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Clinical and molecular characterization of Turkish patients with familial hypomagnesaemia: novel mutations in *TRPM6* and *CLDN16* genes

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Abstract

Background. Recent identification and characterization of novel renal Mg^{2+} transporters and ion channels have greatly increased our understanding of the normal physiology of renal magnesium handling.

Methods. The present study deals with the clinical and molecular characterization of eight Turkish children (median age 10.6 years, range 3–16.2 years, five boys and three girls) with primary hypomagnesaemia from six families.

Results. All patients initially presented with tetany and convulsions. Laboratory evaluation yielded severely low serum magnesium levels and low serum calcium levels in all patients. While six patients exhibited inadequately low parathyroid hormone levels, the two remaining patients showed hyperparathyroidism, hypercalciuria and nephrocalcinosis. Genetic studies revealed familial hypomagnesaemia with secondary hypocalcaemia (HSH) due to a *TRPM6* mutation in six patients and familial hypomagnesaemia with hypercalciuria and nephrocalcinosis (FHHNC) due to a *CLDN16* mutation in one patient.

Conclusions. Among recently identified magnesium-wasting disorders, HSH and FHHNC represent two major entities also in the Turkish population. Besides clinical course and laboratory diagnosis of hypomagnesaemia, the detection of renal calcium wasting and parathyroid function are crucial to differentiate between these most prevalent forms of hereditary magnesium deficiency. While *TRPM6* mutations underlying HSH almost uniformly lead to a complete loss of function of the TRPM6 protein, the severity of FHHNC phenotype depends on the residual function of the mutated claudin-16 protein.

Keywords: claudin-16; CLDN16; familial hypomagnesaemia; TRPM6

Introduction

Magnesium plays a critical role as a cofactor for numerous enzyme systems in intracellular metabolism; these include the various phosphokinases and phosphatases that are involved in energy storage and utilization. Hypomagnesaemia in children occurs secondarily in diverse clinical conditions such as polycythaemia in infancy, intestinal malabsorption and short bowel syndrome, infants of diabetic mothers but also primarily in familial hypomagnesaemic disorders [1].

Symptoms of hypomagnesaemia do not necessarily correlate with serum magnesium levels. Among the first symptoms of hypomagnesaemia are abdominal pain, nausea, vomiting, lethargy and weakness. In more pronounced magnesium depletion, symptoms of increased neuromuscular excitability predominate, such as tremor, carpopedal spasms, muscle cramps, tetany and finally generalized seizures. Cardiac manifestations include atrial or ventricular tachycardia, premature contractions, a prolonged QT interval and torsades de pointes [2].

Familial hypomagnesaemia presenting in the neonatal period and infancy is a genetically determined condition. With advances in molecular genetics, a number of hereditary disorders leading to hypomagnesaemia have been found, with the most important of these being the Gitelman syndrome, familial hypomagnesaemia with hypercalciuria and nephrocalcinosis (FHHNC), familial hypomagnesaemia with secondary hypocalcaemia (HSH) and autosomal dominant hypomagnesemia [2–4]. The mutational analysis of affected individuals with familial hypomagnesaemia together with the functional analysis of mutations in heterologous expression systems represents an attractive approach to gain further insight into the physiology of renal magnesium handling.

Here, we present molecular analyses of eight Turkish patients with familial hypomagnesaemia and provide information regarding phenotypic presentations and clinical courses for this genetically characterized cohort.

Patients and methods

Medical records of eight children with primary hypomagnesaemia from six families who were followed up at the paediatric endocrinology clinic of Marmara University were reviewed. Diagnosis of primary hypomagnesaemia

was established by the presence of low serum magnesium and absence of secondary reasons for hypomagnesaemia and requirement for high doses of magnesium. Malabsorption was excluded on the basis of evaluation of general status of nutrition, average weight and height standard deviation scores (SDS) as well as serum albumin and total protein concentrations and lack of diarrhoea. Bone status was assessed by measuring serum levels of total alkaline phosphatase, parathyroid hormone (PTH), 25-hydroxycholecalciferol (25-OH vitamin D) and bone densitometry (DEXA) Z-scores (Lunar). General laboratory screenings included the following: serum electrolytes, serum creatinine, urine electrolytes and urine creatinine obtained from spot urine samples. Laboratory data furthermore is comprised of the analysis of thyroid function and growth hormone stimulation studies as needed. Diagnoses of seven patients were confirmed genetically (Table 1).

Table 1. Clinical and biochemical data of genetically characterized patients with familial hypomagnesaemia[a]

Patient	F1.1	F2.1	F2.2	F3.1	F4.1	F5.1	P5.2	P6.1
Gender	M	F	M	M	M	M	F	F
Age at presentation	3 months	3 months	1 month	6 years	1 year	1 month	3 months	3 months
Symptoms at presentation	Seizures	Seizures	Seizures	Seizures	Seizures	Seizures	Seizures	Seizures
Follow-up	15 years	18 years	13 years	13 years	5 years	12 years	3 years	12 years
Laboratory findings								
Initial serum Mg^{2+} (mmol/L)	0.16	0.08	0.20	0.26	0.14	0.50	0.5	0.08
Initial serum Ca^{2+} (mmol/L)	1.8	1.8	2.4	1.8	2.6	1.8	1.7	2.1
PTH at manifestation (pg/mL)	<3	<3	5	243	<3	<3	12	108
Serum Mg^{2+} under therapy (mmol/L)	0.39	0.45	0.41	0.50	0.75	0.58	0.66	0.66
Serum Ca^{2+} under therapy (mmol/L)	2.7	2.1	2.6	2.3	2.6	2.4	2.3	2.5
Treatment								
Oral Mg^{2+} (mmol/kg/day)	0.2	0.4	0	3.0	0.8	0.6	0.5	1.3
Intramuscular Mg^{2+} (mmol/kg/day)	0.8	0.9	0.9	0	0	3.7	7	0
Urinary analyses								
FE-Na^{+} (%)	0.7	0.6	0.6	1.2	0.6	0.6	0.5	1.0
FE-K^{+} (%)	9.4	7.7	3.8	12	9.2	8.9	2.6	6.3
FE-Mg^{2+} (%)	0.1	0.1	0.8	19	2.7	2.3	1.9	0.7
Ca^{2+}/Creatinine (mg/mg)[a]	0.05	0.01	0.04	0.53	0.24	0.14	0.04	0.49[c]
Nephrocalcinosis	–	–	–	+	–	–	–	+
GFR (mL/min/1.73m^2)	139	164	213	89	173	167	162	151
Bone mineral density (Z-scores in DEXA)	0.7	−0.8	−0.8	0.9	1.1	0.2	1.1	−0.9
Growth (height-H, weight-W, body mass index-BMI SDS)	H: −1.06 W: −0.3 BMI: 0.25	H: −2.8 W: −1.3 BMI: 0.31	H: −1.2 W: −1.8 BMI: −1.8	H: −1.1 W: 0.8 BMI: 1.6	H: −0.3 W: 0.2 BMI: 0.6	H: −2.1 W: 1.1 BMI: 2.0	H: 0.7 W: 0.30 BMI: 0.98	H: −3.5 W: −2.9 BMI: −1.4
Genetic diagnosis	HSH	HSH	HSH	FHHNC	HSH	HSH	HSH	unknown
Affected gene	*TRPM6*	*TRPM6*	*TRPM6*	*CLDN16*	*TRPM6*	*TRPM6*	*TRPM6*	unknown
Zygosity	homozygous	homozygous	homozygous	homozygous	heterozygous	homozygous	homozygous	unknown
Nucleotide level	c.3556C>T	c.3556C>T	c.5775A>G = IVS36−2 A>G	c.290A>G	c.1444-1 G>T = IVS12−1 G>T	c.5775A>G = IVS36−2 A>G	c.5775A>G = IVS36−2 A>G	unknown
Consequence/protein level	p.Q1186X (stop mutation)	p.Q1186X (stop mutation)	Splice site mutation	p.D97G (amino acid exchange)	Splice site mutation	Splice site mutation	(splice site mutation)	unknown

[a]GFR, glomerular filtration rate.
[b]Hypercalciuria defined as a Ca^{2+}/creatinine ratio >0.25 mg/mg according to Matos *et al.* [5].
[c]Value obtained before initiation of thiazide treatment.

Studies were performed with approval of the Ethics Committee of the Marmara University Faculty of Medicine, Istanbul, Turkey. Families of each participant provided written informed consent, and all studies were conducted in accordance with the principles of the Declaration of Helsinki.

Genetic studies

Haplotype analysis. Due to the effort and cost of *TRPM6* mutational screening, a haplotype analysis of the *TRPM6* locus was performed prior to single-strand conformation polymorphism (SSCP) analysis and sequencing in consanguineous and multiplex families F1, F2 and F5 to confirm genetic linkage. Microsatellite markers D9S1806, D9S1115, 8580-1540, 23938-194, D9S175 and D9S284 linked to the *TRPM6* gene on chromosome 9q22 were amplified by polymerase chain reaction (PCR) (primer sequences available upon request). Fragments were separated on 6% polyacrylamide gels under denaturing conditions in an ALFexpress DNA sequencer (Pharmacia Biotech, Uppsala, Sweden), and data were analysed 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 crossing-over events.

Mutational analysis. Extraction of DNA from blood leukocytes was performed using standard protocols. *TRPM6* mutational screening was performed by SSCP analysis [6]. For that purpose, an overlapping set of PCR primers based on the sequence of the human *TRPM6* gene (genomic contig GenBank accession number AL354795) was used to amplify the complete coding sequence 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). Subsequently, exons with conformational variants were directly sequenced from both strands (Applied Biosystems 310 Genetic Analyser). For *CLDN16* and *CLDN19* mutational analysis, the complete coding sequence and the intron/exon boundaries of both genes were amplified using overlapping sets of PCR primers based on human reference sequences (genomic contigs GenBank accession numbers AC009520 and AC098484) and directly sequenced from both strands. Patient 6.1 was additionally screened for mutations in the *CASR* gene coding for the calcium-sensing receptor (CaSR). Again, the complete coding sequence as well as intron/exon boundaries were amplified using overlapping sets of PCR primers based on human reference sequences (genomic contigs GenBank accession numbers AC068630 and AC068754) and directly sequenced from both strands.

Mutagenesis

A pEGFP-C1 vector (Clontech, Mountain View, CA) containing full-length CLDN-16 (GenBank accession number AF152101) with an N-terminally located green fluorescence protein (GFP)-tag was kindly provided by Jianghui Hou, Renal Division, Washington University Medical School, St Louis. Site-directed mutagenesis was performed with a PCR-based mutagenesis method (QuikChange Mutagenesis Kit; Stratagene, La Jolla, CA). The molecular clones for claudin-16 Mutant D97G were verified by DNA sequencing.

Cell culture and transfection

MDCK cells were grown in cell culture plates containing Earle's minimum essential medium (PAA laboratories, Dartmouth, MA), supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (100 μg/mL, each). Cells were cultured at 37°C in a 5% CO_2-containing atmosphere. MDCK cells were transiently transfected with claudin-16 wild-type and mutant constructs using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The expression and cellular localization of N-terminally GFP-tagged Claudin-16 wild-type and mutant proteins were verified using a fluorescence microscope (Zeiss, Jena, Germany).

Results

Eight Turkish children (median age 10.6 years, range 3–16.2 years, five boys and three girls) with primary hypomagnesaemia participated in the study. All of the subjects except one (F4.1) were the product of consanguineous marriages. There were two pairs of siblings in our study group originating from two different families with consanguineous parents. Pedigrees of the families are shown in Figure 1.

All patients presented with tetany and convulsions in early infancy (before 1 year of age) except F3.1 who first had convulsions at 6 years of age. However, the diagnosis of HSH in two patients (F1.1 and F5.1) was made at later ages due to presence of hypocalcemia and inadequately low PTH levels at the same time (F1.1 was diagnosed at 7 years of age and F5.1 was diagnosed at 5 years of age). Both were formerly diagnosed as having hypoparathyroidism and treated with 1,25-dihydroxycholecalciferol and calcium. Because they remained symptomatic and had recurrent seizures despite calcium and vitamin D treatment, hypomagnesaemia was finally suspected. However, their younger siblings (F1.2 and F5.2) were started on magnesium treatment earlier due to previous index cases. All patients were on maintenance magnesium treatment. Type of treatment and doses were adjusted primarily according to gastrointestinal tolerance and rate of seizures and secondarily to serum magnesium levels. All patients except F2.1 were treated with both oral and parenteral magnesium. As symptoms could not be controlled with oral treatment alone, weekly intramuscular MgSO4 injections were added at doses described in Table 1. F2.1 could not tolerate oral magnesium at all due to diarrhea so he was only treated with maintenance intramuscular MgSO4 injections at weekly intervals. All patients and families preferred parenteral magnesium substitutions and were compliant with the treatment. No adverse effects were observed during parenteral magnesium therapy. All patients experienced recurrence of symptoms during accompanying illnesses such as common infections of childhood.

Two patients with HSH (F1.2 and F5.1) and one patient with yet undefined genetic diagnosis (F6.1) had short stature with height SDS below −2. Their SDSs of midparental heights were −0.7, 0.2 and −1.0, respectively. However, their growth hormone secretion (evaluated by growth hormone stimulation tests, clonidine, L-dopa) and IGF1 and IGFBP3 levels were normal. Short statute could not be explained by malnutrition either. Serum albumin and total protein levels of all patients were within the normal range of 3.5–4.5 and 5.5–8 g/dL, respectively. Furthermore, body mass index SDS values were in the range of −1.4 to 1.7.

No patient had rickets neither clinically nor biochemically under treatment. Their alkaline phosphatase levels and X-rays of hands were normal. DEXA Z-scores of the patients ranged between −0.7 and 1.1 and mean 25-OH vitamin D levels were 93 nmol/L (20–120 nmol/L). Serum PTH levels at initial manifestation were found to be suppressed (<3–12 pg/mL) in HSH patients but elevated in our patient with FHHNC (F3.1) and in Patient F6.1 (243 and 108 pg/mL, respectively). Additionally, patients F1.1, F1.2, and F5.1 were treated with active vitamin D at doses of 0.25–0.5 mcg/day due to low PTH levels and oral calcium doses of 30 mg/kg/day intermittently during follow-up. Moreover, patients were given 25-OH vitamin D as needed according to serum 25-OH vitamin D levels.

Renal ultrasound was normal in cases with a clinical diagnosis of HSH but showed grade four medullary nephrocalcinosis in the patient with FHHNC (F3.1) and the patient

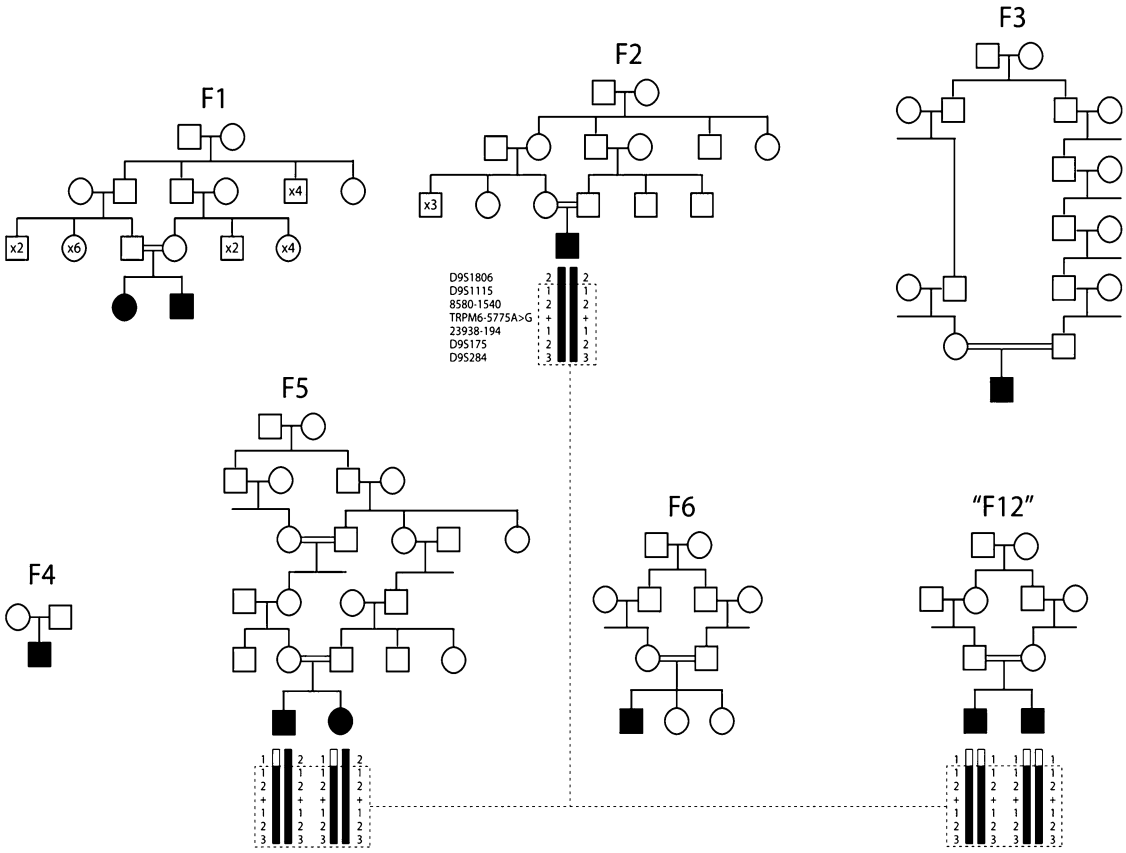

Fig. 1. Pedigrees of families with primary hypomagnesaemia, haplotype analyses in unrelated families F2, F5 as well as family 'F12' published before [6] revealed a common core haplotype (dashed boxes).

with a yet undefined genetic diagnosis (F6.1). Urinary calcium/creatinine levels were routinely followed up in all patients and were within the normal range except for Patients F3.1 and F6.1 who exhibited hypercalciuria and consecutive nephrocalcinosis (Table 1). Renal function was normal in all patients except Patient F3.1 who exhibited a marginally reduced renal function. Our patients' serum sodium and potassium levels were all within normal limits, fractional excretions of sodium and potassium did not indicate renal salt and/or potassium wasting (Table 1).

The diagnosis of primary hypomagnesaemia was confirmed genetically in seven cases, whereas unfortunately, the genetic diagnosis in Patient F6.1 could not be clarified (Table 1). Six patients in our cohort were found to carry mutations in the *TRPM6* gene causing 'familial hypomagnesaemia with secondary hypocalcaemia (HSH)'. Patient F3.1 shows a homozygous mutation in *CLDN16* (paracellin-1) causing 'familial hypomagnesaemia with hypercalciuria/nephrocalcinosis (FHHNC)', while in Patient F6.1 with clinically suspected FHHNC mutations in the genes encoding claudin-16, claudin-19 as well as the CaSR were excluded (Table 1).

A haplotype analysis of the *TRPM6* locus was performed in families F1, F2 and F5 confirming linkage to the *TRPM6* locus (data not shown). The mutational analysis of the *TRPM6* gene revealed three different mutations in affected patients from families F1, F2, F4 and F5. Mutant *TRPM6* alleles were detected in homozygous state in affected individuals from families F1, F2 and F5 with parental consanguinity, whereas in Patient F4.1 originating from a family without parental consanguinity only one disease-causing mutation could be identified. Table 1 depicts the consequent changes in TRPM6 amino acid sequence/structure.

Interestingly, patients from families F2 and F5 are carriers of the same mutation 5775A>G that has already been described by our group [7]. This mutation does not alter the corresponding glutamine at amino acid Position 1925 of the TRPM6 protein but is presumed to interfere with correct splicing as the residue (−2) is conserved at a high percentage (58%) of donor splice sites [8]. Accordingly, the splice site prediction tool NNSPLICE0.9 (http://www.fruitfly.org/seq_tools/splice.html) shows a reduction in splice site score from 0.53 to 0.24 (threshold 0.4).

Haplotype data from families F2 and F5 as well as the previously published family (F12 in [6]) revealed from a common core haplotype in all patients bearing the 5775A>G mutation pointing to the existence of a common ancestor (Figure 1).

In addition to the previously reported mutation 5775A>G, two novel *TRPM6* mutations were identified (Table 1). The mutations comprise one preterminal stop mutation (Q1186X) leading to a truncation of the TRPM6 protein behind the ion channel domain and a splice site mutation [IVS12(−1)G>T] affecting the acceptor splice site prior to Exon 13. All mutations cosegregate with the phenotype and none of the single-nucleotide exchanges were observed in 102 control chromosomes.

In Patient F3.1 with a clinical diagnosis of FHHNC, a homozygous nucleotide exchange was identified in the *CLDN16* gene (nt358A>G), leading to a nonconservative exchange from an aspartate to glycine at Position 97 of the CLDN16 protein (D97G). The mutation has not been described before. It is located directly after the first transmembrane domain at the beginning of the first extracellular loop of the claudin-16 protein. The aspartate at Position 97 is conserved between species [9]. The mutation is considered 'possibly damaging' by PolyPhen (genetics.bwh.harvard.edu/pph). Heterologous expression of GFP-tagged claudin-16 constructs in MDCK cells demonstrated a regular membrane trafficking of wild-type claudin-16 to the cell surface (Figure 2A). In contrast, claudin-16 D97G showed a mistargeted localization with a perinuclear distribution pattern compatible with a retention of the mutant protein in the endoplasmatic reticulum (Figure 2B).

Unfortunately, we were unable to identify a disease-causing mutation in Patient F6.1 with a suspected clinical diagnosis of FHHNC. The mutational screening did not identify mutations in *CLDN16* and *CLDN19*, the genes in which mutations have been identified in FHHNC [10, 11]. In addition, pathogenic mutations were excluded in the *CASR* gene encoding the CaSR. Activating CaSR mutations are known to cause autosomal dominant hypocalcaemia (ADH) also leading to combined renal calcium and magnesium wasting and consecutive nephrocalcinosis [12].

Table 1 summarizes clinical and laboratory data of the patients.

Discussion

In this study, eight Turkish children from six families with primary hypomagnesaemia have been evaluated. The clinical diagnosis of hereditary renal magnesium wasting was genetically confirmed in seven children from five families. Two novel mutations in the *TRPM6* gene and one novel mutation in the *CLDN16* gene are described. Molecular analyses genetically confirmed that six of eight patients with primary hypomagnesaemia have familial hypomagnesemia with secondary hypocalcaemia (HSH) which is a rare autosomal recessive disease caused by mutations in a gene located on 9q22 called *TRPM6*. *TRPM6* codes for a member of the transient receptor potential family of cation channels which was characterized as a critical component of a magnesium-permeable ion channel expressed along the intestine as well as in the distal convolute of the kidney tubule [13, 14]. The apically located magnesium-permeable ion channel responsible for magnesium uptake into epithelial cells represents a critical component of the active transcellular magnesium transport pathway in the intestine and kidney. HSH patients with loss of function mutations in *TRPM6* therefore not only exhibit a defect in intestinal magnesium uptake but also show renal magnesium wasting [14–16].

The common TRPM6 mutation described in families F2 and F5 [5775A>G = IVS36(−2)A>G splice site mutation] has been previously reported in another Turkish family (F12) [7]. There is no known consanguinity between these families. Therefore, we identified a first recurrent mutation in Turkish patients with HSH. Microsatellite analyses in the affected patients indeed point to a common ancestor arguing for a founder effect of this mutation in Turkish patients with HSH.

The preterminal stop mutation as well as the splice site mutation identified in Patients F1.1, F1.2 and F4.1, both presumably truncate the TRPM6 protein leading to a complete loss of function. This finding confirms a previous assumption that pathogenic mutations in HSH predominantly lead to a complete loss of function of the TRPM6 protein irrespective of the location of the truncating mutation [7].

In our cohort, one patient (F3.1) had a homozygous *CLDN16* mutation, whereas in patient F6.1, no pathogenic mutation was identified. *CLDN16* codes for the tight junction protein claudin-16 (paracellin-1) expressed in the thick ascending limb of Henle's loop. Here, magnesium is reabsorbed passively via the paracellular route together with calcium through specialized tight junctions driven by a favorable transepithelial potential [2]. Loss of function mutations of claudin-16 and its close relative claudin-19 lead to an identical renal phenotype with combined renal calcium

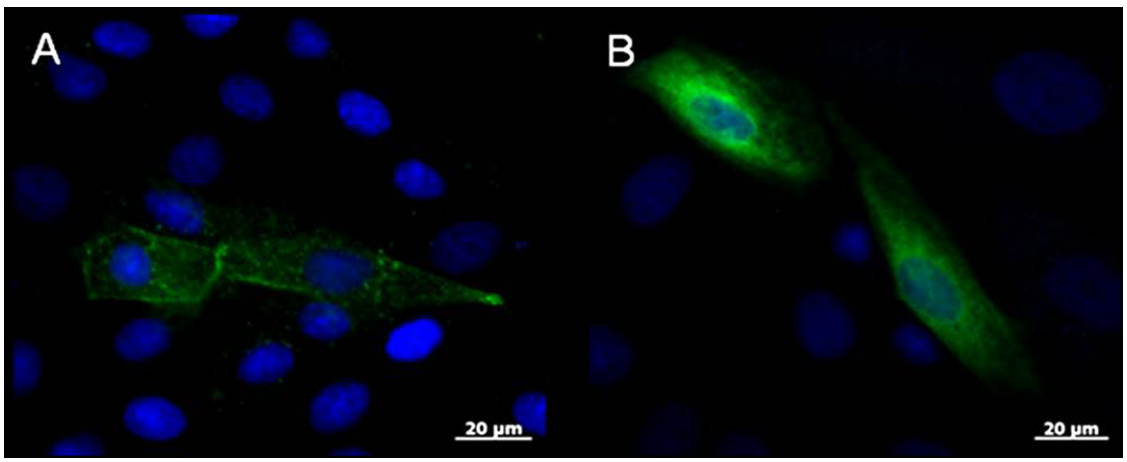

Fig. 2. The claudin-16 mutation D97G results in a trafficking defect. Fluorescence images after expression of GFP-tagged claudin-16 in MDCK cells show a regular membrane trafficking of wild-type claudin-16 to the cell surface (**A**). In contrast, mutant claudin-16 G97D shows a lack of membrane transport and is retained in the endoplasmatic reticulum indicated by a perinuclear distribution pattern (**B**).

and magnesium wasting and nephrocalcinosis called familial hypomagnesaemia with hypercalciuria and nephrocalcinosis (FHNNC). Patients with claudin-19 mutations in addition exhibit a significant ocular involvement with macular colobomata and severe vision impairment [11]. None of our patients demonstrated pathological findings in ocular examination. FHHNC patients usually present during early childhood with recurrent urinary tract infections, polyuria/polydipsia and nephrolithiasis [9]. Signs of severe hypomagnesaemia such as cerebral convulsions and muscular tetany as observed in our Patients F3.1 and F6.1 are less common. The concomitant hypocalcemia that is observed in HSH only during phases of severe hypomagnesaemia also occurs in a small subset of patients with *CLDN16* mutations. It is not a secondary phenomenon as in HSH, but the result of the combined defect in renal magnesium and calcium handling and might even lead to hypocalcaemic tetany and convulsions as observed in F3.1 and F6.1 [9].

As another well-known difference in clinical presentation between patients with HSH and FHHNC, serum PTH levels are inappropriately suppressed (<3 pg/mL) or below normal (5–12 pg/mL) in HSH patients during phases of severe hypomagnesaemia, whereas they are usually elevated in patients with FHHNC [2], which can also be seen in our cohort. This finding can be used for the clinical differentiation of patients with familial hypomagnesemia.

The prognosis of FHHNC patients is rather poor with progression to chronic renal failure early during adolescence [17]. The most recent renal function in Patient F3.1 is slightly reduced (Table 1) possibly indicating the beginning of the expected progression to chronic renal failure. The short stature observed in F6.1 cannot be explained by chronic renal failure as she still exhibits a normal renal function at her actual age. Short stature in this patient could neither be explained by any linkage to Gitelman syndrome, nor could any improvement in height be demonstrated by magnesium replacement therapy. The D97G CLDN16 mutation found in Patient F3.1 has not been described before. It is not found in healthy controls, occurs at an amino acid residue conserved between species, and is considered possibly damaging by PolyPhen (see above). Heterologous expression of GFP-tagged mutant claudin-16 D97G in MDCK cells demonstrated a trafficking defect with an intracellular retention of the mutant protein. Therefore, a complete loss of function can be assumed for the D97G mutant present in homozygous state in Patient F3.1. Defective membrane trafficking caused by claudin-16 mutations represents a common pathomechanism leading to disturbed paracellular magnesium and calcium transport in FHHNC [17, 18]. As described previously, a more severe clinical course with early progression to chronic renal failure has to be assumed [19].

In contrast, we were unable to confirm the clinically suspected diagnosis of FHHNC in Patient F6.1. Moreover, we also excluded ADH by screening of the *CASR* gene encoding the CaSR, activating mutations in which also lead to combined renal calcium and magnesium wasting with consecutive nephrocalcinosis [12].

Laboratory evaluation of HSH patients usually reveals extremely low serum magnesium and serum calcium levels. The mechanism leading to hypocalcaemia is still not completely understood. Impaired synthesis and/or release of PTH and end-organ resistance to PTH seem to contribute to the disturbance in calcium metabolism. In addition, PTH-induced release of calcium from bone is substantially impaired in the presence of hypomagnesaemia, as magnesium depletion interferes with the generation of cAMP in response to PTH [2, 3]. PTH levels in HSH patients are found to be inappropriately low at initial presentation as in our patients. Therefore, these patients tended to be misdiagnosed as having hypoparathyroidism. This also caused delay in correct diagnosis and proper treatment in F2.1 and F5.1.

The most common manifestation of magnesium deficiency in humans is neuromuscular hyperexcitability that becomes clinically evident as generalized convulsions, which only respond to magnesium therapy. In our cohort, seizures were the exclusive common initial finding of hypomagnesaemia preceding the diagnoses of either familial hypomagnesaemia with secondary hypocalcemia (HSH) or familial hypomagnesaemia with hypercalciuria (FHHNC). Administration of high doses of oral magnesium is successful in achieving at least subnormal serum magnesium levels in HSH patients. The substitution of magnesium in hypomagnesaemia is primarily aimed at the relief of clinical symptoms. Unfortunately, especially in cases of hereditary renal magnesium wasting, normal values for total serum magnesium are hardly achieved by oral substitution without considerable side effects, mainly resulting from the cathartic effects of magnesium salts [2]. In our cohort, we aimed at serum magnesium levels that enabled our patients to resume to a normal life being convulsion free while at the same time provoking only a tolerable degree of gastrointestinal disturbance. Patients fortunately experienced no manifestation of malabsorption or rickets after treatment. Despite high dose oral and/or parenteral magnesium supplementation, serum magnesium levels remained low in the majority of patients leading to recurrent convulsions during follow-up even though the incidence of convulsions was significantly decreased to 1–2/year under treatment. Low serum magnesium levels also explain the low fractional excretions for magnesium observed in the majority of our patients, as renal magnesium wasting usually represents a shift in the renal threshold for magnesium and only occurs if serum magnesium levels exceed a critical value [7].

Growth disturbance in HSH has been rarely described and the underlying mechanism is unclear [19]. The finding of short stature detected in two patients with *TRPM6* mutations in our cohort may be a feature of the disease itself especially in Patient F1.2 who had been diagnosed and treated properly since early infancy. Alternatively, short stature could also be explained by noncompliance with the treatment or late diagnosis as in the case of F5.1. However, both patients had normal growth hormone axis as shown with dynamic tests excluding growth hormone deficiency.

In conclusion, evaluation of our cohort with familial hypomagnesaemia revealed a homogenous clinical picture at manifestation with onset in early infancy with generalized cerebral convulsions and tetany but revealed heterogenous molecular findings including three TRPM6 mutations and

one novel CLDN16 mutation. The 5775A>G splice site mutation represents the first founder mutation identified in the *TRPM6* gene so far and might facilitate the genetic screening in Turkish HSH patients in the future.

As a correct and early diagnosis is important for neurological outcome, measurement of serum magnesium as well as serum calcium and PTH is crucial. Guidance for the differential diagnosis of different forms of renal magnesium wasting is obtained by the analysis of urinary calcium and renal ultrasound. Finally, growth disturbances might represent an additional clinical finding in patients with hereditary magnesium wasting.

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Conflict of interest statement. None declared.

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