GC**G**GACTT**ACG**AATTGCTACAG**C**GA-P | |

Upper case and underlined letters indicate the locations of primers and probes, respectively. Inserted DNA is shown in parenthesis. Nucleotides in boldface were used for mutation detection.
F: forward, R: reverse, UP: upstream, DW: downstream, N: normal allele, ins: insertion allele, FITC: fluorescein isothiocyanate, P: phosphate.

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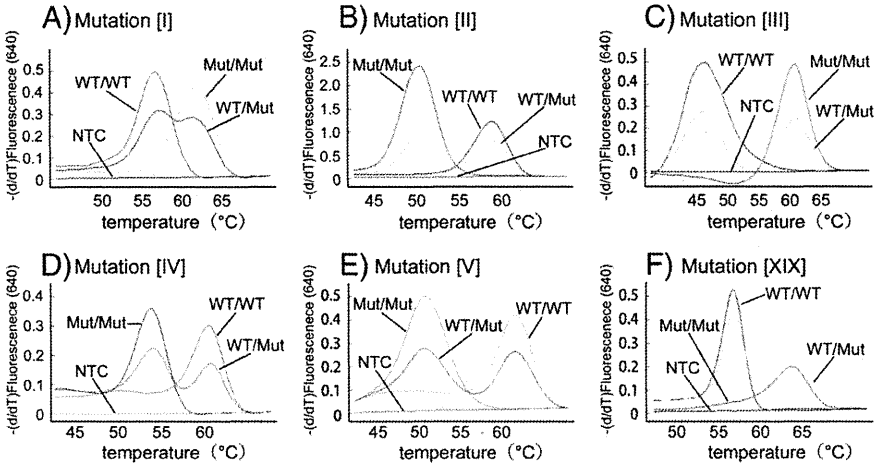


**Fig. 2.** Typical melting curves used in the detection of mutations [I–V] and [XIX]. Each assay using primer/probe sets A–F is displayed in a separate graph (A–F). WT: wild-type allele, Mut: mutant allele, NTC: no DNA template control.

### 2.3. Validation of the mutation detection system

After establishing the protocol for detecting the 11 prevalent mutations, 50 DNA samples from patients' blood were sent from Kagoshima University to Tohoku University for the validation of this system in a single-blind manner. Similarly, 26 DNA samples purified from paper-filter blood samples were analyzed in the same manner as the blood DNA samples.

### 2.4. Estimation of the carrier frequency

For the estimation of the heterozygous carrier frequency, 420 genomic DNA samples from healthy volunteers were screened using the HybProbe analysis for the 11 prevalent mutations. All detected mutations were confirmed by direct sequencing.

### 2.5. Ethics

This study was approved by the Ethical Committees of Tohoku University School of Medicine and Kagoshima University. Written informed consent was obtained from all participants or their guardians.

## 3. Results

### 3.1. Development of the mutation detection system

In primer/probe sets B, D, and E, the reporter probes were designed to be complimentary to the wild-type allele (Fig. 1A). To allow for an improved detection of the mutations, primer/probe sets A and C were designed to be complementary to the mutant allele (Figs. 1B, C). In the primer/probe set F, two forward PCR primers, which were specific to the wild-type and the mutant alleles, were used with a common reverse primer for the co-amplification of the wild-type and 3-kb insertion alleles (Fig. 1E). Two reporter probes, which had a common anchor probe, were used for the detection of the wild-type and mutant alleles. Because the two reporter probes had different melting temperatures, we were able to identify the allele that was amplified. Fig. 2 shows representative results of the melting curve analyses using the primer/probe sets A–F, in which all of the mutant alleles generated distinct peaks corresponding to the wild-type alleles.

In the primer/probe set G, we used a reporter probe that was complementary to the mutant [XXI] allele (Fig. 1D). All five mutations in exon 17 were successfully differentiated from the wild-type allele (Figs. 3A–E). The [XXIX] mutation is an additional mutation in exon

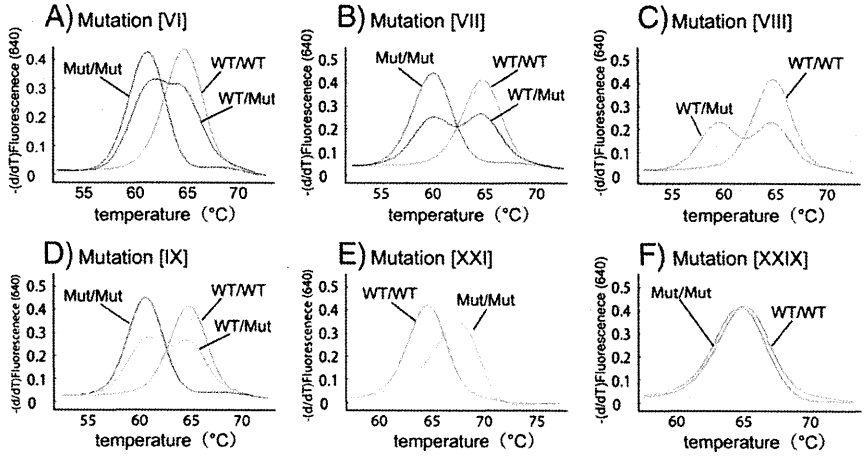


**Fig. 3.** Typical melting curves used in the detection of mutations [VI–XI], [XXI], and [XXIX] on exon 17. Genotyping was performed using primer/probe set G. Each melting curve for a target mutation is displayed in a separate graph (A–F). Note that mutation [XXIX] (F) is a non-target mutation on the anchor probe. WT: wild-type allele, Mut: mutant allele.

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17 that is not listed in Table 1. The [XXIX] mutation is located in the anchor-probe binding site and not on the reporter-probe binding site (Fig. 1D). To examine the effect of mutations on the anchor probe, we genotyped a patient with a heterozygous [XXIX] mutation using primer/probe set G (Fig. 3F). We found no change in the melting curves between the wild-type allele and the [XXIX] allele, thereby suggesting that point mutations within the anchor probe sequence have little effect on the melting curve analysis.

### 3.2. Validation

The genotypes determined at Tohoku University using the proposed method and those determined at Kagoshima University using a previously published method were identical for the 11 common mutations (Table S1 in supplementary material). We performed a similar test using DNA samples purified from filter-paper blood samples to determine if this method could be used for newborn screening. The genotypes determined in both laboratories were identical for all 26 DNA samples (Table S2 in supplementary material).

### 3.3. Frequency of eleven prevalent mutations

We found four heterozygous carriers of mutation [I], three of mutation [II], and two of mutation [V]. In addition, primer/probe set G detected one heterozygous mutation, which was confirmed as mutation [VIII] by direct sequencing. Altogether, 10 mutations were detected in 420 Japanese healthy controls.

## 4. Discussion

We developed a simple and rapid genetic test using real-time PCR combined with the HybProbe system for the 11 prevalent mutations in *SLC25A13*: mutations [I], [II], [III], [IV], [V], [VI], [VII], [VIII], [IX], [XIX], and [XXI]. This genetic test is a closed-tube assay in which no post-PCR handling of the samples is required. In addition, the genotyping is completed within 1 h. This test can utilize DNA samples purified from both peripheral blood and filter-paper blood. The reliability of the test was confirmed by genotyping 76 blind DNA samples from patients with citrin deficiency, including 50 peripheral blood and 26 filter-paper blood DNA samples. Because screening for the 11 targeted mutations would identify 95% of mutant alleles in the Japanese population [19], both, one, and no mutant alleles are expected to be identified in 90.4%, 9.3%, and less than 0.3% of patients, respectively. This genetic test would be useful not only in Japan but also other East Asian countries, including China, Korea, Taiwan and Vietnam, in which the same mutations are prevalent. Our test is expected to detect 76–87% of the mutant alleles in the Chinese population [12,19,25], 95–100% in the Korean population [12,19,26], 60–68% in the Taiwanese population [27,28], and 100% in the Vietnamese population [12,19]. If we were to prepare a primer/probe set for mutation [X]:g.IVS6+5G>A [12], which is prevalent in Taiwan, the estimated sensitivity would exceed 90% in the Taiwanese population [27,28].

Recently, the high resolution melting (HRM) method was reported to be suitable for the screening of mutations in the diagnosis of citrin deficiency [28]. HRM analysis is a closed-tube assay that screens for any base changes in the amplicons. The presence of SNPs anywhere on the amplicons can affect the melting curve, thereby suggesting that HRM is not suitable for screening for known mutations, but rather, is best suited to screening for unknown mutations. When we detected one heterozygous prevalent mutation, we performed HRM screening for all 17 exons of *SLC25A13*. After HRM screening, only the HRM-positive exons were subjected to direct sequencing analysis. Several mutant alleles were identified using this approach.

The frequency of homozygotes, including compound heterozygotes, presenting *SLC25A13* mutations in the population at Kagoshima (a prefecture in the southern part of Japan) has been calculated to be 1/17,000 based on the carrier rate (1/65) [19]. The prevalence of NICCD has been also reported to be 1/17,000–34,000 [29]. In this study, the carrier rate in Miyagi (a prefecture in northern Japan) was 1/42 (95% confidential interval, 1/108–1/26), thereby yielding an estimated frequency of patients with citrin deficiency of 1/7,100. Our result, together with the previous report [19], suggests that a substantial fraction of the homozygotes or compound heterozygotes of SLC25A13 mutations was asymptomatic during the neonatal period.

The early and definitive diagnosis of citrin deficiency may be beneficial for patients with citrin deficiency by encouraging specific dietary habits and avoiding iatrogenic worsening of brain edema by glycerol infusion when patients develop encephalopathy [30,31]. Because the screening of blood citrulline levels by tandem mass analysis at birth does not detect all patients with citrin deficiency, the development of a genetic test would be welcomed. In this study, we demonstrated that genomic DNA extracted from filter paper blood samples was correctly genotyped, thereby indicating the feasibility of newborn screening using this genetic test. If 100,000 babies in the northern part of Japan were screened by this method, we would detect 14 homozygotes or compound heterozygotes with *SLC25A13* mutations and 2400 heterozygous carriers. In 2400 heterozygous carriers, we would expect to observe only 1 to 2 compound heterozygotes with one target and one non-target mutation. The estimated frequency of babies with two non-target mutations is 0.04/100,000. Our genetic method would therefore allow us to screen newborn babies efficiently. If we performed this genetic test in a high-throughput real-time PCR system, such as a 384- or 1,536-well format, the cost per sample could be lowered.

In conclusion, we have established a rapid and simple detection system using the HybProbe assay for the 11 prevalent mutations in *SLC25A13*. This system could be used to screen newborns for citrin deficiency and may facilitate the genetic diagnosis of citrin deficiency, especially in East Asian populations.

Supplementary materials related to this article can be found online at doi:10.1016/j.ymgme.2011.12.024.

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The authors acknowledge the contribution of Dr. Keiko Kobayashi, who passed away on December 21th, 2010. Dr. Kobayashi discovered that the *SLC25A13* gene is responsible for citrin deficiency and devoted much of her life to elucidating the mechanism of citrin deficiency. This work was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology and the Ministry of Health, Labor, and Public Welfare.

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ORIGINAL PAPER

# Proportion of malformations and genetic disorders among cases encountered at a high-care unit in a children's hospital

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**Abstract** Genetic disorders and birth defects account for a high percentage of the admissions in children's hospitals. Congenital malformations and chromosomal abnormalities are the most common causes of infant mortality. So their effects pose serious problems for perinatal health care in Japan, where the infant mortality is very low. This paper describes the reasons for admissions and hospitalization at the high-care unit (HCU) of a major tertiary children's referral center in Japan. We retrospectively reviewed 900 admission charts for the period 2007–2008 and found that genetic disorders and malformations accounted for a significant proportion of the cases requiring admission to the HCU. Further, the rate of recurrent admission was higher for patients with genetic disorders and malformations than for those with acquired, non-genetic conditions. Over the past 30 years, admissions attributed to genetic disorders and malformations has consistently impacted on children's hospital and patients with genetic disorders and malformations form a large part of this facility. These results reflect improvements in medical care for patients with genetic disorders and malformations and further highlight the large proportion of cases with genetic disorders, for which highly specialized management is required. Moreover, this study emphasizes the need for involvement of clinical geneticists in HCUs at children's hospitals.

**Keywords** Malformation · Genetic disease · High-care unit · Children's hospital · Mortality

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## Introduction

Genetic disorders and birth defects account for a high percentage of the admissions to children's hospitals [4, 13]. In 2008 [5], the Ministry of Health, Labor and Welfare in Japan reported that congenital malformations, chromosomal abnormalities, and genetic diseases are the leading causes of death in children during the first year of life. As per that report, 999 infants under the age of 1 year died of congenital malformations and chromosomal abnormalities; this corresponds to 35.7% of the total number of deaths in this age group. Since 1985, congenital malformations and chromosomal abnormalities have remained the leading causes of infant mortality in Japan [5]. Indeed, in USA it

has been found that patients with genetic disorders had a greater need for hospital admission and were hospitalized for longer durations than were those without genetic disorders [14].

However, recent advances in treatment are likely to improve the survival of individuals with congenital malformations, which, in turn, is likely to increase the rates of readmission to pediatric intensive care units (PICUs) [16]. Several studies have assessed the role of genetic disorders in pediatric mortality and hospitalization [2, 6, 7, 16]. Congenital malformations and chromosomal abnormalities pose serious challenges for perinatal health care in this country, as they are the leading contributors to the infant mortality rate in Japan.

In this study, we assessed the reasons for admissions and hospitalization to the high-care unit (HCU) of a major tertiary children's referral center in Kanagawa Prefecture, Japan, and compared our findings to those of a study of this unit 30 years ago. To elucidate the impact and contribution of birth defects and genetic diseases on pediatric hospitalization, we studied the reason for hospitalization, underlying diagnoses, and duration of hospitalization in this children's hospital in Japan.

## Materials and methods

Permission for the study was obtained from the Ethical Committee of our medical center.

We retrospectively analyzed the cases of children hospitalized at the HCU of Kanagawa Children's Medical Center (KCMC) between June 2007 and December 2008. KCMC is a major tertiary children's referral center for pediatric cardiology, surgery, and cancer cases and serves a large area in Kanagawa Prefecture, Japan. It has an institute for the severely handicapped, a PICU, a neonatal intensive care unit, and an HCU. In contrast to the PICU, which admits patients who have undergone cardiovascular or neurosurgery, the HCU specializes in pediatric patients with other acute conditions. All of the patients were included if they were admitted to the HCU from the emergency room, operating room, or inpatient ward. KCMC, with 419 beds, is the only specialized pediatric hospital in Kanagawa Prefecture, where the total number of births is 80,000 annually [8, 9]. About 8,500 patients (male/female, 1:1) were admitted to KCMC in 2007, and the average of hospital stay was 15.3 days.

We summarized and reviewed the medical charts of all patients admitted to the HCU. The charts and summaries were reviewed for age, sex, duration of hospitalization, underlying disease, and reason for admission. Subcategories were created for the underlying diseases and reason for admission.

The underlying disease was classified into two main categories: genetic conditions and acquired (non-genetic) conditions. Genetic conditions were considered to include chromosomal abnormalities, recognizable malformation and dysplasia, multiple malformations, isolated malformations (e.g., those related to the heart, central nervous system (CNS), and respiratory and gastrointestinal tracts), other single-gene defect-related conditions, mitochondrial diseases, and metabolic disorders (Table 1). All cases of chromosomal abnormalities and multiple malformations were examined using standard karyotyping. Cases of recognizable malformation/dysplasia were ascertained by clinical dysmorphologists (H.Y., N.F., and K.K.). Acquired conditions were considered to include perinatal complications, trauma, neoplasm, and sequelae of severe infectious conditions.

The reasons for admission were classified as problems of the respiratory system, CNS, heart, gastrointestinal tract, kidneys and urinary tract, infectious diseases, post-operative management, and unknown condition. Those cases that did not fall into these categories were placed into a category called "others."

Statistical analyses were performed to compare the duration of hospitalization and the age distribution, using StatView version 5.0 (SAS Institute, Inc; Cary, NY). Categorical data were reported as counts and percentages, and continuous data as mean (SD) or median values. Statistical differences for categorical variables were determined by using chi-squared analyses. Median differences were compared by Mann–Whitney $U$ test.

## Results

A total of 900 admissions, consisting of 687 individual cases with 200 recurrent admissions, were reviewed. Sixteen admissions were excluded from the study because of insufficient information regarding the underlying causes for admission.

The median age at admission was 3.5 years (range, 1 day–32.5 years), and the sex ratio was 1.36 (396 males and 291 females). The median lengths of hospitalization in the HCU were 4 days. Table 2 shows the distribution of the 884 admissions across the different categories of causes for admission. Most patients were admitted for common medical problems, including respiratory problems, post-operative management, and CNS problems. Of the 298 admissions for respiratory problems, most cases involved respiratory infection, including pneumonia and bronchitis. Admissions for post-operative management accounted for 30.7% cases (271 of 884 admissions), while CNS problems such as convulsions, encephalitis, and meningitis accounted for 16.3% (144 of 884 admissions).

**Table 1** Definitions of categories

| Category | Examples |
|---|---|
| Chromosomal syndromes | Down syndrome, trisomies 13 and 18, cri du chat syndrome, and Wolf–Hirschhorn syndrome |
| Recognizable malformation/dysplasia | 22q11.2 deletion syndrome, CHARGE syndrome, and VATER association, Lowe syndrome, achondroplasia, Crouzon syndrome, Noonan syndrome, and Treacher–Collins syndrome |
| Multiple malformations | |
| Isolated malformations | |
| Congenital heart diseases | VSD ASD, AVSD, TGA, and DORV |
| Central nervous system malformations | Schistorrhachis, hydrocephalus, and meningoencephalocele |
| Gastrointestinal malformations | Diaphragmatic hernia, biliary atresia, and congenital intestinal obstruction |
| Respiratory system malformations | CCAM and tracheal stenosis |
| Other isolated malformations | Cleft palate and cleft lip |
| Single-gene defect | Metabolic diseases, spinal muscular atrophy, and spinocerebellar degeneration |
| Mitochondrion | |

The classification of the underlying conditions of the 687 patients is shown in Table 3. In 13 cases, the data for identifying the underlying disease were insufficient (e.g., charts were missing). These cases were categorized as "unknown condition." Of the total 687 patients, 372 (54.1%) had genetic disorders and the remaining 302 (44.0%) had acquired conditions unrelated to genetic disorders, including perinatal complications, neoplasm, and trauma. Among the 372 patients with genetic disorders, 72 had chromosomal abnormalities, with Down syndrome (29 cases) being the most common underlying disorder. Seventy patients had recognizable malformations and dysplasia, with conditions such as osteogenesis imperfecta, 22q11.2 deletion syndromes, CHARGE syndrome, and VATER association. Multiple malformations with unrecognizable patterns were present in 38 cases while isolated malformations, including CNS malformation, congenital heart disease, and gastrointestinal malformation were present in 160 cases.

We also summarized the reasons for the total of 884 admissions, according to the underlying condition (genetic or acquired). Of these admissions, 200 were readmissions. Patients with genetic disorders and malformations had a greater tendency to be hospitalized repeatedly as compared with those with acquired conditions (Fig. 1). In both genetic and acquired condition categories, respiratory disease, post-operative management, and CNS problems were the major medical problems leading to admission.

We further compared age distribution and the lengths of hospitalization between the groups with genetic and acquired disorders (Table 4). The patients with genetic

**Table 2** Medical problems for admission ($N$=884)

| Causes for admission | Number | Percent |
|---|---|---|
| Respiratory problems | 298 | 33.7 |
| Post-operative management | 271 | 30.7 |
| CNS problems | 144 | 16.3 |
| Gastrointestinal problems | 35 | 4.0 |
| Cardiac diseases | 23 | 2.6 |
| Other infectious state | 23 | 2.6 |
| Examination | 21 | 2.4 |
| Kidney and urinary tract problems | 14 | 1.6 |
| Other | 55 | 6.2 |
| Total | 884 | 100.0 |

**Table 3** Classification of underlying diseases in 678 patients

| Underlying diseases | Number | Percent |
|---|---|---|
| Genetic disorders and malformations (subtotal) | 372 | 54.1 |
| Chromosomal abnormalities | (72) | 10.5 |
| Recognizable malformation/dysplasia | (70) | 10.2 |
| Multiple malformations | (38) | 5.5 |
| Isolated malformations | (subtotal:160) | 23.3 |
| Central nervous system malformation | (71) | 10.3 |
| Congenital heart disease | (35) | 5.1 |
| Gastrointestinal malformation | (32) | 4.7 |
| Respiratory system malformation | (9) | 1.3 |
| Other isolated malformations | (13) | 1.9 |
| Single-gene defect | (26) | 3.8 |
| Mitochondrion | (6) | 0.9 |
| Acquired non-genetic conditions (subtotal) | 302 | 44.0 |
| Perinatal complications | (66) | 9.6 |
| Neoplasm | (38) | 5.5 |
| Trauma(non-accidental and accidental) | (27) | 3.9 |
| Infection | (16) | 2.3 |
| Other | (155) | 22.6 |
| Unknown | 13 | 1.9 |
| Total | 687 | 100.0 |

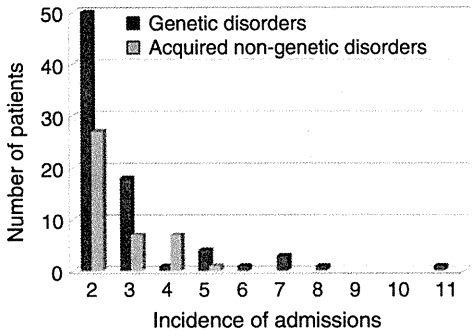


**Fig. 1** Comparison of the incidence of admission between the groups with genetic disorders and acquired disorders. In both groups, a total of 200 patients were readmitted. The group with genetic disorders generally required frequent readmission

disorders were significantly younger than those with acquired conditions (median age, 2.0 vs. 4.9 years; $P<0.0001$). There is no significant difference in the length of hospitalization between the patients with genetic disorders and those with acquired conditions (median, 4 vs. 4 days; $P=0.26$), but some patients with genetic disorders had much longer hospitalization (mean, 13.0 vs. 7.0 days; $P=0.007$; range, 1–979 days). Among the reasons for admission, respiratory problems tended to have a longer duration of hospitalization for patients with genetic disorders than for those with acquired conditions (median, 7 vs. 5 days; $P=0.17$).

## Discussion

Our study shows that genetic disorders and malformations account for a significant proportion of cases requiring admission to the HCU. Additionally, the rate of recurrent admission was higher among patients with genetic disorders and malformations than among those with acquired non-genetic conditions. This finding is in agreement with those of previous reports for other countries [4, 13].

Several studies from different countries have previously suggested that genetic conditions and malformations and the associated mortality and morbidity have a significant impact on the cost burden for society and the patients' families. Cunniff et al. reported that 19% of deaths in a PICU were in cases of heritable disorders [1]. Stevenson and Carey reported that the 34.4% of deaths in a children's hospital were due to malformations and genetic disorders [15]. On the basis of a population-based study, Yoon et al. reported that the overall rate of hospitalization was related to birth defects and genetic diseases, and varied with age and race/ethnicity [16]. McCandless et al. reported the enormous impact of genetic disease on inpatient pediatrics and the health care system in both admission rates and the total hospital charges [11]. These studies emphasize the importance of understanding the impact that genetic diseases have on mortality and healthcare strategies [15]. Furthermore, it is also clear that early recognition of the underlying disorders is necessary for optimal management of patients with genetic disorders.

Our study highlights another aspect related to the impact of genetic disorders and malformations. In 1981, Matsui et al. analyzed the cases of 18,736 children of total admission during 1975–1979 to KCMC and found that 44% had genetic disorders and malformations [10]. Although our study period and ward are limited to those in the HCU, the patients with genetic disorders and malformations had consistently significant impact in KCMC during the ensuing three decades. Further, it emphasizes that medical care for acute conditions and surgical procedures frequently requires highly specialized knowledge of unusual disease conditions and should be provided in consultation with specialists such as clinical geneticists.

**Table 4** Comparison of patients with genetic disorder vs. acquired condition on ages at admission and lengths of stay

| | Genetic disorders | | Acquired conditions | | |
|---|---|---|---|---|---|
| | Median (range) | *n* | Median (range) | *n* | *P* |
| Ages | 2.0 years (1 day–27.0 years) | 372* | 4.9 years (9 days–32.5 years) | 302* | <0.0001 |
| Length of hospitalization (days) | | | | | |
| Respiratory problem | 7 (1–979) | 182 | 5 (1–97) | 109 | 0.17 |
| CNS | 4 (1–54) | 73 | 4 (1–207) | 68 | 0.61 |
| Cardiovascular | 4 (2–11) | 13 | 4 (2–24) | 8 | 0.94 |
| Gastrointestinal | 5.5 (1–37) | 22 | 5 (2–15) | 12 | 0.60 |
| Kidney and urinary tract | 3 (2–12) | 5 | 8 (2–12) | 9 | 0.32 |
| Sepsis | 3.5 (2–9) | 14 | 7 (2–20) | 9 | 0.19 |
| Post-operative care | 2 (1–49) | 174 | 2 (1–62) | 93 | 0.18 |
| Total | 4 (1–979) | 518 | 4 (1–207) | 366 | 0.26 |

*For the patients who have recurrent admissions, the only first admission was calculated

Although the strategies for management of respiratory infection, by means of newly developed antibiotics and mechanical ventilators, and surgical intervention for infants with malformations, have improved, the general strategies for the medical treatment of genetic disorders and malformations remain to be clarified. Hall commented on the report by Yoon et al. [16] and emphasized the significance of basic research on the human genome and developmental genetics [3]. As shown in Table 2, genetic disorders and malformations include rare diseases, which, although uncommon, remain an important public-health issue and a challenge for the medical community [12].

Our study had the limitations of genetic studies and evaluation in cases with multiple malformations and other isolated malformations. The underlying conditions of most patients in this study were ascertained by clinical geneticists, but high-resolution genome analysis with arrays using comparative genomic hybridization was applied in only limited cases. Recently, research attention has focused to a large extent on rare genetic disorders and Mendelian diseases, because of their significant effect on human health, with the aim of identifying disease-related genetic variations. Re-evaluation and classification of underlying disorders, especially in the case of multiple congenital anomalies in undiagnosed patients, are required for further analysis.

Another limitation of our study is estimation of the financial burden of the group of patients with a genetic background. McCandless et al. showed that the disorders with genetic determinant account for 81% of the total hospital charges [11]. Their results are consistent with those of Hall et al. in 1978 [4]. Further analysis of financial burden in our study may provide useful information for improvement of health care systems.

In conclusion, we report here the proportion of genetic disorders and malformations among cases encountered at the HCU of a tertiary children's medical center in Japan. Over 30 years, the proportion of admissions attributed to genetic disorders and malformations has impact and currently accounts for more than half of admissions to this facility. These results firstly indicate improvements in medical care for patients with genetic disorders and malformations and further highlight the large proportion of cases with genetic disorders. As these cases require highly specialized management, the involvement of clinical geneticists in HCUs at children's hospitals is crucial. Eventually, a better fundamental understanding of genetic disorders and malformations may lead to further improvements in medical care and may reduce the impact of these conditions on the patients and their families.

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**Conflict of interest** The authors declare no conflict of interest.

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ORIGINAL INVESTIGATION

# Novel intragenic duplications and mutations of *CASK* in patients with mental retardation and microcephaly with pontine and cerebellar hypoplasia (MICPCH)

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**Abstract** The *CASK* gene encoding a member of the membrane-associated guanylate kinase protein family is highly expressed in the mammalian nervous system of both adults and fetuses, playing several roles in neural development and synaptic function. Recently, *CASK* aberrations caused by both mutations and deletions have been reported to cause severe mental retardation (MR), microcephaly and disproportionate pontine and cerebellar hypoplasia (MICPCH) in females. Here, mutations and copy numbers of *CASK* were examined in ten females with MR and MICPCH, and the following changes were detected: nonsense mutations in three cases, a 2-bp deletion in one case, mutations at exon–intron junctions in two cases, heterozygous deletions encompassing *CASK* in two cases and interstitial duplications in two cases. Except for the heterozygous deletions, each change including the intragenic duplications potentially caused an aberrant transcript, resulting in *CASK* null mutations. The results provide novel mutations and copy number aberrations of *CASK*, causing MR with MICPCH, and also demonstrate the similarity of the phenotypes of MR with MICPCH regardless of the *CASK* mutation.

## Introduction

The *CASK* gene (OMIM: *300172) encoding a member of the membrane-associated guanylate kinase (MAGUK) protein family is highly expressed in the mammalian ner-

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vous system of both adults and fetuses (Stevenson et al. 2000), and plays several roles in neural development and synaptic function (Hsueh 2006). Recently, we have reported the possible involvement of the decrease of intact *CASK* expression induced by a microdeletion at Xp11.4 in the pathogenesis of mental retardation (MR) and microcephaly (Hayashi et al. 2008). Concurrently, Najm et al. (2008) reported that mutations of *CASK* caused severe MR, microcephaly, and disproportionate pontine and cerebellar hypoplasia (MICPCH, OMIM: #300749). To examine the involvement of point mutations and/or copy number variations (CNVs) of *CASK* in patients with MR and MICPCH, we performed DNA sequencing and molecular cytogenetic analyses including array-based comparative genomic hybridization (aCGH) in ten patients showing such phenotypes. Pathogenic alterations of *CASK* were detected in all ten cases, and each change including intragenic duplications caused a truncation of the gene.

## Subjects and methods

### Subjects

From three hospitals in Japan, we recruited ten Japanese females (patients 1–10) suspected of having *CASK* mutations based on phenotypic criteria: female, MR and MICPCH. All patients were examined and evaluated by clinical dysmorphologists in each hospital. Their clinical features are shown in Table 1.

Briefly, their ages at the last follow-up ranged from 11 months to 14 years. All were born by normal full-term deliveries. At birth, patients 1, 2, 3, 5 and 7 revealed obvious signs of microcephaly (<−2.0 SD) and at present all the patients show severe microcephaly; their development has been markedly retarded. Only patients 5 and 6 can walk, and only patient 9 can speak any words at all. Patients 1, 2, 7, 9 and 10 show muscular hypotonia. Magnetic resonance imaging (MRI) of the brain demonstrated similar aberrations; all patients revealed hypoplasia of the cerebellum, mesencephalon and pons (Fig. 1; Table 1). Conventional karyotyping of peripheral blood lymphocytes with approximately 400–550 bands revealed a normal female karyotype, 46,XX, in each patient.

All samples were obtained with prior written informed consent from the parents and approval by the local ethics committee and all the institutions involved in this project. A lymphoblastoid cell line (LCL) was established by infecting lymphocytes of the patients and patients' parents with Epstein–Barr virus, as described previously (Saito-Ohara et al. 2002).

### Mutation analysis

Mutations within all coding sequences of *CASK* were analyzed by exon amplification and direct sequencing using primer combinations designed for genomic sequences around each exon (Najm et al. 2008). Any base changes detected in samples were confirmed by sequencing each product in both directions.

### aCGH analysis

For patients in whom no mutation of *CASK* was detected, we performed aCGH to estimate the genomic copy number variant (CNV) around *CASK*. We employed an in-house bacterial artificial chromosome (BAC)-based array, the 'MCG X-tiling array' (X-Array) (Inazawa et al. 2004), which contains 1001 BAC/PACs throughout the X-chromosome other than pseudoautosomal regions for aCGH analysis (Hayashi et al. 2007). Hybridization was performed as described elsewhere (Hayashi et al. 2007). For two patients, we also applied an oligonucleotide array (Agilent Human Genome CGH Microarray 244K; Agilent Technologies, Santa Clara, CA, USA) to determine the boundaries of the CNVs identified with the X-Array. DNA labeling, hybridization and washing of the arrays were performed according to the directions provided by the manufacturer. The hybridized arrays were scanned using an Agilent scanner (G2565BA), and the CGH Analytics program version 3.4.40 (Agilent Technologies) was used to analyze CNVs after data extraction, filtering and normalization by Feature Extraction software (Agilent Technologies).

### Fluorescence in situ hybridization (FISH)

FISH was performed as described elsewhere (Hayashi et al. 2005) using BAC clones located around the region of interest to confirm all the detected CNVs.

### Genomic PCR and reverse transcription-PCR

Genomic PCR was performed on genomic DNA of the ten patients and an unaffected female as a control. Reverse transcription-PCR (RT-PCR) was performed on cDNA extracted from LCLs of patients 5, 6, 9 and 10 and an unaffected female as a control. The sequences of the primer combinations are provided in Supplementary Table 1.

### Real-time quantitative PCR

To determine the boundaries of the duplications more precisely, real-time quantitative PCR (qPCR) was performed

**Table 1** Clinical features and analysis of *CASK*

| Patient | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| Gender | F | F | F | F | F | F | F | F | F | F |
| Age at last follow-up | 2 years, 8 months | 2 years, 0 month | 2 years, 8 months | 11 months | 7 years, 9 months | 14 years | 1 years, 9 months | 2 years, 0 months | 12 years | 7 years, 2 months |
| Gestational age (weeks) | 41 | 41 | 40 | 41 | 40 | 41 | 36 | 41 | 41 | 41 |
| Height at birth (SD) | *NA* | 0.2 | −2.6 | −0.8 | −3.8 | −0.8 | −2.2 | −0.9 | −2.0 | 0.2 |
| Weight at birth (SD) | −1.9 | −0.1 | −1.6 | −0.7 | −2.2 | −0.3 | −2.9 | −1.9 | −1.1 | 0.4 |
| OFC at birth (SD) | −3.2 | −2.3 | −2.8 | −0.8 | −3.4 | −0.4 | −4.3 | −1.3 | −1.9 | −1.5 |
| Height at last follow-up (SD) | −1.5 | −2.7 | −1.9 | −1.7 | −3.7 | −4.9 | −0.9 | −3.0 | −3.5 | −3.0 |
| Weight at last follow-up (SD) | −2.5 | −2.2 | −2.1 | −1.4 | −2.4 | −3.3 | −2.0 | −2.5 | −2.3 | −2.0 |
| OFC at last follow-up (SD) | −4.3 | −3.5 | −4.0 | −3.2 | −4.5 | −6 | −4.6 | −4 | −5.4 | −5.2 |
| Mental retardation | Severe | Moderate | Severe | Severe | Severe | Moderate | Severe | Moderate | Severe | Severe |
| Development (month) | | | | | | | | | | |
| Holding one's head | 6 | 4 | 4 | 5 | 3 | 4 | 6 | 4 | + | 3 |
| Sitting | – | 9 | – | – | 12 | 9 | 12 | 15 | – | 22 |
| Walking | – | – | – | – | 36 | 30 | – | – | – | – |
| Speech | – | – | – | – | – | – | – | A few words spoken | – | – |
| Muscular hypotonia | + | + | – | – | – | NA | + | – | + | + |
| Seizure | – | – | – | – | – | – | – | – | + | + |
| EEG | Spike and slow | *NP* | Normal | *NP* | Normal | Normal | Normal | Normal | Spike | Abnormal |
| MRI[a] | CE, ME, PO | CE, ME, PO | CE, ME, PO | CE, ME, PO | CE, ME, PO | CE, ME[b], PO[b] | CE, ME, PO | CE, ME, PO | CE, ME, PO | CE, ME, PO |
| Other abnormalities | Bilateral sensory deafness | Craniofacial dysmorphisms, bilateral hydronephrosis, deafness | | | | | Bilateral sensory deafness | | Severe scoliosis | |
| Karyotype | 46,XX | 46,XX | 46,XX | 46,XX, inv (9)(p12q13)[c] | 46,XX | 46,XX | 46,XX | 46,XX | 46,XX | 46,XX |
| X-tiling array | NP | NP | – | NP | – | – | del(X) (p11.3p11.4)dn | del(X) (p11.3p11.4)dn | dup(X)(p11.4) | dup(X) (p11.4), dup(X) (p11.21) |
| Size of CNV (Mb) | | | | | | | 3.0 | 1.1 | 0.2 | Two times 0.2 |

**Table 1** continued

| Patient | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| *CASK* mutation | | | | | | | | | | |
| Exon | 2 | 4 | 27 | 3 | Intron 4 | Intron 21 | None | NP | NP | NP |
| Nucleotide change | c.79C>T | c.316C>T | c.2632C>T | c.243_244delTA | c.357-1G > A | c.2040-1G > C | | | | |
| Protein change | *p.R27X* | *p.R106X* | *p.Q878X* | *p.Y81X* | *p.S119Rfs7X* *p.H120Pfs22X* | *p.W680Cfs29X* *p.W680Cfs3X* | | | | |
| *CASK* aberration in parents | NP | None | None | NP | None | None | None | None | NP | None |

*NA* not available, *NP* not performed, *SD* standard deviation

+, present; –, absent

[a] Showing hypoplastic region: *CE* cerebellum, *ME* mesencephalon, *PO* pons

[b] The hypoplasia is mild

[c] Normal variation

using genomic DNA of patient 10, four unaffected females and one unaffected male using the 7500 Real-Time PCR System (Applied Biosystems) and KAPA SYBR® FAST qPCR Master Mix (KAPA Biosystems) according to the manufacturers' instructions. Primers were designed using Primer3 software. Four primer combinations, Primer 1–4, were designed for the duplication at Xp11.4 between A_16_P21451772 and A_16_P21451795, which were oligonucleotides on the nucleotide array. Six primer combinations, primer 5–10, were designed for the duplication at Xp11.21 between A_16_P03708491 and A_14_P101900. The sequences of the primer combinations are provided in Supplementary Table 2.

## Results

### Mutation analysis

The analysis detected the mutations probably responsible for the phenotypes in six cases (Table 1; Fig. 2). Nonsense mutations were detected in three cases: c.79C>T (*p.R27X*), c.316C>T (*p.R106X*) and c.2632C>T (*p.Q878X*) in patients 1, 2 and 3, respectively. A deletion of two nucleotides in exon 3 was detected in patient 4: c.243_244delTA (*p.Y81X*). Mutations in intron were detected in two cases: c.357-1G>A of intron 4 in patient 5 and c.2040-1G>C of intron 21 in patient 6. Both intronic mutations were located at the splice acceptor sites and possibly affected the splicing of *CASK*. None of the mutations has been reported previously (Najm et al. 2008; Tarpey et al. 2009). In patients 2, 3, 5 and 6, we did not detect the mutation in either of the parents (data not shown), suggesting the mutations to be de novo.

### aCGH analysis and FISH

In four patients without possible causative mutations in *CASK*, CNVs involving *CASK* were detected by aCGH (Table 1; Fig. 3a). Heterozygous deletions including the gene were detected in patients 7 and 8. Duplications were detected in two patients: a 0.2-Mb intragenic duplication within *CASK* was detected in patient 9, and two 0.2-Mb duplications were detected at Xp11.4 within *CASK* and at Xp11.21, including part of *AX747041*, the function of which was unclear, in patient 10. All the CNVs were confirmed by FISH (Fig. 3b). Notably, FISH demonstrated that the duplication was a tandem duplication in patient 9, and that the two duplications in patient 10 were caused by a paracentric inversion between Xp11.4 and Xp11.21. In patients 7, 8 and 10, FISH detected the same CNV in neither of the parents, suggesting the CNVs to be de novo (data not shown). Parental samples of patient 9 were not

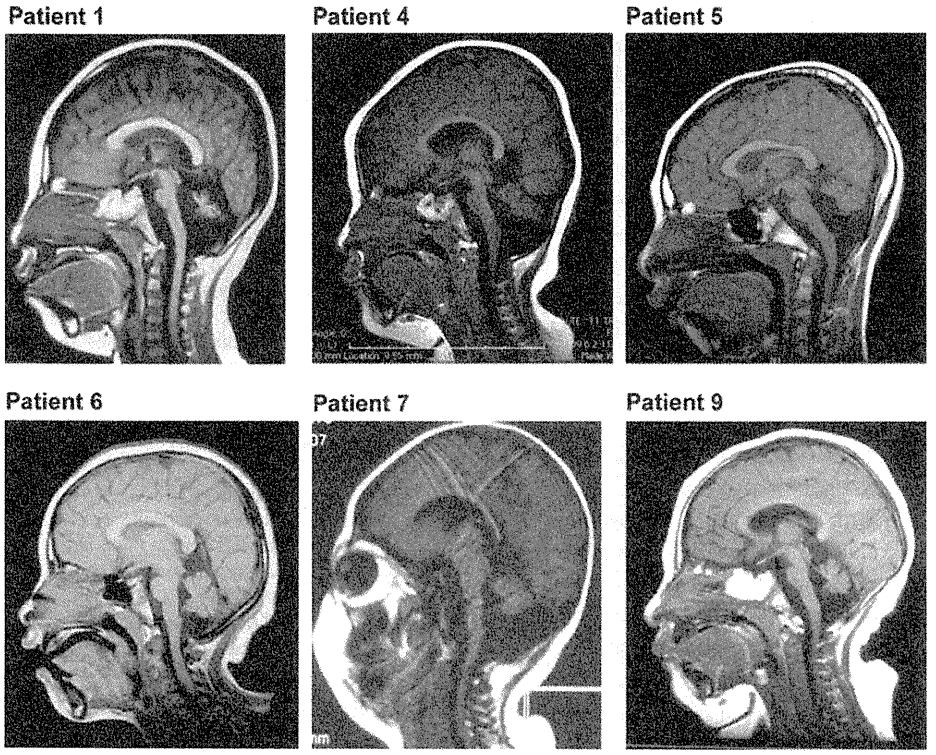


**Fig. 1** Representative brain MRI scans in six patients. All of them showed a hypoplastic cerebellum, mesencephalon and pons

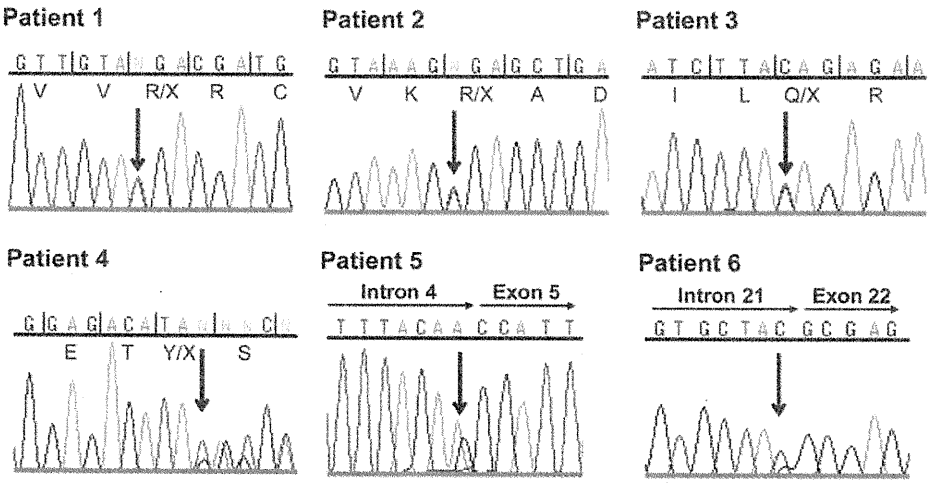


**Fig. 2** Partial electropherograms depicting the CASK mutations; c.79C>T in patient 1, c.316C>T in patient 2, c.2632C>T in patient 3, c.243_244delTA in patient 4, c.357-1G > A in patient 5 and c.2040-1G > C in patient 6. Each *arrow* indicates the mutated nucleotide. The translated amino acid sequences are shown under the nucleotides. The *black vertical lines* indicate reading frames

available. A scheme of the deletions and duplications is shown in Fig. 3c.

## Transcript analysis

Subsequently, we estimated the effect of the mutations at the splicing acceptor sites. For patient 5, we performed RT-PCR using primer combinations designed to target the area between exon 3 and exon 8 and obtained two products; one was the same size as the wild-type amplicon and the other was a smaller product (Fig. 4a; Supplemental Table 1). The smaller product was cloned and sequenced to show three different-sized transcripts (clone 1–3, Fig. 4b). Clone 1 was a transcript missing all of exon 5 (Fig. 4c) and clone 3 was a transcript in which exon 5 was absent and alternatively a part of intron 5 was inserted (Fig. 4d). Both were aberrant *CASK* transcripts leading to a frameshift and a premature stop codon, *p.S119Rfs7X and p.H120Pfs22X*,

respectively (Table 2). Clone 2 was a normal transcript. For patient 6, we also performed RT-PCR using primer combinations designed between exon 19 and exon 27 to obtain a smaller product than the control (Fig. 5a; Supplemental Table 1). The product was cloned and sequenced to show transcripts of three different sizes (clone 1–3, Fig. 5b). Clone 1 was a transcript without the initial 8 bp of exon 22 (Fig. 5c) and clone 3 was a transcript missing all of exon 22 (Fig. 5d). Both were aberrant *CASK* transcripts leading to a frameshift and a premature stop codon, *p.W680Cfs29X* and *p.W680Cfs3X*, respectively (Table 2). Clone 2 was a normal transcript; therefore, both of the mutations at the splicing acceptor sites caused *CASK* null mutations.

We also estimated the effects of the duplication of *CASK* in patients 9 and 10. Since the results of FISH suggested that an interstitial part of *CASK* was duplicated in patient 9 (Fig. 3b), we precisely mapped the duplication using the oligonucleotide array (Fig. 6a). We predicted the structure of the intragenic duplication and performed RT-PCR using primer combinations designed between exon 7 and exon 4, that is, specific to the duplication, and obtained a product (Fig. 6; Supplementary Fig. 1a; Supplementary Table 1). Sequencing showed the product of the RT-PCR to consist of exon 7 followed by exon 3 (Fig. 6c). These results indicated that the duplication in patient 9 probably caused an aberrant *CASK* transcript leading to a frameshift and a premature stop codon.

In patient 10, FISH suggested that the duplications were a result of a paracentric inversion (Fig. 3b). We also determined the precise size of each duplication with an oligonucleotide array (Fig. 7a) and qPCR (Supplementary Fig. 1b) and predicted a rearrangement in which exon 1, exon 2 and the duplicated exons 3–9 of *CASK* were at Xp11.21. (Fig. 7b). Inversion-specific genomic PCR using a primer combination between intron 8 of *CASK* and intron 1 of *AX747041* supported such a rearrangement (Fig. 7c; Supplementary Table 1). They were potentially transcribed to produce an aberrant transcript with exon 2 of *AX747041* which was originally located at Xp11.21 (Fig. 7b); thus, an inversion-specific RT-PCR using primer combinations targeting between exon 7 of *CASK* and exon 2 of *AX747041* was performed and a product was generated only in patient 10 (Fig. 7d; Supplementary Table 1). Sequencing of this product demonstrated that following exon 9 of *CASK*, exon 2 of *AX747041* was transcribed to produce a stop codon (Fig. 7e). These results clearly proved that the inverted duplication in patient 10 also resulted in *CASK* null mutations.

**Fig. 3** Array-CGH analysis and FISH. **a** Results of the X-array analyses. Clones are ordered according to the UCSC mapping position. Each *spot* represents the test/reference value after normalization and $\log_2$ transformation in each BAC clone. The *gray* vertical bar indicates the position of a centromere. *Black arrows* indicate each CNV. An approximately 3.0-Mb deletion at Xp11.4p11.3 was detected in patient 7 and an approximately 1.1-Mb deletion at Xp11.4p11.3 was detected in patient 8. In patient 9, an approximately 0.2-Mb duplication at Xp11.4 was detected based on the increased ratios of one BAC clone. In patient 10, two approximately 0.2-Mb duplications were detected at Xp11.4 and at Xp11.21. **b** Representative results of FISH and enlarged X chromosomes in each patient. *Yellow arrows* denote aberrant signal(s). Of the enlarged chromosomes, the *left* one is intact and the right one is affected, and *white* bands indicate the position of the centromere. In patients 7 and 8, FISH using a probe at Xp11.4 (RP11-95C16, *red*) and a reference probe at Xq28 (RP11-119A22, *green*) confirmed each deletion (*yellow arrow*). In patient 9, FISH using a probe at Xp11.4 (RP11-1069J5, *red*) and the same reference probe delineated tandem duplication (*yellow arrow*). In patient 10, both probes at the two duplications, RP11-1069J5 (*red*) and RP11-1106F5 (*green*), were hybridized at the same position (two *yellow arrows* in the *left enlarged panel*). On the right side, a schematic representation is shown. In the *right enlarged panel*, the probe combination located within the two duplications, RP11-95C16 at Xp11.4 (*red*) and RP11-179I23 at Xp11.21 (*green*), showed an inverted orientation in the affected chromosome X (two *yellow arrows*, *right*) compared with the intact chromosome X (*left*). On the right, a schematic representation is shown. **c** Scheme of the region around *CASK*, BAC clones, genes, deletions and duplications. *Open double-headed arrows* indicate the deletions and *filled double-headed arrows* indicate the duplications. *Horizontal black bars* indicate BAC clones (RP-11 series). *Thin horizontal arrows* indicate genes and their directions. In concordance with ISCN 2005 (Shaffer and Tommerup 2005), this result was described as follows: patient 7, arr cgh Xp11.4p11.3(RP11-829G10 → RP11-469F12)x1; patient 8, arr cgh Xp11.4p11.3(RP11-1069J5 → RP11-52P6)×1; patient 9, arr cgh Xp11.4(RP11 → 1069J5)x3; and patient 10, arr cgh Xp11.4 (RP11-1069J5)×3,Xp11.21(RP11-54I5 → RP11-1106F5)×3 (color figure online)

## Discussion

Among the ten cases with phenotypic criteria, female, MR and MICPCH, we detected genomic aberrations of *CASK* in all cases, nonsense mutations in three cases, a 2 bp-deletion in one case, mutations at the splice acceptor sites in two cases, heterozygous deletions encompassing *CASK* in two cases and intragenic duplications in two cases. Not only the nonsense mutations but also mutations at the splice acceptor sites generated aberrant *CASK* transcripts, leading to a frameshift and a premature stop codon. The intragenic tandem duplication in patient 9 and the intragenic inversion/duplication probably due to inv(X)(p11.4p11.21) in patient 10 also produced an aberrant transcript causing a frameshift, leading to a premature stop codon within *CASK*. We previously reported that the heterozygous deletion containing *CASK* probably reduced the expression of intact *CASK* in a female patient (Hayashi et al. 2008). The current study extends the variety of genomic alterations causing *CASK* null mutations. The incidence of *CASK* mutations and of CNVs involving *CASK* was almost the same, and there was no mutational hot spot in *CASK* according to a previous report (Najm