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Original article

New TRPM6 mutation and management of hypomagnesaemia with secondary hypocalcaemia

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Abstract

Background: TRPM6 gene mutation has been reported to cause hypomagnesemia with secondary hypocalcemia (HSH). However, the genotype–phenotype correlation for TRPM6 gene mutations has not been clarified.

Objective: To elucidate the factors underlying the severe neurological complications in HSH and evaluate the potential association between the location of TRPM6 gene mutations and clinical data of HSH.

Methods: A Japanese patient diagnosed with HSH at 10 weeks of age exhibited neurological damage and failed to thrive. Magnesium supplements were therefore started at 12 weeks of age. Mutational analysis of the TRPM6 gene was performed using a direct sequencing method to determine the position and type of mutation. Using the data of 29 HSH patients reported in the literature, linear regression analysis was also performed to examine the association between TRPM6 gene mutation location and HSH onset age, initial serum magnesium and calcium concentrations, and dose of oral magnesium.

Results: A novel stop-codon homozygous mutation [c.4190 G > A] W1397X was identified in exon 26 of the patient's TRPM6 gene. No statistical correlation was found between the location of mutations in the TRPM6 gene and the clinical data for 4 clinical indicators of HSH.

Conclusions: We identified the first Japanese HSH patient with a novel nonsense mutation in the TRPM6 gene. Regression analysis of mutation locations in the protein-coding region of TRPM6 and the reported clinical data for 4 clinical indicators of HSH in 30 HSH patients did not detect a genotype–phenotype correlation.

Keywords: TRPM6; HSH; Genotype–phenotype correlation; Magnesium; Mental retardation; Failure to thrive

1. Introduction

Hypomagnesemia with secondary hypocalcemia (HSH, OMIM #602014) is a rare autosomal recessive disorder that is characterized by the development of neurological symptoms, including tetany, muscle spasms, and seizures, in early infancy due to low serum magnesium [1]. The low serum magnesium levels

Abbreviations: HSH, hypomagnesemia with secondary hypocalcemia; TRPM6, transient receptor potential channel melastatin 6

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associated with HSH result from defective intestinal magnesium absorption and increased renal magnesium clearance, which lead to secondary hypocalcemia due to insufficient secretion and resistance to parathyroid hormone (PTH) [2,3]. To help prevent the negative outcomes of PTH deficiency, which include severe neurological damage and even death, HSH patients require life-long oral magnesium supplementation [4,5]. In 2002, an association was identified between HSH and mutations in the gene encoding the transient receptor potential channel melastatin 6 (TRPM6), which is involved in transepithelial magnesium transport and belongs to the transient receptor potential (TRP) family of cation channels [6,7]. To date, at least 38 *TPRM6* mutations have been reported and include stop codon, frame-shift, and splice-site mutations, and exon deletions [4]. Although an apparent association exists between the development of HSH and *TRPM6* abnormality, no definitive genotype–phenotype correlation has been established between TRPM6 gene mutations and disease severity [5].

2. Patient

The patient was an infant female born as the first child of consanguineous parents of Japanese origin. The patient's family pedigree is presented in Fig. 1A. The patient was delivered without problems and had a birth weight of 2795 g. At 72 days of age, the patient had a generalized tonic seizure with apnea, and was then admitted to a local hospital. Blood examination showed hypocalcemia (1.98 mmol/l; reference value: 2.2–2.7 mmol/l); however, the symptoms improved without any specific treatment after 1 week.

At 81 days of age, the patient was referred to our hospital because of recurrent seizure. On admission, the patient presented with seizure, muscle hypotonia, and severe irritability. Although blood examination showed hypocalcemia (1.55 mmol/l), the level of parathyroid hormone (PTH) was normal (17 pg/ml, reference value: 12–92 pg/ml). Blood urea nitrogen, creatinine, electrolytes, blood glucose and urinalysis were within normal ranges. Electroencephalogram and magnetic resonance imaging of the brain did not show any specific findings. Midazolam (0.2 mg/kg/h) for seizure and calcium gluconate (calcium; 35.2 mg/kg/day) for hypocalcemia were administered. After admission to our hospital, hypomagnesemia was detected (0.10 mmol/l; reference value: 0.75–1.25 mmol/l). Fractional magnesium excretion ($FEMg^{+2}$) was 2.7% (reference value: 1.0–8.0%), indicating that the renal absorption of magnesium was impaired. For this reason, magnesium was intravenously administered (4.37–5.47 mg/kg/day) at 84 days of age. All symptoms disappeared in accordance with normalization of the serum magnesium level. Creatinine clearance and urinary electrolytes, including calcium excretion, were within normal ranges. Renal ultrasonography was normal. Based on these findings, the patient was clinically diagnosed with HSH.

At 93 days of age, the patient was orally administered magnesium (26.73 mg/kg/day) four times daily in place of intravenous magnesium treatment. After 2 days of oral magnesium, the patient had mild diarrhea and irritability due to hypomagnesemia (0.49 mmol/l). The patient's irritability was reduced by increasing the dose of oral magnesium to 53.46 mg/kg/day, and the mild diarrhea also improved following the administration of oral magnesium with probiotics six times daily. The

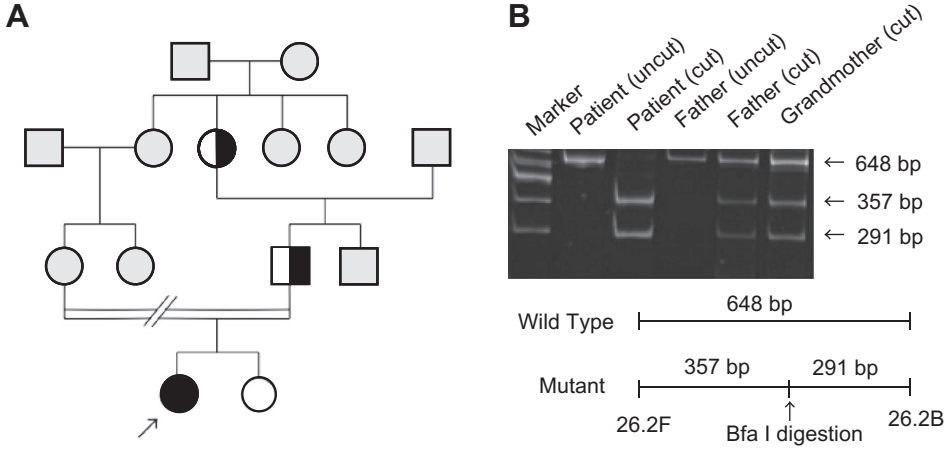

Fig. 1. Patient's family pedigree and genetic analysis of the TRPM6 gene. (A) Patient's family tree. The parents and sister of the proband did not have symptoms of HSH. Filled symbols, study patient; open symbols, wild-type haplotype; semi-filled symbols, heterozygous mutation carriers; grey symbols, unknown genotype; female; square, male; double slash, divorce. (B) Identification of the W1397X mutation in the TRPM6 gene. PCR-RFLP analysis was performed to identify mutations in the patient's TRPM6 gene using SDS–PAGE separated on an 0.8% gel. The patient has a homozygous W1397X mutation in the TRPM6 gene, and the patient's father and grandfather are heterozygous for this mutation. The restriction enzyme *Bfa*I was used in the analysis.

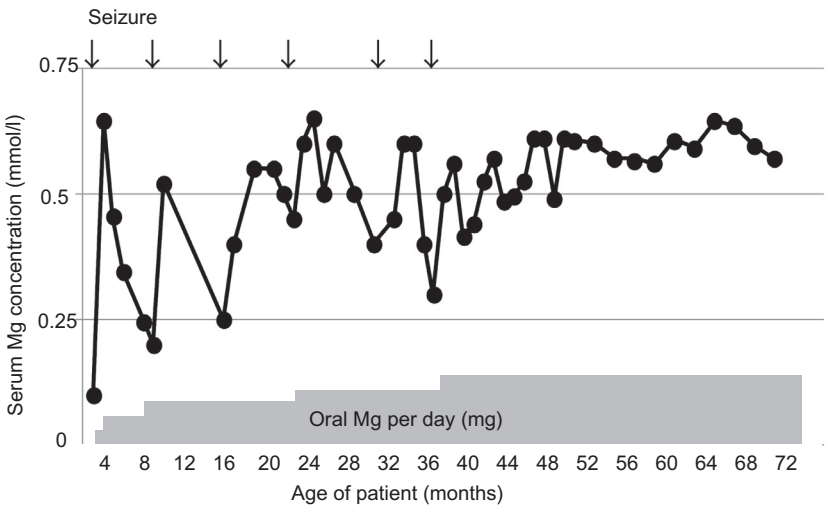

Fig. 2. Patient's clinical course. With increasing oral magnesium (Mg) administration over time, the patient's serum Mg concentration reached or exceeded the lower normal limit (0.75–1.25 mmol/l) and the number and frequency of seizures gradually decreased. Arrows indicate the occurrence of a seizure. Gray bars indicate the dose (mg/day) of orally administered Mg. The patient received a Mg dose of 101.45 mg/day at 3 months of age; 202.91 mg/day between 4 and 8 months of age; 242.40 mg/day between 8 and 24 months of age; and a maximum dose of 568.62 mg/day from 24 to 38 months of age.

patient was discharged after her serum magnesium level became stable (0.53–0.74 mmol/l) at 120 days of age. The patient's clinical course is shown in Fig. 2.

Due to poor drug compliance, the patient experienced several symptoms, including seizure, irritability, and photophobia, related to low magnesium serum levels and occasionally required intravenous infusion of magnesium until 3 years of age. The patient experienced mild diarrhea as a side effect of oral magnesium. The last reported seizure occurred at 3 years of age (Fig. 2). At 4 years of age, the patient scored 52 on an intelligence quotient test (Tanaka-Binet test) (normal > 85) and had a height of 93.0 cm (-2.7 SD for the standard height of Japanese girls). The patient was evaluated for growth hormone (GH) deficiency at 4 years and 8 months of age by performing an arginine infusion text. The patient's peak plasma GH concentration increased from 2.3 to 17.7 ng/ml after arginine infusion, suggesting that GH deficiency was not a factor.

The patient's parents provided written informed consent for the genetic analysis to be performed and for the patient's clinical data to be published. The study protocol was approved by the Ethics Committee of the Kurume University Graduate School of Medicine.

3. Methods

3.1. Mutational analysis

Extraction of DNA from white blood cells was performed using standard protocols. We designed 41 primer sets (primer sequences are available upon request) based on the sequence of the human TRPM6 gene (genomic contig GenBank accession No. NC_000009) to amplify the complete coding sequence (exons 1–39; exon 26 required 3 primer sets to cover the entire exon) and intron/exon boundaries of the TRPM6 gene from genomic DNA. Both strands of the amplified products were directly sequenced using a CEQ Dye Terminating Cycle Sequencing Kit (Beckman Coulter, Inc., Fullerton, CA) on a CEQ™ 8000 Genetic Analysis System (Beckman Coulter, Inc.). The sequences were assembled into a contig using the DNASIS Pro program (Hitachi Software Engineering Co., Ltd., Japan), and the resulting contig was aligned to the sequence of the human TRPM6 gene. To determine if the W1397X mutation was present in the patient's family members, PCR-RFLP analysis was performed using the primer set TRPP6.26.2F and TRPM6.26.2B, and the restriction endonuclease *Bfa*I.

3.2. Statistical analysis

Linear regression analysis was performed to investigate the association between the location of mutations in the TRPM6 gene and four clinical indicators of HSH: age of disease onset, initial serum magnesium and calcium concentrations (mmol/l), and dose of oral magnesium supplement (mg/kg/day) for 29 patients reported in the literature (Table 1). Patients with mutations in introns were excluded from the analysis. A total of 30 patients, which included the present study patient, were included in the analysis (Table 1) [5,6,8–12]. The level of statistical significance was set at $p > 0.05$. All analyses were performed using SPSS statistical software (SPSS Inc., Chicago, IL).

Table 1
Clinical data and *TRPM6* mutations of the 30 HSH patients analyzed in the present study.

Case	Exon	*TRPM6* mutation	Onset age (day)	Initial serum Mg/Ca (mmol/l)	Amount of oral Mg (mg/kg/day)	Refs.
1	25	Q1186X	90	0.16/1.8	4.86	[10]
2	25	Q1186X	90	0.08/1.8	9.72	[10]
3	12	R474X	16	0.32/1.78	250	[9]
4	5	E157X	35	0.16/1.43	20	[11]
5	16	S590X	60	0.21/1.63	25.03	[5,6]
6	4	S141L	120	0.1/2.50	32.81	[5,6]
7	11/26	H427fsX429 + 1260fsX283	35	0.41/1.88	75.35	[5,6]
8	17	R736fsX737	35	0.17/1.5	19.44	[5,8]
9	17	R736fsX737	35	0.22/1.6	18.23	[5,8]
10	32/33	Del exons 31 + 32	150	0.15/1.94	11.91	[5]
11	32/33	Del exons 31 + 32	35	0.22/1.73	10.21	[5]
12	22/23	Del exons 23 + 24	90	ND/1.74	9.97	[5]
13	30	L1673fsX1675	60	0.2/1.31	13.61	[5]
14	21	I944fsX959	42	ND/	13.37	[5]
15	26	Fs + preterm stp	60	0.1/1.66	72.92	[5]
16	26	Fs + preterm stp	120	0.19/	94.79	[5]
17	36	Loss of splice site/exon skipping	9	0.09/1.6	13.12	[5]
18	36	Loss of splice site/exon skipping	120	0.16/1.75	22.85	[5]
19	21	R928X	21	0.2/1.35	24.79	[5]
20	16	P599fsX609	35	0.29/1.45	42.05	[5]
21	21	Del exon 21	28	0.44/1.7	60.03	[5]
22	5	E157X	90	0.1/1.45	48.61	[5]
23	26	R1533X	60	/	17.26	[5]
24	6	D223fsX263	180	0.3/1.75		[5]
25	23	Y1053C		0.05/1.78	23.51	[12]
26	5/34	E157X + S1754 N	120	0.2/1.6	12.15	[12]
27	17	L708P + Loss of splice site	42	0.12/1.6	45.2	[12]
28	19/29	E872G + Q1663R	270	0.1/1.47	36.45	[12]
29	25	L1143P + Loss of splice site	60	0.08/1.94	18.23	[12]
30	26	W1397X	72	0.1/1.98	53.46	Present
Median	21.75		60.0	0.16/1.70	22.85	
Range	4–36		16–270	0.05–0.44/1.31–2.50	4.86–250	
p Value (vs. exon)			0.65	0.29/0.82	0.15	

Mg, magnesium; Ca, calcium; ND, not detectable; Del, deletion; Fs, frame shift.

4. Results

4.1. Mutational analysis

A novel homozygous stop-codon mutation, [c.4190 G > A] W1397X, was detected in exon 26 of the patient's TRPM6 gene based on the results of PCR-RFLP analysis (Fig. 1B). The patient's father and paternal grandmother were heterozygous for this mutation, whereas the patient's younger sister had wild-type *TRPM6* alleles (Fig. 1A). No member of the patient's immediate family had any clinical symptoms of HSH or abnormal laboratory findings related to serum magnesium, calcium, phosphate, and intact PTH levels. Unfortunately, the genotype of the patient's mother could not be determined as she was separated from the family due to divorce. Based on the genotype of the patient and the patient's father, the mother was likely heterozygous for the W1397X mutation. However, because no molecular analyses for chromosomal abnormalities were performed, we cannot exclude the possibility of uniparental disomy from the patient's father.

4.2. Association between TRPM6 gene mutation location and clinical data of HSH

To investigate if a correlation exists between the degree of loss of function of the TRPM6 protein

resulting from gene mutation and HSH severity we retrospectively analyzed the reported clinical data of 30 HSH patients, including the present study patient, with mutations in exons of the TRPM6 gene (Table 1). Linear regression analysis was performed for several clinical parameters of HSH, specifically disease onset age, initial serum magnesium and calcium concentrations, and oral magnesium dose. However, no significant correlations between the total number of exons in the TRPM6 gene and each of the four examined parameters of HSH severity were detected.

5. Discussion

We identified the first Japanese HSH patient with a novel homozygous stop-codon mutation in exon 26 of the TRPM6 gene. The patient manifested recurrent seizures at 10 weeks of age, leading to the diagnosis of HSH, and suffered from both physical and mental impairment in the form of short stature and mental retardation despite magnesium supplementation. One of the underlying causes of the complications was possibly due to poor drug compliance as a result of family problems. The retrospective analysis of 29 HSH patients with TRPM6 gene mutations did not detect a relationship between mutation location and any of the four indicators of HSH. Our findings suggest that preventing severe complications of HSH requires early diagnosis and good drug compliance to avoid recurrent seizures and permanent mental damage. In addition, clinical data for serum calcium and magnesium levels is not expected to aid in the genetic screening of nonsense mutations in the TRPM6 gene.

Our HSH patient exhibited early signs of mild mental retardation and had a low IQ test score at 4 years of age. Although HSH patients typically have normal psychomotor development, delayed diagnosis and repeated convulsions can cause mild to severe mental retardation as a result of neurological damage [4,5]. In the present patient, mental retardation may have resulted from the slightly delayed diagnosis of HSH and unstable serum magnesium levels associated with frequent diarrhea, which resulted in repeated convulsions over a 2.5-years period. In addition, family problems led to inadequate nurturing and low drug compliance, which likely contributed to the patient's low serum magnesium levels, convulsions, and mental impairment.

The patient's symptoms, particularly the frequency of seizures, gradually improved with increasing serum magnesium levels. Several additional factors may have influenced the patient's clinical improvement, including the oral administration of probiotics, avoidance of allergic foods, and improved family care. Although the mechanisms by which hypomagnesemia causes neurological damage are unknown, impaired voltage-dependent magnesium gating of the N-methyl-D-aspartate receptor may induce seizures [13,14]. In TRPM6 knockout mice, abnormal development and neural tube defects were observed [15], suggesting that TRPM6 affects nerve development. Mental retardation has also been reported in HSH patients due to the delayed administration of magnesium [4,5]. Our patient experienced seizures when serum magnesium fell below 0.4 mmol/l as a result of poor drug compliance. Thus, the rapid diagnosis of HSH and supplementation of magnesium above a threshold serum level are important to prevent psychomotor impairment.

Our patient had short stature and failed to thrive. Although growth disorders are rarely observed in HSH patients and the underlying mechanisms remain unclear [5,14], inadequate nutrition might be a major contributing factor. It is also possible that our patient's unstable family environment impacted her development, as short stature and failure to thrive have been reported for children who have suffered from physical and mental abuse [16–19]. At the time of writing, the patient had stable serum magnesium levels, was free from family problems, and had normal GH secretion. Thus, it is possible that young HSH patients with short stature and failure to thrive may improve with continued magnesium supplementation.

Schlingmann et al. [5] first reported that no genotype–phenotype correlation exists for HSH, and since this publication, a definitive genotype–phenotype correlation has not been demonstrated for this disorder. Most of the mutations in the HSH patients included in the present analyses (Table 1) would theoretically result in nonsense-mediated decay of TRPM6 mRNA and caused reduced TRPM6 protein translation [20]. Moreover, due to the large size of the TRPM6 gene, which consists of 39 exons spanning 167 kb of genomic sequence and codes for a protein of 2022 amino acids (Fig. 3), mutational screening is time consuming and expensive. If nonsense mutations leading to protein dysfunction or rapid mRNA decay could be predicted from clinical data, it would increase the efficiency of mutational screening and detection. We speculated that the total number of exons encoding the protein may be correlated with the clinical parameters of HSH. However, our present analyses of 30 HSH patients suggest that no correlation exists between TRPM6 genotype and the clinical parameters of HSH, demonstrating that clinical data are not useful for the estimation of the exon mutation site.

Several limitations of the present study warrant mention. First, as only one HSH patient with a stop-codon mutation in exon 26 of the TRPM6 gene was analyzed, our findings need to be confirmed in other patients who also possessed the identified mutation. Second, the linear regression analysis only included the data of 30 patients, a sample size that may have been too small to

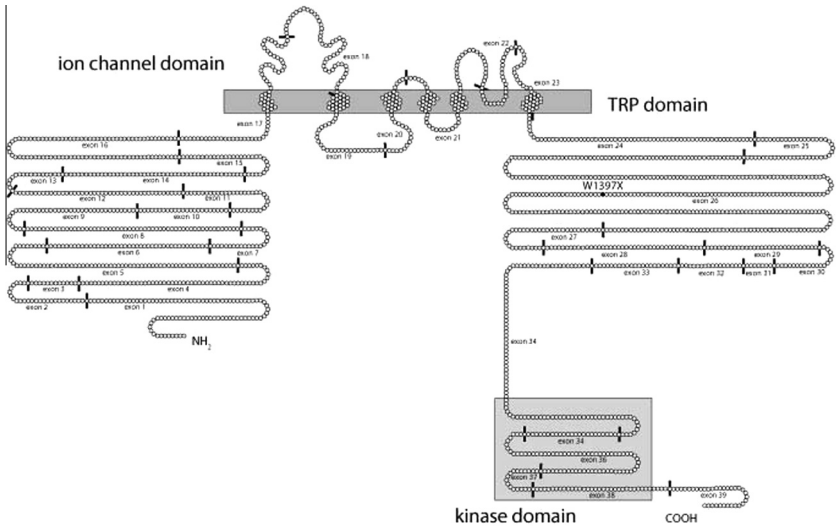

Fig. 3. Structure of the TRPM6 gene and exons. The TRPM6 gene consists of 39 exons spanning 167 kb of genomic sequence and coding for a protein of 2022 amino acids. The TRPM6 protein harbors an ion channel region with six transmembrane domains and a putative pore region between the fifth and sixth transmembrane domain, a long N-terminus conserved within the TRPM family, a TRP domain of unknown function located C-terminally of the ion channel domain, and a C-terminal kinase domain with sequence similarity to atypical α kinases. The structural organization of the TRPM6 gene presented in this figure was adapted from Ref. [20].

detect a relationship between exon number of the genetic abnormality and serum magnesium levels. Finally, the trend of increasing magnesium levels with the increasing level of TRPM6 gene impairment may have been influenced by differences in the therapeutic strategies between patients.

6. Conclusions

We describe here a Japanese HSH patient with a novel mutation in exon 26 of the TRPM6 gene. Despite the administration of magnesium at 12 weeks of age, the patient experienced family problems that may have adversely affected drug compliance, leading to short stature and mental retardation. The location analysis of mutations in the protein-coding region (exon number) of TRPM6 in 30 HSH patients and the 4 examined clinical parameters of disease showed that no genotype–phenotype correlation exists for HSH, as has been reported previously.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.braindev.2014.06.006.

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