Novel TRPM6 Mutations in 21 Families with Primary Hypomagnesemia and Secondary Hypocalcemia

Karl P. Schlingmann,* Martin C. Sassen,* Stefanie Weber,*† Ulla Pechmann,* Kerstin Kusch,* Lutz Pelken,* Daniel Lotan,‡ Maria Syrrou,§ Jeffrey J. Prebble,‖ David E.C. Cole,¶ Daniel L. Metzger,# Shamima Rahman,** Toshihiro Tajima,†† San-Ging Shu,†† Siegfried Waldegger,* Hannsjoerg W. Seyberth,* and Martin Konrad*§§

**Department of Pediatrics, Philipps-University Marburg, Marburg, Germany; †Pediatric Nephrology, University Children's Hospital Heidelberg, Heidelberg, Germany; ‡Pediatric Nephrology, The Chaim Sheba Medical Center, Tel-Hashomer, Israel; §Laboratory of General Biology, Medical School, University of Ioannina, Ioannina, Greece; ‖Department of Pediatrics, Toowoomba Base Hospital, Toowoomba, Australia; ¶Department of Laboratory Medicine & Pathobiology, University of Toronto, Ontario, Canada; #Pediatric Endocrinology, British Columbia Children's Hospital, Vancouver, British Columbia, Canada; **Hospital for Children Great Ormond Street, London, United Kingdom; ††Department of Pediatrics, Hokkaido University Medical School, Sapporo, Japan; ‡‡Department of Pediatrics, Taichung Veterans General Hospital, Taichung, Taiwan; and §§Pediatric Nephrology, University Children's Hospital, Inselspital Bern, Switzerland*

Primary hypomagnesemia with secondary hypocalcemia is a rare autosomal recessive disorder characterized by profound hypomagnesemia associated with hypocalcemia. Pathophysiology is related to impaired intestinal absorption of magnesium accompanied by renal magnesium wasting as a result of a reabsorption defect in the distal convoluted tubule. Recently, mutations in the *TRPM6* gene coding for TRPM6, a member of the transient receptor potential (TRP) family of cation channels, were identified as the underlying genetic defect. Here, the results of a *TRPM6* mutational analysis of 21 families with 28 affected individuals are presented. In this large patient cohort, a retrospective clinical evaluation based on a standardized questionnaire was also performed. Genotype analysis revealed *TRPM6* mutations in 37 of 42 expected mutant alleles. Sixteen new *TRPM6* mutations were identified, including stop mutations, frame-shift mutations, splice-site mutations, and deletions of exons. Electrophysiologic analysis of mutated ion channels after heterologous expression in *Xenopus* oocytes proved complete loss of function of TRPM6. Clinical evaluation revealed a homogeneous clinical picture at manifestation with onset in early infancy with generalized cerebral convulsions. Initial laboratory evaluation yielded extremely low serum magnesium levels, low serum calcium levels, and inadequately low parathyroid hormone levels. Treatment usually consisted of acute intravenous magnesium supplementation leading to relief of clinical symptoms and normocalcemia, followed by lifelong oral magnesium supplementation. Serum magnesium levels remained in the subnormal range despite adequate therapy. This is best explained by a disturbed magnesium conservation in the distal convoluted tubule, which emerged in all patients upon magnesium supplementation. Delay of diagnosis resulted in permanent neurologic damage in three patients.

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Primary hypomagnesemia with secondary hypocalcemia (HSH) was first described by Paunier *et al.* (1). Patients usually present in early infancy with generalized convulsions or signs of increased neuromuscular excitability. Laboratory evaluation at manifestation reveals severely reduced serum magnesium levels accompanied by hypocalcemia and barely detectable parathyroid hormone (PTH) levels. The unexpected finding of hypoparathyroidism is thought to result from an inhibition of PTH synthesis and secretion induced by extreme hypomagnesemia (2).

Contrasting all other known forms of hereditary hypomagnesemia, pathophysiologic studies in affected patients using radioactive magnesium isotopes pointed to a primary defect in intestinal magnesium absorption (3,4). The presence of an additional renal magnesium leak in HSH was controversially discussed (4–6).

By using a DNA pooling strategy, Walder *et al.* (7) had mapped a gene locus for HSH on chromosome 9q22. Recently, mutations in the *TRPM6* gene have been identified as the underlying genetic defect in patients with HSH (8,9).

TRPM6 codes for TRPM6, a new member of the transient receptor potential (TRP) family of cation channels. Within the

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Address correspondence to: Dr. Karl Peter Schlingmann, University Children's Hospital Marburg, Deutschhausstrasse 12, 35037 Marburg, Germany. Phone +49-6421-286-2483; Fax: +49-6421-286-5724; E-mail schlingm@staff.uni-marburg.de

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TRP family, TRPM6 belongs to the TRPM subfamily, whose eight members exhibit a significant diversity in domain structure as well as cation selectivity and activation mechanisms (10). TRPM6 is closely related to TRPM7, both sharing the unique feature of a serine/threonine kinase domain c-terminally fused to their ion channel domain (11).

The ubiquitously expressed TRPM7 has been characterized as a constitutively active ion channel that is permeable for a variety of divalent cations, including calcium and magnesium, whose basal activity is regulated by intracellular levels of magnesium and Mg-ATP (12,13). TRPM7 was shown to play a crucial role in cellular magnesium homeostasis (14).

In contrast to TRPM7, the expression pattern of TRPM6 seems to be more confined, with expression mainly along the gastrointestinal tract as well as in kidney predominantly in the distal convoluted tubule (DCT) (8), where it is presumed to be involved in the apical entry of magnesium into epithelial cells (15). Functional data on TRPM6 are contradictory. Whereas one study succeeded in heterologous expression of TRPM6 in mammalian cells and showed channel properties similar to those observed for TRPM7 (15), another study failed to detect measurable currents upon TRPM6 expression (16). Instead, it was demonstrated that heteromultimerization with TRPM7 is essential for correct membrane targeting of TRPM6. In this study, TRPM7-induced currents were significantly increased by co-expression of TRPM6.

As *TRPM6* mutations that are found in patients with HSH are the only naturally occurring human knockout for a member of the TRPM family described so far, the mutational analysis of affected individuals together with the functional analysis of mutations in heterologous expression systems represents an attractive approach to gain further insight into TRPM ion channel function. Here, we present comprehensive genotypic data on 28 patients with HSH and provide detailed information regarding phenotypic presentations and clinical courses for this genetically characterized cohort.

Materials and Methods

Patients and Families

Twenty-one families that have HSH, are of different ethnic origin, and have 28 affected individuals were analyzed (Table 1). Family pedigrees are shown in Figure 1. Parental consanguinity was noted in 12 families, and six families had two or more affected siblings. Families F1 to F5 were included in the original report on the involvement of TRPM6 in HSH by our group (8). Clinical aspects of families F4, F5, F6, F7, F15, and F17 were reported previously (6,17–21). For all patients, the diagnosis was based on manifestation in early infancy with generalized convulsions or muscular tetany, profound hypomagnesemia at the time of presentation accompanied by hypocalcemia, and relief of clinical symptoms and normocalcemia upon administration of magnesium salts.

Mutation Analysis

Extraction of DNA from blood leukocytes was performed using standard protocols. *TRPM6* mutational screening was performed by

Table 1. Clinical data of genetically characterized patients with HSH[a]

Patient	Gender	Age at Manifestation	Age at Diagnosis	Follow-Up (yr)	Symptoms at Manifestation	Initial Serum Mg^{2+} (mM)	Initial Serum Ca^{2+} (mM)	Oral Mg^{2+} (mmol/kg per d)	Mg^{2+} under Therapy (mM)	Fe_{Mg} (%)	Diarrhea	Additional Findings
F1.1	F	2 mo	2 mo	4	Seizures	0.21	1.63	1.03	0.59	2.6	−	
F2.1	M	3 wk	6 yr	10	Seizures	ND	1.29	0.62	0.57	2.8	−	Mental retardation
F3.1	F	4 mo	4 mo	3	Seizures	0.10	2.50	1.35	0.55	3.6	+	
F4.1	M	5 wk	5 wk	25	Seizures	0.41	1.88	3.10	?	?	+	
F5.1	F	5 wk	5 wk	14	Seizures	0.17	1.50	0.80	0.55	5.1	?	
F5.2	F	5 wk	5 wk	13	Seizures	0.22	1.60	0.75	0.55	2.1	?	
F6.1	M	5 mo	5 mo	15	Seizures	0.15	1.94	0.49	0.86	3.9	−	
F6.2	M	5 wk	5 wk	12	Seizures	0.22	1.73	0.42	0.85	?	−	Cardiac arrhythmia
F7.1	M	6 wk	10 mo	25	Seizures	0.21	1.63	3.00	0.60	?	+	
F8.1	F	3 mo	3 mo	8	Seizures	ND	1.74	0.41	0.60	?	−	
F9.1	F	2 mo	2 mo	3	Tetany	0.20	1.31	0.56	0.56	?	+	Failure to thrive
F10.1	F	6 wk	6 wk	15	Seizures	ND	?	0.55	0.51	2.4	+	
F11.1	F	2 mo	2 mo	6	Seizures	0.10	1.66	3.00	0.44	?	+	Failure to thrive
F11.2	M	4 mo	4 mo	3	Seizures	0.19	?	3.90	?	?	+	Failure to thrive
F12.1	M	3 mo	[b]	7	Seizures	0.09	1.60	0.54	0.33	?	−	
F12.2	M	4 mo	[b]	3	Asymp[c]	0.16	1.75	0.94	0.53	?	−	
F13.1	F	6 mo	6 mo	10	Seizures	0.30	1.75	?	0.61	3.2		Hyperactivity
F13.2	F	?	?	8	Asymp[c]	?	?	?	?	?	+	
F13.3	M	?	?	2	Asymp[c]	?	?	?	?	?	+	
F14.1	M	7 mo	7 mo	1.5	Seizures	0.29	1.60	0.63	0.50	5.1	+	
F15.1	F	3 wk	8 wk	28	Seizures	0.20	1.35	1.02	0.78[d]	?	+	
F16.1	F	5 wk	5 wk	6	Seizures	0.29	1.45	1.73	0.49	4.9	+	Failure to thrive
F17.1	M	2 wk	2 yr	14	Seizures	0.37	?	?	0.58	2.5	+	Mental retardation
F17.2	F	4 mo	4 mo	9	Seizures	?	?	?	?	?	?	
F18.1	F	4 wk	4 wk	0.5	Seizures	0.44	1.70	2.47	0.45	14.3	?	
F19.1	M	3 mo	4 mo	0.5	Seizures	0.10	1.45	2.00	0.50	3.7	?	
F20.1	F	2 mo	2 mo	18	Seizures	?	?	0.71	0.62	?	−	
F21.1	F	3 wk	3 wk	3	Seizures	0.20	1.72	0.93	0.52	?	−	

[a]F, female; M, male; asymp, asymptomatic; ND, not detectable; Fe_{Mg}, fractional excretion of magnesium.
[b]Suspected for Gitelman syndrome.
[c]Siblings of index patients, laboratory diagnosis of HSH while still asymptomatic.
[d]Patient F15.1 receives subcutaneous magnesium.

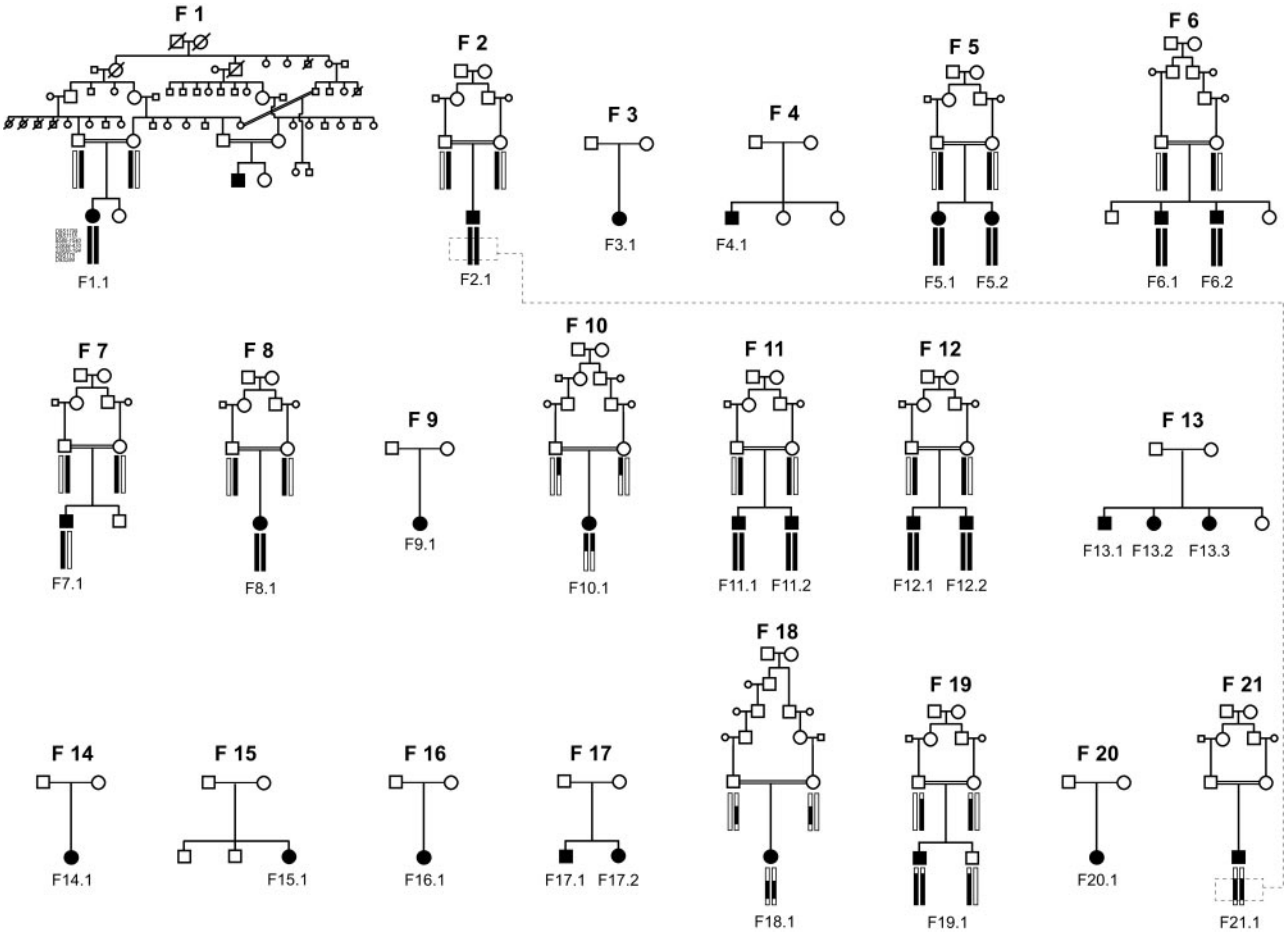

Figure 1. Family pedigrees for families F1 to F21 and results of haplotype analyses. Filled symbols, affected individuals; slash mark, deceased individuals; double lines, consanguinity; black bars, haplotypes segregating with primary hypomagnesemia with secondary hypocalcemia (HSH); white bars, wild-type haplotypes; dashed boxes, common haplotype identified in patients F2.1 and F21.1.

single-strand conformation polymorphism analysis (22). An overlapping set of PCR primers based on the sequence of the human *TRPM6* gene (genomic contig GenBank accession no. AL354795) was used to amplify the complete coding sequence (exons 1 to 39) and the intron/exon boundaries from genomic DNA (primer sequences available upon request). Amplified products were separated on polyacrylamide gels by electrophoresis (Multiphor II; Pharmacia Biotech, Uppsala, Sweden). Subsequently, exons with conformational variants were sequenced directly from both strands (Applied Biosystems 310 Genetic Analyzer; Foster City, CA).

Haplotype Analysis

The microsatellite markers D9S1799, D9S1115, 8580-1540, 23938-470, 23938-194 (8), and D9S175, linked to the *TRPM6* gene on chromosome 9q22, were amplified by PCR (primer sequences available upon request). Fragments were separated on 6% polyacrylamide gels under denaturing conditions in an ALFexpress DNA sequencer (Pharmacia Biotech), and data were analyzed using Fragment Manager software version 1.2 (Pharmacia Biotech). Alleles were numbered according to their order in gel electrophoresis, and haplotypes were constructed from the genotypic data. The most likely haplotypes were inferred by minimizing the number of crossover events in each family.

Heterologous Expression of TRPM6 in Xenopus *oocytes*

For functional analysis of mutant TRPM6, selected mutations (H427fsX429, R928X, Y1533X, L1673fsX1675, and delEx32-33) were introduced into full-length TRPM6 by site-directed mutagenesis. TRPM cDNA were subcloned into the pOGII vector (a pBluescript derivative with the 5′- and 3′-untranslated regions of *Xenopus* β globin). A total of 10 ng of *in vitro* transcribed cRNA (mMessage mMachine kit; Ambion, Austin, TX) for each TRPM6 construct was injected together with 10 ng of TRPM7-cRNA into defolliculated *Xenopus* oocytes, which were kept in ND96 solution that contained 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES (pH 7.4), 2.5 mM sodium pyruvate, 0.5 mM theophylline, and 20 μg/ml gentamicin at 16°C. Two to 5 d after injection, two-electrode voltage-clamp measurements were performed with a GeneClamp 500 amplifier (Axon Instruments, Burlingame, CA) at room temperature. Currents were recorded in ND96 solution without sodium pyruvate, theophylline, and gentamicin. Data were reproduced in at least two different batches of oocytes derived from different frogs. Statistical analysis was performed on current recordings derived from one batch of oocytes with at least 10 oocytes per data point using *t* test (assuming significance if $P < 0.05$).

Clinical and Laboratory Data

Serum biochemical parameters were analyzed using standard techniques. The GFR was calculated using the Schwartz formula (23). The ultrafiltrable fraction of serum magnesium was calculated as $UF_{Mg} = 0.7 \times S_{Mg}$ (24). Renal magnesium handling was assessed by calculating fractional magnesium excretions with [$Fe_{Mg} = (U_{Mg} \times S_{Cr})/(UF_{Mg} \times U_{Cr} \times 100$] as well as by calculating the magnesium excretion index [$GF_{Mg} = U_{Mg} \times S_{Cr}/U_{Cr}$], where Fe is fractional excretion, S_{Mg} is serum Mg, U_{Mg} is urinary Mg, S_{Cr} is serum creatinine, U_{Cr} is urine creatinine, and GF_{Mg} is magnesium excretion index. Urinary magnesium excretions expressed as GF_{Mg} were plotted against the ultrafiltrable fraction of serum magnesium (UF_{Mg}) as described (9). Renal ultrasound was performed to rule out nephrocalcinosis. The clinical course was evaluated retrospectively with a standardized questionnaire. Diarrhea as the mean side effect of high oral magnesium administration was considered as three or more loose or watery bowel movements per day. This study was approved by the local ethics committee, and informed consent was obtained from the patients and/or their parents

Results

Mutation Analysis

Mutation analysis of the *TRPM6* gene revealed 23 different mutations among our cohort of 21 families (Table 2). Both mutant *TRPM6* alleles were detected in affected individuals of 16 families, whereas in affected individuals of five families, only one mutation could be identified.

Table 2 depicts the consequent changes in TRPM6 amino acid sequence/structure. It is interesting that two patients from families who were of Turkish ancestry and living in France (F2.1) and Germany (F21.1) were carriers of the same splice-site mutation. Analysis of polymorphic microsatellite markers in both families revealed haplotype sharing for flanking markers 8580-1540, 23938-470, and 23938-194, pointing to a common ancestor (Figure 1).

In addition to the seven mutations previously reported, 16 novel mutations were identified. Altogether, the mutational spectrum comprises one point mutation, four stop mutations, seven splice-site mutations, seven frame-shift mutations, and four deletions. All mutations co-segregated with the phenotype, and none of the single-nucleotide exchanges was observed in 102 control chromosomes.

Figure 2 shows the distribution of mutations along the *TRPM6* gene as well as the consequent changes in amino acid sequence of the TRPM6 protein. The mutations observed are distributed over the entire TRPM6 protein without specific clustering.

Of the deletions, del exon 21 directly affects the sixth transmembrane domain. The other three deletions are located C-terminally of the ion channel domain. Assuming an intact splicing from the predeletion exon to the postdeletion exon, the deletions of exons 22 to 23 and 25 to 27 would lead to a frame shift and consequently a preterm stop codon. In contrast, the deletion of exons 32 to 33 represents an in-frame deletion, which possibly allows for a correct C-terminus distal to the deletion. The breakpoint of the deletion of exons 32 to 33 was demonstrated to be located within an ALU repetitive sequence

Table 2. Results of the *TRPM6* mutational analysis[a]

Patient	Gender	Origin	Consanguinity	Zygosity	Nucleotide Change	Exon(s)	Consequence of Mutation
F1.1	F	Turkey	Yes	Homo	1769C>G	Ex 16	S590X
F2.1	M	Turkey	Yes	Homo	2667 + 1G>A	IVS 20	Loss of splice site/exon skipping
F3.1	F	Sweden	No	Comp-hetero	[3537-1G>A] + [422C>T]	IVS 25 + Ex 4	Loss of splice site/exon skipping + S141L
F4.1	M	Israel	No	Comp-hetero	[1280delA]+ [3779–91del]	Ex 11 + Ex 26	H427fsX429 + E1260fsX1283
F5.1	F	Albania	Yes	Homo	2207delG	Ex 17	R736fsX737
F5.2	F	Albania	Yes	Homo	2207delG	Ex 17	R736fsX737
F6.1	M	Australia	Yes	Homo	del Ex 31 + 32	Ex 32 + Ex 33	In-frame del
F6.2	M	Australia	Yes	Homo	del Ex 31 + 32	Ex 32 + Ex 33	In-frame del
F7.1	M	Egypt	Yes	Homo	1308(+1)G>A	IVS 11	Loss of splice site/exon skipping
F8.1	F	India	Yes	Homo	del Ex 22 + 23	Ex 22 + Ex 23	Frame shift + preterm stop
F9.1	F	France	No	Hetero	[5017-18delT] + ?	Ex 30 + ?	L1673fsX1675 + ?
F10.1	F	Rumania	Yes	Homo	Del2831_2832insG	Ex 21	I944fsX959
F11.1	F	Pakistan	Yes	Homo	del Ex 25 –Ex 27	Ex 25–Ex 27	Frame shift + preterm stop
F11.2	M	Pakistan	Yes	Homo	del Ex 25 –Ex 27	Ex 25–EX 27	Frame shift + preterm stop
F12.1	M	Turkey	Yes	Homo	5775A>G	Ex 36	Loss of splice site/exon skipping
F12.2	M	Turkey	Yes	Homo	5775A>G	Ex 36	Loss of splice site/exon skipping
F13.1	F	UK	No	Hetero	[668delA] + ?	EX 6 + ?	D223fsX263
F13.2	F	UK	No	Hetero	[668delA] + ?	Ex 6 + ?	D223fsX263
F13.3	M	UK	No	Hetero	[668delA] + ?	Ex 6 + ?	D223fsX263
F14.1	M	Germany	No	Hetero	[1208(−1)G>A] + ?		Loss of splice site/exon skipping + ?
F15.1	F	Germany	No	Hetero	2782C>T + ?	Ex 21 + ?	R928X + ?
F16.1	F	Japan	No	Comp-hetero	[del1796_1797] + [5057(+2)T>C]	Ex 16 + IVS 30	P599fsX609 + loss of splice site/exon skipping
F17.1	M	Taiwan	No	Hetero	[2537(−2)A>T + ?	IVS 19+ ?	Loss of splice site/exon skipping + ?
F17.2	F	Taiwan	No	Hetero	[2537(−2)A>T + ?	IVS 19 + ?	Loss of splice site/exon skipping + ?
F18.1	F	Greece	Yes	Homo	del Ex 21	Ex 21	Frame shift + preterm stop
F19.1	M	Turkey	Yes	Homo	469G>T	Ex 5	E157X
F20.1	F	Germany	No	Comp-hetero	Ex 26 + IVS 32	Ex 26 + IVS 32	R1533X + loss of splice site
F21.1	F	Turkey	Yes	Homo	2667 + 1G>A	IVS 20	Loss of splice site/exon skipping

[a]Homo, homozygous; hetero, heterozygous; comp-hetero, compound-heterozygous; Ex, exon; IVS, intervening sequence or intron; del, deletion; fs, frame shift.

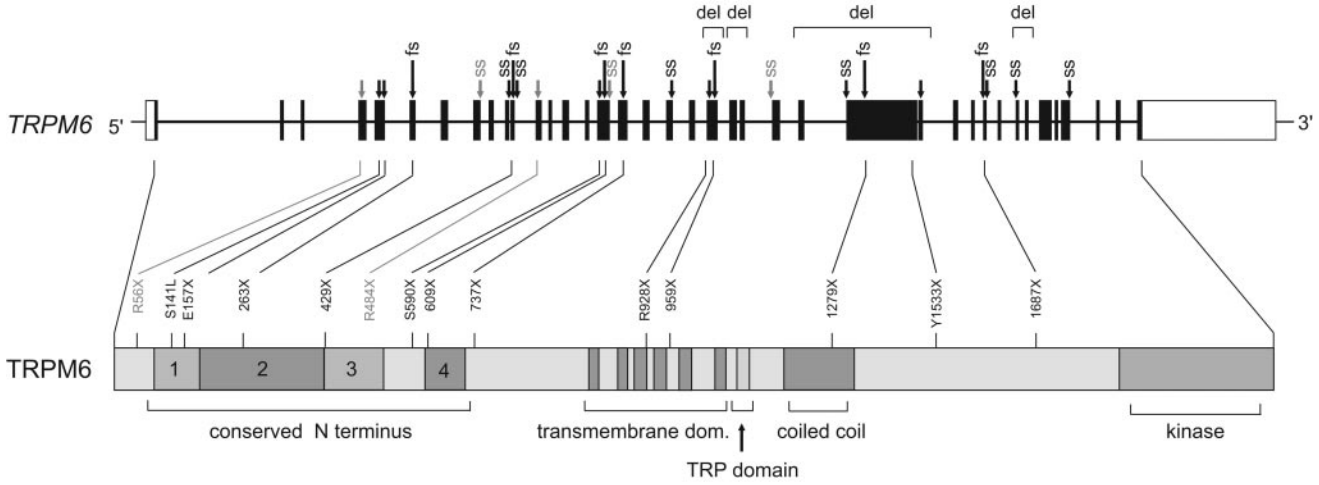

Figure 2. mRNA structure of the *TRPM6* gene and domain structure of the TRPM6 protein. The *TRPM6* gene consists of 39 coding exons (■) spanning approximately 163 kb of genomic DNA. Functional TRPM6 domains are deduced from the TRPM7 model as described previously (13). The mutations detected in HSH, along with the corresponding exon number and their functional consequences for the TRPM6 protein, are indicated. fs, frameshift; ss, splice site.

by PCR-based generation of a specific fusion fragment (data not shown).

The splice-site mutations mainly affect the invariably conserved residues at positions +/−1 and +/−2. We also observed one mutation (nt5775 A>G) located two bp proximal to the donor splice site at exon 36, which does not alter the corresponding tryptophane at amino acid position 1925 of the TRPM6 protein. Instead, it is thought to interfere with correct splicing as the residue (−2) is conserved at a high percentage (58%) of donor splice sites (25).

Functional Analysis in Xenopus *oocytes*

As described previously (16), TRPM6 depends on its close homologue TRPM7 for correct membrane trafficking and formation of functional channel complexes. We therefore co-expressed cRNA of mutant and wild-type TRPM6 with TRPM7 cRNA in *Xenopus* oocytes and compared the resulting current amplitude with currents elicited by isolated expression of TRPM7. Isolated expression of TRPM6 did not entail ion currents significantly different from uninjected control oocytes (Figure 3). As described previously (16), expression of TRPM7 alone resulted in the development of inward and outward currents at negative and positive voltages, respectively. Co-expression of wild-type TRPM6 together with TRPM7 significantly increased current amplitude.

In contrast, none of the tested mutant TRPM6 constructs exerted a significant effect on TRPM7 current amplitude (Figure 4), compatible with a complete loss-of-function effect of the tested TRPM6 mutations. Currents observed for frame-shift and stop mutations before and after the putative TRPM6 ion channel domain were not significantly different from each other. The deletion mutant (deletion of exons 32 to 33) even displayed slightly lower current amplitudes.

Clinical Presentation

Table 1 summarizes the clinical data. There were 16 female and 12 male patients in the cohort. Ages at time of initial presentation ranged from 2 wk to 7 mo (median 2 mo), and the diagnosis of hereditary hypomagnesemia was made at a median age of 2 mo (range 3 wk to 6 yr). All patients presented with generalized seizures except for one patient, who presented with tetany (F9.1). In multiplex families, diagnosis of the disease for one sibling generally prompted the search for hereditary hypomagnesemia in the other sibling(s) (F5, F6, F11, F12, F13, and F17).

Initial serum magnesium levels ranged from not detectable values to 0.44 mmol/L, with a median of 0.20 mmol/L. Initial serum calcium levels ranged from 1.29 to 2.50 mmol/L, with a median at 1.65 mmol/L. PTH levels were measured in only seven patients before initiation of treatment. Levels ranged from not detectable values to 36 pg/ml, with a median at 13 pg/ml. Serum phosphate levels were measured in 13 patients before initiation of treatment, with levels ranging from 1.10 to 3.17 mmol/L and a median at 2.38 mmol/L (reference range 1.9 to 3.0 mmol/L for full-term newborns [26]).

Initial treatment for seizures consisted of anticonvulsives in nine patients; in five patients, hypocalcemia was suspected as the primary cause of convulsions, which resulted in intravenous administration of calcium alone. The detection of hypomagnesemia together with hypocalcemia led to in instant magnesium administration in 16 patients, mostly together with intravenous calcium. In two patients, no data concerning the initial manifestation and therapy could be obtained.

Data on urinary magnesium excretion expressed as fractional excretions (FE_{Mg}) and magnesium excretion indices (GF_{Mg}) were available for 13 patients. Values ranged from 2.1 to 14.3% (median 3.6%; normal range 3 to 5% for normomagnesemic individuals). When plotting urinary magnesium excretions (ex-

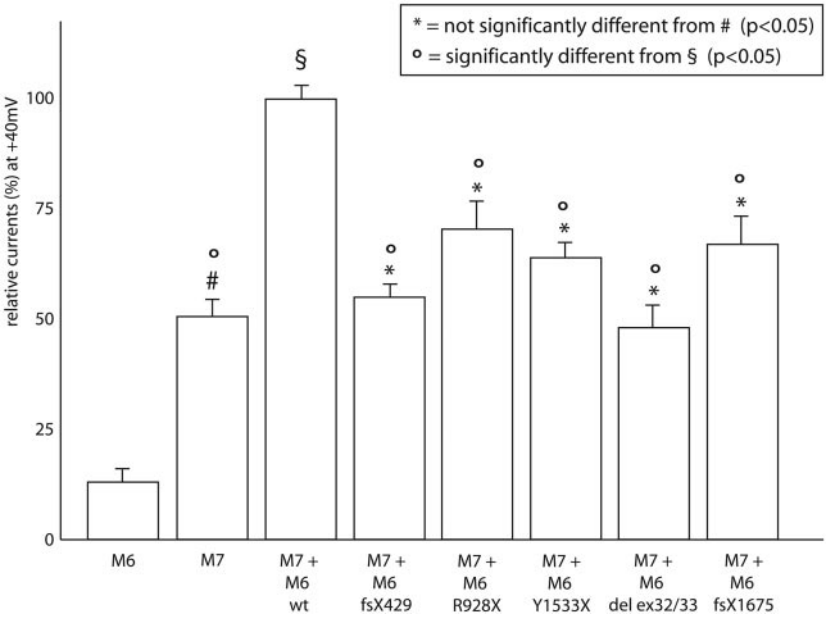

Figure 3. Heterologous expression of TRPM6/7 in *Xenopus* oocytes. Comparison of currents obtained at 40 mV in oocytes that were injected with 10 ng of wild-type or mutant TRPM6 and TRPM7 cRNA as described (16). Current-voltage relationships were determined on at least 10 oocytes per current-voltage curve. The currents obtained after co-injection of wild-type TRPM6 and TRPM7 cRNA (§) are set 100% per definition. Co-injection of wild-type TRPM6 cRNA (§) led to a significant amplification of TRPM7 currents (#), whereas co-injection of different TRPM6 mutants (*) yielded currents that were not significantly different from expression of TRPM7 alone. Injection of TRPM6 cRNA alone produces currents that are not significantly different from noninjected control oocytes. Bars indicate relative percentages of current densities expressed as means (±SEM).

pressed as GF_{Mg}) against the UF_{Mg}, patients exhibited significantly higher magnesium excretions compared with the rate obtained for normal children (27) (Figure 4).

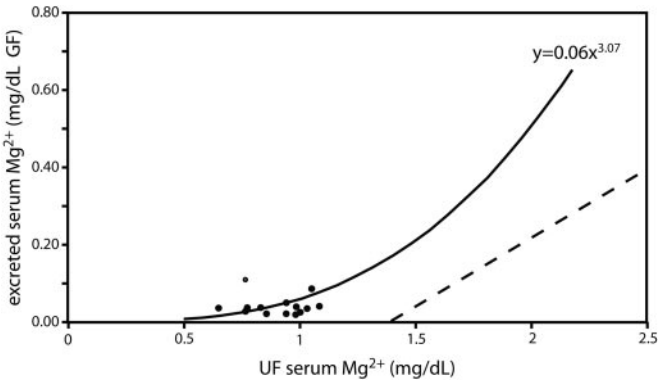

Figure 4. Urinary excretion of magnesium under oral substitution therapy (data of 13 patients shown). The urinary magnesium excretion index (GF_{Mg}) is plotted against the ultrafiltered magnesium load (UF_{Mg}). With the exception of one patient's excretion (F18.1; empty symbol), which might be falsely high, the values observed in our patients are normally distributed around the curve calculated by Walder *et al.* ($y = 0.06x^{3.07}$) after performing magnesium loading tests in their patients ($P < 0.05$). The dashed line represents the rate in normal children (27).

Follow-Up

Clinical symptoms and laboratory data recorded during the follow-up period are summarized in Table 1. The median follow-up was 8 yr, with a range from 0.5 to 28 yr.

In 23 (82%) patients, hereditary magnesium deficiency was diagnosed within days during the first hospitalization, and maintenance magnesium therapy was prompted. In four patients, serum magnesium levels were not measured or hypomagnesemia was not noticed, which resulted in persistent or recurrent episodes of cerebral convulsions. The delay of diagnosis and repeated convulsions resulted in mild to moderate mental retardation in three patients.

After initiation of magnesium administration, serum calcium levels rapidly returned to normal values, with a median of 2.4 mmol/L during maintenance therapy. PTH levels also normalized with a median at 32 pg/ml under maintenance therapy; however, PTH levels are available for only a small number of patients (11 of 28). Serum magnesium levels failed to reach normal values under oral substitution and remained in the subnormal range (median 0.55 mmol/L).

Maintenance therapy generally consisted of an oral supplementation with different magnesium salts. Daily oral magnesium doses ranged from 0.41 to 3.90 mmol/kg per d, with a median of 0.93 mmol/kg per d. The main side effect of oral doses of magnesium was diarrhea, which was observed in a considerable number of cases (14 of 28). One patient (F10.1) receives monthly intravenous infusions in addition to oral supplementation; another patient (F15.1) requires continuous subcutaneous infusion *via* insulin pump as described before (28). In one well-documented case, continuous nocturnal nasogastric

magnesium infusions were introduced as an alternative to parenteral applications (19).

Except for three patients with mild to moderate mental retardation, the psychomotor development was normal in all other cases. In patient F15.1, an episode of prolonged seizures at the age of 27 yr lead to transient dysarthria, which lasted over several months. Severe cardiomyopathy was documented in a patient with prolonged, untreated hypomagnesemia (F17.1) (21); cardiac arrhythmia was observed in patient F6.2 during the initial presentation (18). Although the majority of patients show normal growth, four patients (F9.1, F11.1, F11.2, and F16.1) with pronounced diarrhea exhibit severe failure to thrive.

Discussion

Genotype Analysis

In our cohort of 28 patients with HSH from 21 families, we detected 16 novel mutations in the *TRPM6* gene in addition to the seven known mutations in families F1 to F5 described before (8). In all patients with HSH, the mutational analysis of the *TRPM6* gene yielded at least one mutant allele. Together with the mutations reported by Walder *et al.* (9), who identified three splice-site and two stop mutations, 28 different *TRPM6* mutations have been identified so far. These comprise six different stop mutations, 10 splice site mutations, seven frameshift mutations, four deletions of exons, and one point mutation (S141L), summarized in Figure 2.

The mutations identified in patients with HSH are distributed over the entire TRPM6 protein. Mutations that lead to premature stops of translation are located before as well as behind the ion channel domain. As patients who bear mutations located in the C-terminus of the TRPM6 protein show no phenotypic differences compared with patients with early stop mutations, loss of function of TRPM6 seems to be independent from the location of the truncating mutation. The same holds true for splice-site mutations and deletion of exons located C-terminally of the ion channel domain. Taken together, these findings point to an important role of the kinase for TRPM6 ion channel function. This observation is of particular interest when compared with *in vitro* characteristics of TRPM7. Heterologous expression of wild-type TRPM7 induces currents that are sensitive to intracellular magnesium and Mg-ATP. Although its kinase activity is obviously not required for channel activation, deletion of the kinase domain leads to a suppression of ion channel activation at physiologic intracellular magnesium levels (14). Truncation of TRPM6 before the kinase domain might lead to a loss of ion channel function by a similar mechanism.

Regarding the larger deletions and presuming a correct splicing from the exon before to that behind the deleted sequence, three of the deletions would result in a shift of the reading frame and a premature stop of translation. In contrast, the deletion of exons 32 and 33 in patients F6.1 and F6.2 represents an in-frame deletion and could possibly allow for a correct translation of the following exons. It is interesting that these two patients are unique in our cohort in that they exhibit normal serum magnesium levels under supplementation therapy. As the deletion of exons 32 and 33 would affect only a small part of the TRPM6 protein between ion channel and kinase domain, one could speculate that kinase activity and ion channel function might partially remain intact in these patients.

Electrophysiologic analysis of mutated TRPM6 after heterologous expression together with TRPM7 in *Xenopus* oocytes proved the expected complete loss of function of TRPM6. None of the examined TRPM6 mutants exerted a significant effect on TRPM7 current amplitude as shown for wild-type TRPM6 irrespective of the localization of the mutation before or after the ion channel domain. Finally, the functional analysis of S141L-TRPM6 demonstrated an intracellular retention of the mutant protein upon co-expression with TRPM7 and an abrogation of proper tetrameric TRPM6/TRPM7 complex assembly in the endoplasmic reticulum, which seems to be critical for TRPM6 membrane targeting (16).

Taken together, *TRPM6* mutations identified in patients with HSH invariably result in a complete loss of function of the TRPM6 protein as proved by heterologous expression. Obviously, complete lack of TRPM6 ion channel activity is required for the development of the typical HSH phenotype. It is intriguing to speculate whether minor changes in TRPM6 function by single-point mutations could result in a less severe clinical picture or even subclinical magnesium deficiency.

Phenotype Analysis

Phenotypic evaluation of affected individuals revealed disease onset in early infancy. Repeated measurements of serum magnesium in patients F5.2 and F19.1 point to a continuous decline from birth onward. As magnesium is freely exchanged at the placenta level, patients are probably adapted to their mother's magnesium levels and are depleted progressively until magnesium deficiency becomes clinically manifest after several weeks to months.

The predominant symptom at initial presentation consisted of generalized seizures (96%). At manifestation, serum magnesium measurements were performed in the majority of cases. However, hypomagnesemia was not always recognized as the primary cause of clinical symptoms or misinterpreted as transitory, which led to recurrence of clinical symptoms in a number of patients. Hypomagnesemia was in the majority of cases accompanied by hypocalcemia as a consequence of a functional hypoparathyroidism (2). The clinical and laboratory findings observed here are in good agreement with a previous study that also showed manifestation predominantly with seizures and found similar values for serum magnesium and calcium at manifestation (29).

Magnesium uptake studies using radioactive magnesium isotopes have been used to demonstrate the primary defect in intestinal magnesium absorption in HSH but are not available on a routine basis (20). Measurement of urinary magnesium excretion therefore is an important diagnostic tool. In HSH, data on urinary magnesium excretion and the contribution of a renal magnesium leak to HSH pathophysiology were contradictory (4,5,30).

Physiologically, the kidney, in the presence of hypomagnesemia, aims at preserving magnesium by lowering fractional excretions below 0.5 to 1%, with the physiologic range being 3

to 5% (31). Rodriguez-Soriano *et al*. (27) suggested a renal threshold for magnesium with virtually absent renal magnesium excretion when serum magnesium levels decrease below 0.7 mmol/L (equivalent to approximately 0.5 mmol/L [approximately 1.2 mg/dl] ultrafiltrable magnesium). Therefore, in patients with HSH and serum magnesium levels almost continuously in the subnormal range, fractional excretions would be expected to be below 1%, presuming an intact renal magnesium conservation.

At initial manifestation during severe hypomagnesemia (S_{Mg} approximately 0.2 mmol/L), fractional magnesium excretions were found to be low in single patients with HSH (data not shown). However, fractional magnesium excretions observed during oral magnesium supplementation are increased inadequately (median Fe_{Mg} 3.6%) considering that patients are still overtly hypomagnesemic (median S_{Mg} 0.55 mmol/L). This renal magnesium leak was also demonstrated by plotting urinary magnesium excretions (expressed as GF_{Mg}) against the UF_{Mg}. Patients with HSH displayed significantly higher magnesium excretions compared with healthy control subjects (Figure 3, dashed line). Except for one patient (F18.1), the values for urinary magnesium excretions in relation to serum magnesium levels observed in our patients are normally distributed around the curve calculated by Walder *et al*. (9) ($y = 0.06x^{3.07}$) after performing magnesium loading tests in their patients ($P < 0.05$).

Therefore, a significant renal magnesium leak could be demonstrated in HSH, which clearly contributes to the pathogenesis of the disease and probably prevents reaching physiologic serum values under adequate therapy in most cases. From these findings together with the immunohistologic data (15), it can be concluded that TRPM6 is a crucial component of the apical magnesium channel not only in intestine but also in the DCT.

Standard treatment in HSH consists of an exclusive administration of magnesium. At time of manifestation, intravenous or intramuscular administrations are preferred, whereas the maintenance therapy usually consists of an oral administration of high doses of magnesium. However, because of gastrointestinal side effects, some patients require additional parenteral magnesium.

The mean oral magnesium dose in our patient cohort (0.93 mmol/kg per d) is considerably lower than the one reported by Shalev *et al*. (29) (mean 1.6 mmol/kg per d). However, the dosage in our study greatly varies between patients and centers (range 0.41 to 3.90 mmol/kg per d). Whether this variation reflects different individual requirements or is the consequence of differences in therapeutic strategies remains unknown. As reported previously (29), delay of diagnosis and prolonged seizures can lead to neurologic impairment or may even be fatal. Neurologic deficits were not evaluated in a standardized manner for this study but were clinically evident in three (11%) of our patients. Laboratory evaluation of serum magnesium levels therefore should represent an indispensable part of the workup in infantile seizures. After correction of hypomagnesemia, serum levels should be monitored closely to prevent recurrence of symptoms in the presence of hereditary magnesium deficiency.

In summary, we have clinically characterized a large cohort of 21 families with genetically proven HSH. The molecular analysis of the *TRPM6* gene identified mutations that uniformly lead to a complete loss of function of the TRPM6 protein. Mutations C-terminally of the ion channel domain suggest an important role of the kinase domain for TRPM6 ion channel function as reported for TRPM7 (14).

Patients with HSH require lifelong high oral magnesium supplementation, which leads to relief of clinical symptoms and normalization of calcium metabolism. Early diagnosis of hereditary hypomagnesemia as causative for infantile seizures is critical for neurodevelopmental outcome. A renal magnesium leak that is evident in all patients with HSH, which is barely detectable at initial presentation, not only contributes to the development of hypomagnesemia but also prevents an adequate conservation of the absorbed magnesium under supplementation.

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