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ORIGINAL RESEARCH

Familial Hypomagnesemia with Hypercalciuria and Nephrocalcinosis Due to *CLDN16* Gene Mutations: Novel Findings in Two Cases with Diverse Clinical Features

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Abstract

Biallelic loss of function mutations in the *CLDN16* gene cause familial hypomagnesemia with hypercalciuria and nephrocalcinosis (FHHNC), and chronic kidney disease. Here we report two cases of FHHNC with diverse clinical presentations and hypercalcemia in one as a novel finding. Pt#1 initially presented with urinary tract infection and failure to thrive at 5.5 months of age to another center. Bilateral nephrocalcinosis, hypercalcemia (Ca: 12.2 mg/dl), elevated parathyroid hormone (PTH) level, and hypercalciuria were detected. Persistently elevated PTH with high/normal Ca levels led to subtotal-parathyroidectomy at the age of 2.5. However, PTH levels remained elevated with progressive deterioration in renal function. At 9-year-old, she was referred to us for evaluation of hyperparathyroidism and, hypomagnesemia together with hypercalciuria, elevated PTH with normal Ca levels, and medullary nephrocalcinosis were detected. Compound heterozygosity of *CLDN16* variants (c.715G>A, p.G239R; and *novel* c.360C>A, p.C120*) confirmed the diagnosis. Pt#2 was a 10-month-old boy, admitted with irritability and urinary crystals. Hypocalcemia, hypophosphatemia, elevated PTH and ALP, low 25(OH)D levels, and radiographic findings of rickets were detected. However, additional findings of hypercalciuria and bilateral nephrocalcinosis were inconsistent with the nutritional rickets. Low/normal serum Mg levels suggested the diagnosis of FHHNC which was confirmed genetically as a homozygous missense (c.602G > A; p.G201E) variant in *CLDN16*. Yet, hypocalcemia and hypomagnesemia persisted in spite of treatment. In conclusion, FHHNC may present with diverse clinical features with mild hypomagnesemia leading to secondary hyperparathyroidism with changing Ca levels from low to high. Early and accurate clinical and molecular genetic diagnosis is important for proper management.

Keywords *CLDN16* · Claudin 16 · Familial hypomagnesemia with hypercalciuria and nephrocalcinosis · FHHNC · Hypomagnesemia · Hypercalcemia · Hyperparathyroidism · Rickets

Introduction

Familial hypomagnesemia with hypercalciuria and nephrocalcinosis (FHHNC) is a rare autosomal recessive disorder characterized by renal calcium and magnesium loss, medullary nephrocalcinosis, and progressive chronic kidney disease (CKD) [1]. Patients with FHHNC usually present with recurrent urinary tract infections (UTI), polyuria, polydipsia, and failure to thrive. Hypomagnesemia, hypercalciuria, elevated PTH levels, and hypocitraturia are the most common biochemical findings.

Significant decrease in glomerular filtration rate (GFR) may be observed in FHHNC at the time of diagnosis and about one-third of these progress to CKD during adolescence. Supportive treatment with magnesium and thiazide

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diuretics plausibly delays the progression of end-stage CKD [2]. Current treatment for end-stage renal dysfunction in this disease is renal transplantation [1]. Increased parathyroid hormone (PTH) concentrations, regardless of GFR, have been described in most cases [3]. Inherited defects of dental enamel formation such as amelogenesis imperfecta are also observed [4, 5].

FHHNC is caused by biallelic loss of function mutations in *CLDN16* (FHHNC-1, MIM #248250) or *CLDN19* genes (FHHNC-2, MIM #248190) [6, 7]. *CLDN16* gene (formerly *PCLN1*; MIM *603959) encodes the Claudin 16 tight-junction protein localized in the thick ascending limb of Henle's loop (TALH) and, is responsible for paracellular calcium and magnesium transport. Absence of severe ocular involvement differentiates patients with *CLDN16* gene mutations from *CLDN19* gene mutations [8].

Up to date, 69 different mutations (54 missense/nonsense, 5 splicing, 4 small deletion, 2 small insertion, 2 small indel, 1 gross deletion, 1 complex re-arrangement) have been reported in the *CLDN16* gene [9]. It has been shown that *CLDN16* gene mutations can cause complete or partial loss of protein function. In mutations causing complete loss of protein function, the symptoms begin earlier and progress more rapidly [10, 11]. Nevertheless, a genotype–phenotype correlation was not observed in all cases [12].

Herein, we report two different cases with FHHNC-1 with diverse clinical characteristics at diagnosis and with a *novel* nonsense variant in *CLDN16* gene.

Patients and Methods

Patient 1 (Pt #1)

A 9.5-year-old girl was referred to our pediatric endocrinology unit for evaluation of hyperparathyroidism. She was born at term with a birth weight of 3150 g (0.2 SDS) and was the second child of non-consanguineous parents. History revealed that she was evaluated for failure to thrive and recurrent UTI in another center at the age of 5.5 months. Bilateral nephrocalcinosis, hypercalcemia, and hypercalciuria were found on evaluation. It was learned that vitamin D prophylaxis had been stopped due to hypercalcemia at the age of 4 months. At initial examination, PTH level was 55 pg/mL (N: 15–65) while serum Ca level of 12.2 mg/dL (N: 8.7–11.0), serum phosphate level of 4.5 mg/dL (N: 5.0–7.8), and urinary Ca/Creatinine (Cr) ratio of 1.14 mg/mg (N: < 0.8). Fluid replacement for dehydration and potassium citrate had been commenced. PTH levels had been increased to 213 pg/mL and 220 pg/mL while serum Ca level was 10.8 mg/dL in the following days. During follow-up, parathyroid scintigraphy was performed due to persistently elevated PTH concentrations [PTH: 104–577 pg/mL] with high–normal levels of serum Ca (11.2 to 10.5 mg/dL) and hypercalciuria. There was diffuse hyperplasia with more prominent uptake at lower parathyroid glands. Multiple endocrine neoplasia type 2a (MEN2A) had been suspected on the basis of hyperparathyroidism and elevated calcitonin levels [125 and 169 pg/mL (N: 0–10)], however, genetic analysis for *ret* protooncogene was found to be normal. Calcitonin levels were reduced but remained mildly elevated on follow-up (19–27 pg/ml). Subtotal parathyroidectomy and thyroid biopsy had been performed at the age of 2.5 years which demonstrated parathyroid hyperplasia and normal thyroid histology. However, hypercalciuria and hypocitraturia were persistent after parathyroidectomy hence, hydrochlorothiazide was added to the treatment. PTH elevation with progressive deterioration in renal function was observed during follow-up. She has also received cinacalcet for about a year, between the 8 and 9 years of age, for elevated PTH levels.

Patient 2 (Pt #2)

A 10-month-old boy was referred for the investigation of hypocalcemia. He was born at term with a birth weight of 3850 g (1.6 SDS). Parents were first cousins once removed. He had received routine Vitamin D prophylaxis of 400 IU/day up to 1 month of age. Urinary white crystals were first recognized at 3 months of age.

Molecular Analyses

Genomic DNA was isolated from peripheral blood using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturers' protocols. For clinical exome sequencing (CES) analysis, all coding regions of 4490 genes related to rare inherited diseases were sequenced on the Illumina NovaSeq Platform using the SOPHiA Clinical Exome Solution kit (Sophia Genetics SA, Switzerland). Data were analyzed by using Sophia DDM® platform (Sophia Genetics SA, Switzerland). To determine pathogenic variants, we filtered in: (1) All nonsynonymous, missense, nonsense, frameshift, splice-site, no-stop, no-start, indels, and in frame variants; (2) synonymous or intronic variants affecting the consensus splice sites; (3) variants with minor allele frequency (MAF) < 1.0% in population studies (1000 Genomes, Exome Aggregation Consortium database, Exome Sequencing Project, Genome Aggregation Database); (4) variants with variant frequency (VF) is between 20 and 100%. Candidate variants were evaluated via Human Gene Mutation Database (HGMD®) and ClinVar databases. To evaluate the pathogenicity of novel variants, in silico prediction tools (DANN, DEOGEN2, EIGEN, MVP, MutationAssessor, PrimateAI, REVEL, SIFT, PROVEAN, LRT, GERP, FATHMM-MKL, and MutationTaster), segregation analysis, allele frequencies in population studies (1000G,

gnomAD, ExAC), and ACMG criteria were used [13]. For segregation analysis, targeted candidate regions were amplified using custom design primers in patient 1's parents. The polymerase chain reaction (PCR) products were sequenced via ABI PRISM® 3130xl genetic analyzer (Applied Biosystems, Foster City, CA, USA). Patient 2's parents' segregation analysis was performed via next-generation sequencing with Miseq Platform.

Results

When **Pt #1** was referred to our unit at the age of 9.5 years, her weight and height were at − 0.5 and + 0.65 SDS, respectively. Physical examination was unremarkable except for discoloration of teeth with pitting and enamel hypoplasia. Laboratory investigations revealed normal total and ionized calcium, phosphorus, ALP and 25-hydroxy-vitamin D [25(OH)D], and elevated PTH concentrations. Additionally, serum magnesium (Mg) level was 1.4 mg/dL (N: 1.5–2.6). She had inappropriately elevated fractional excretion of Mg (FEMg) at 14% (N: 0.5–4%) despite having hypomagnesemia. Hypercalciuria, hypocitraturia, proteinuria, and increased serum creatinine with low GFR (52.87 mL/min/1.73m^2) were other findings. Bilateral grade 3 medullary nephrocalcinosis was also detected. From the review of the patient's records retrospectively, we found out that she took single day of oral magnesium supplementation at 6 months of age due to low/normal Mg level (1.3 mg/dL) and normalized (Mg: 1.6 mg/dL) in the next day. Other than this, no other laboratory evaluation for Mg had been performed until she presented to our clinic. Anthropometric and biochemical characteristics at diagnosis and during follow-up, are provided in Table 1. The ocular examination of the patient was normal. Nephrocalcinosis, hypercalciuria, hypomagnesemia, and CKD led to the clinical diagnosis of FHHNC-1. Molecular genetic analyses confirmed FHHNC-1 as a previously reported heterozygous missense variant (NM_006580: c.715G>A, p.Gly239Arg, p.G239R) and a *novel* heterozygous nonsense variant (NM_006580: c.360C>A, p.Cys120*) in the *CLDN16* gene. Parental segregation analysis confirmed the compound heterozygosity of these variants. The *novel* nonsense (c.360C>A, p.Cys120*) variant was classified as pathogenic according to the American College of Medical Genetics and Genomics (ACMG) criteria (PVS1, PM2, PP3).

IGV (Integrative Genomics View) visualization of the *CLDN16* gene variants in patient 1 and chromatogram of parental segregation analyses are illustrated in Supplementary Fig. 1.

On the first evaluation of **Pt #2** at the age of 10 months; his weight and height were at − 0.7 and − 1.0 SDS, respectively. Caput quadratum, large anterior fontanelle, and wide wrists were remarkable findings on physical examination. Hypocalcemia (Ca: 7.8 mg/dL, N: 8.7–11.0) and hypophosphatemia (P: 3.3 mg/dL, N: 5.0–7.8), with elevated PTH (PTH: 499 pg/mL) and ALP (593 U/L, N: 123–377) were detected on laboratory. Cupping and mild fraying of the metaphysis on the hand X-ray supported the diagnosis of rickets (Fig. 1). Initial findings of the patient were consistent with nutritional rickets, based on biochemical and radiological findings together with low 25(OH)D level (5 ng/mL). However, urine analysis and renal ultrasonography which were performed for the irritability and urinary crystals, revealed pyuria, hypercalciuria, and bilateral medullary nephrocalcinosis. Yet, hypercalciuria and nephrocalcinosis were inconsistent with the clinical picture of nutritional rickets. When the serum Mg concentration was evaluated, it was found to be at low/normal ranges (1.3 mg/dL, N: 1.2–2.6) and a diagnosis of FHHNC was considered. FHHNC was further supported by normal tubular function tests other than hypocitraturia, microalbuminuria, and molecular genetic analyses of *CLDN16* gene which revealed a known homozygous missense variant, c.602G>A; p.Gly201Glu (p.G201E). Parents were heterozygous for this variant (Supplementary Fig. 2). Ocular examination was normal. Thus, the patient was considered to have vitamin D deficiency rickets on the background of FHHNC.

Sodium citrate and hydrochlorothiazide for nephrocalcinosis and hypercalciuria, oral calcium carbonate and vitamin D supplements for rickets, and antibiotic for UTI were commenced. While serum phosphorus, ALP, and PTH concentrations improved in the follow-up, serum Ca concentrations were low or at the lower limit of normal. Magnesium supplementation was initiated when Mg levels reduced to 0.8 mg/dL at the age of 15 months. At the 5th month of Mg treatment, the patient presented with an afebrile convulsion due to hypocalcemia (7.0 mg/dL) with inappropriately normal PTH levels (59 pg/mL) and hypomagnesemia (0.8 mg/dL). Calcitriol treatment (0.25 mcg/day) was added to Mg supplementation which corrected hypocalcemia (9.5–10.1 mg/dL). However, Mg levels remained to be low (in the ranges of 1.0 to 1.2 mg/dL) despite high doses of oral Mg replacement (50–100 mg/kg/day). PTH levels normalized initially after treatment of nutritional rickets, nonetheless, increased and remained to be elevated with low normal serum Ca concentrations after the age of 2 $^{4/12}$ years despite calcitriol treatment (Fig. 2). The patient was lost to follow-up after the age of 3 and then returned at the age of 8.5 years. The patient discontinued his medications during this period, yet, biochemical evaluation showed higher Ca level with high PTH and similar Mg levels as he had been on treatment. Anthropometric and biochemical characteristics, and treatments used at diagnosis and during follow-up, are provided in Table 1 and Fig. 2.

Table 1 Anthropometric and biochemical characteristics of the patients at diagnosis and during follow-up

Age	Patient 1								Patient 2		
	5.5 months	6.0 months	2.5 years	3.0 years	4.5 years	7.0 years	9.5 years	12.0 years	10 months	17.0 months	8.5 years
Specific periods of evaluation			Preoperative	Postoperative	Before thiazide		At admission	Last visit		After vit D supp	Last visit
Weight (kg/SDS)	–	6.5/– 0.96	11.3/– 1.07	–	–	–	28.2/– 0.5	35.6/– 0.85	8.7/– 0.7	11.7/0.13	25.6/– 0.06
Height (cm/SDS)	–	65.0/– 0.39	87.0/– 0.93	–	–	–	139/0.65	152/0.38	70.5/– 1.0	82/– 0.32	127.5/– 0.27
Target height (cm/SDS)		173.0/1.69		–	–	–	–	–	174.5/– 0.28	–	–
Calcium (mg/dL)*	**12.2**	**10.9**	10.5	8.5	9.3	9.4	9.4	9.6	**7.8**	**7.1**	8.9
Phosphorus (mg/dL)*	**4.5**	**4.7**	5.2	5.4	5.4	5.8	5.2	5.0	**3.3**	5.8	5.0
Magnesium (mg/dL)*	–	1.3	–	–	–	–	**1.4**	2.1	1.3	**0.7**	**1.1**
ALP (U/L)*	**470**	384	320	–	–	347	342	396	**593**	176	234
Creatinine (mg/dL)*	0.39	0.2	0.84	0.8	0.87	**1.19**	**1.48**	**2.98**	0.28	0.32	0.7
PTH (pg/mL) (15–65)	55	**209**	**577**	**307**	**143**	**263**	**220.8**	**271.4**	**499**	59.1	**191.2**
25(OH)D (ng/mL) (20–100)	–	–	**15**	46	–	24.5	23.89	24	**5**	25	**16.79**
1.25(OH)D (pg/mL) (25–154)	**174**	–	–	–	–	–	–	–	33	–	–
Albumin (g/dl) (3.5–5.2)	–	5.0	–	–	–	–	4.4	4.8	4.0	4.2	–
Urinary Mg excretion											
24-h urine Mg (mg/kg/day)	–	–	–	–	–	–	**2.5**	–	–	–	–
FEMg (0.5–4%)	–	–	–	–	–	–	**14**	–	–	–	–
Urinary Ca excretion											
24-h urine Ca (mg/kg/day) (< 4)	–	–	–	–	–	–	**4**	2	**6.5**	–	**8**
Urine Ca/Cr (mg/mg)*	**1.14**	–	0.44	0.25	0.27	0.2	**0.39**	–	**0.91**	**0.54**	**0.28**
Urinary protein excretion											
24-h urine protein (mg/m^2/h) (< 4)	–	–	–	–	–	–	**18.7**	**27**	**10.6**	–	**20**
24-h urine albumin (mg/day)(< 30)	–	–	–	–	–	–	**365**	**452**	**46**	–	**279**
Urinary citrate excretion											
24-h urine (mg/1.73m^2/day)*	–	–	–	–	–	–	**124**	–	–	–	–
Urine Citrate/Cr (mg/mg)*	–	–	–	–	–	**0.04**	–	–	**0.2**	–	–
eGFR (mL/min/1.73m^2)*	–	134	**43**	–	–	–	**39**	**21**	103	103	**75**

GFR was calculated with Creatinine-based 'Bedside Schwartz' equation [0.413 × (height/SCr) if height is expressed in centimeters]: Stage 1: ≥ 90; Stage 2: 60–89; Stage 3: 30–59; Stage 4: 15–29; Stage 5: < 15 (or dialysis)-Stage 1: ≥ 90; Stage 2: 60–89; Stage 3: 30–59; Stage 4: 15–29; Stage 5: < 15 (or dialysis)

SI unit conversion factors for Calcium: mg/dl × 0.25 = mmol/l; for Phosphorus: mg/dl × 0.323 = mmol/l; for Magnesium: mg/dl × 0.411 = mmol/l

Bold values show out-of normal levels of measured parameters in patients

SDS standard deviation score, *ALP* alkaline phosphatase, *PTH* parathyroid hormone, *25(OH)D* 25 hydroxy vitamin D, *1.25(OH)D* 1.25 dihydroxy vitamin D, *GFR* glomerular filtration rate, *Ca* calcium, *Mg* magnesium, *Cr* creatinine, *FEMg* fractional excretion of Mg

*__Age specific reference ranges:__

-Calcium (mg/dL): 0–5 months: 8.7–11.3; 6–12 months: 8.7–11.0; 1–5 years: 9.4–10.8; 6–12 years: 9.4–10.3; 13–20 years: 8.8–10.2

-Phosphorus (mg/dL): 0–5 months: 5.2–8.4; 6–12 months: 5.0–7.8; 1–5 years: 4.6–6.5; 6–12 years: 3.6–5.8; 13–20 years: 2.3–4.5

-ALP (U/L): 0–1 years: 123–377; 1–10 years: 97–362; 10–13 years: 92–418; 13–15 years: 59–275

-Magnesium (mg/dL): Newborn/Infants: 1.2–2.6, Children: 1.5–2.3, Adult: 1.8–2.6

-Creatinine (mg/dL): 0–4 years: 0.03–0.50; 4–7 years: 0.03–0.59; 7–10 years: 0.22–0.59; 10–14 years: 0.31–0.88; > 14 years: 0.50–1.06

Reference ranges of urinary solute excretion:

-Urine Ca/Cr ratio (mg/mg): 0–6 months: < 0.8; 7–12 months: < 0.6; 1–3 years: < 0.53; 3–5 years: < 0.39; 5–7 years: < 0.28; > 7 years: < 0.21

-24-h citrate excretion (mg/1.73m^2/d): > 365 for males and > 310 for females

-Urine Citrate/Cr ratio (mg/mg): 0–5 years: > 0.42; > 5 years: > 0.25

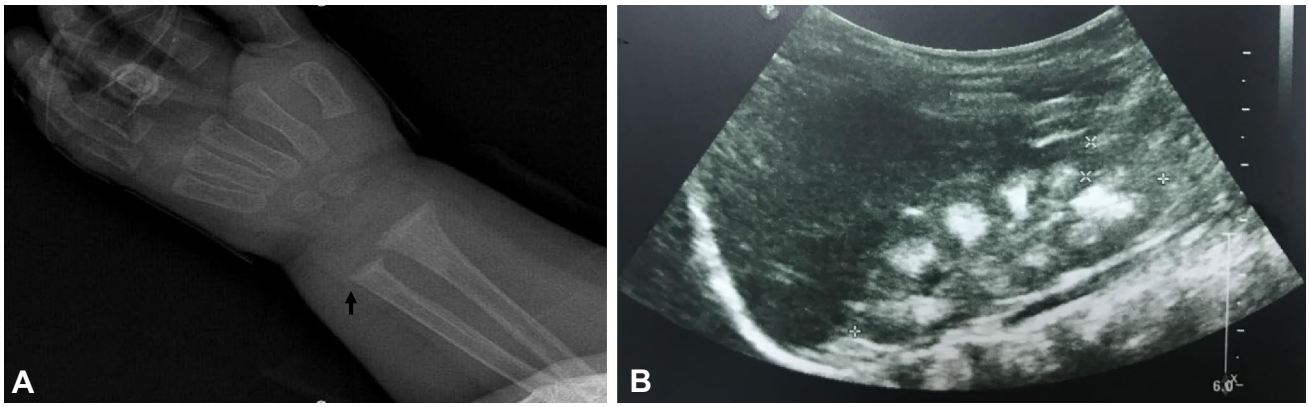

Fig. 1 Hand radiograph of Pt #2 showing cupping and fraying of the metaphysis (**A**) and nephrocalcinosis on ultrasound at initial presentation (**B**)

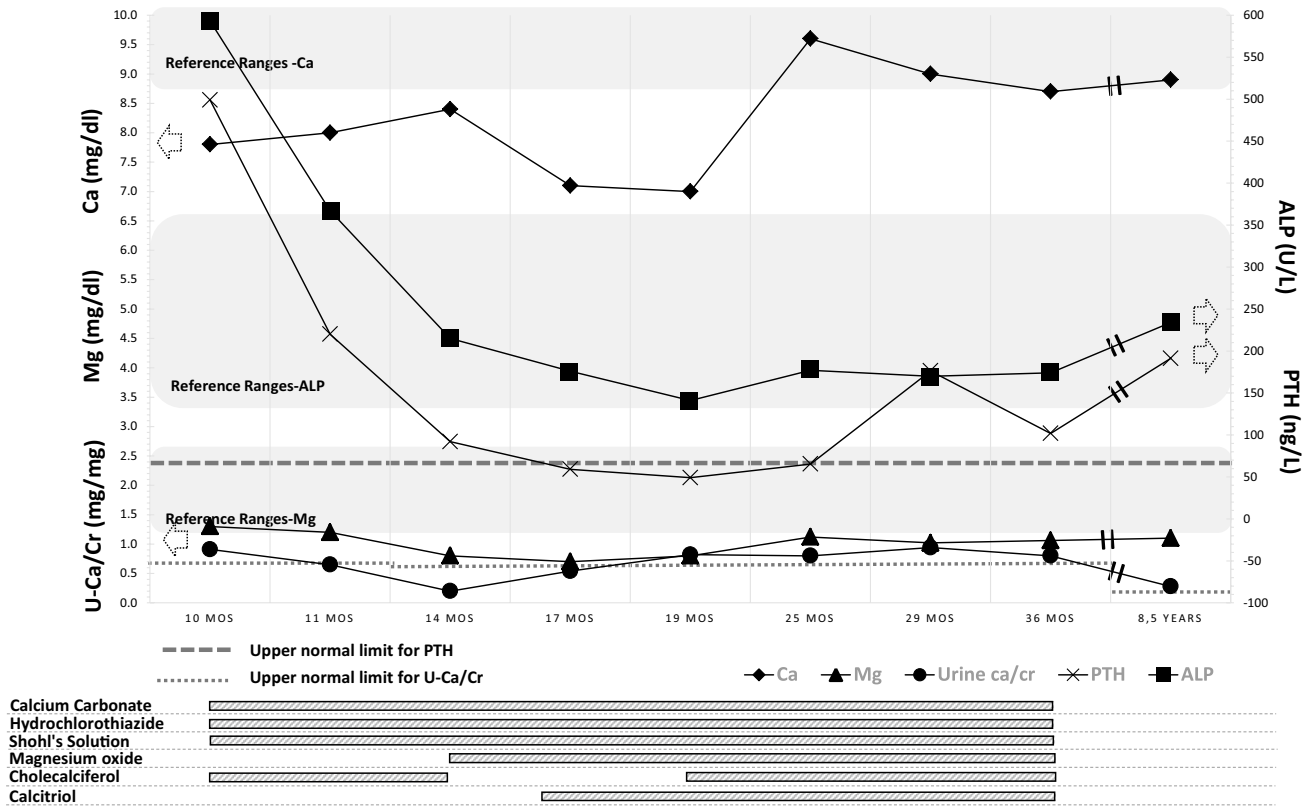

Fig. 2 Graphical demonstration of laboratory parameters of Pt #2 and treatments given. Striped empty boxes on the X-axis indicate treatments. Reference ranges of Ca, Mg, and ALP are shown as shaded gray area

Discussion

Since the first description of the FHHNC more than 150 cases have been reported yet a comprehensive definition of the clinical spectrum of this disorder is still required. Patients with FHHNC present with classical symptoms of hypercalcemia such as polyuria, polydipsia, and failure to thrive, however, they classically do not have hypercalcemia but have hypomagnesemia, hypercalciuria, and nephrocalcinosis. Here we discussed two cases with FHHNC with novel presenting characteristics, one with hypercalcemia mimicking primary hyperparathyroidism and the other with hypocalcemia and rickets, both caused by *CLDN16*

mutations. Although hypomagnesaemia is the main feature of FHHNC, it is absent in 34% of the patients at initial examination [3]. Additionally, hypomagnesemia is usually mild and symptoms related to the acute hypomagnesemia such as convulsions and tetany are usually absent in patients with *CLDN16* mutations which leads to delay in diagnosis as in our 1st case. On the other hand, in our 2nd case, hypomagnesemia worsened after the initiation of thiazide treatment causing a seizure. It must be kept in mind that thiazide diuretics increase urinary Mg excretion and may aggravate hypomagnesemia [14]. Nonetheless, Mg levels may show variation in FHHNC, being normal during early life with developing hypomagnesemia as the patient gets older as in our patients. This pattern in Mg concentrations has also been described in *CLDN16* knockout mice [15].

At TALH where CLDN16 and CLDN19 are expressed, 25% of filtered Ca and 75% of filtered Mg are reabsorbed [16]. In patients with FHHNC, biallelic mutations of *CLDN16* cause a selective defect in paracellular Mg and Ca reabsorption. Paracellular transport of Ca and Mg absorption via claudins are controlled by plasma Ca and Mg through the activation of CaSR in basolateral membrane of TALH cells [16]. Hence, CaSR is not activated only by Ca but Mg and other divalent and trivalent cations [17, 18]. So far, increases in plasma Ca and/or Mg concentrations diminish their own reabsorption by repressing CLDN16 and CLDN19 activity through the activation of CaSR. In addition to kidneys, CaSR also expressed in chief cells of parathyroid gland, calcitonin secreting C cells of thyroid gland, intestine, and bone [19, 20]. PTH secretion is regulated not only by plasma Ca but also plasma Mg through the activation of CaSR. Thus, similar to the regulation of serum Ca on PTH secretion; mild hypomagnesemia lead to inactivation of CaSR and increase in PTH secretion as in hypocalcemia. So far, the patients with *CLDN16* mutations have elevated serum PTH levels, independent from glomerular filtration rate, before the onset of CKD as in our cases [11]. In contrast, hypermagnesemia activates CaSR and inhibit PTH secretion [19]. Additionally, very low Mg concentrations impair PTH secretion due to its role in the adenyl cyclase activity which is important for the synthesis of PTH [20, 21]. Therefore, hypocalcemia with inappropriately low PTH levels can be detected in severe hypomagnesemia which was seen in our Pt #2, not at the presentation but after worsening of hypomagnesemia on thiazide treatment.

Pt #2 presented with features of vitamin D deficiency rickets. Although a concomitant rickets is reported in 7–8% of the cases with FHHNC [2, 3], a detailed explanation about the etiology or type of the rickets was not available in clinical description of these cases. It is conceivable that increased PTH secretion secondary to mild hypomagnesemia induces phosphaturia and accelerated vitamin D metabolism which in-turn cause both rickets and apparent vitamin D deficiency. Moreover, PTH elevation promote bone resorption, increase renal tubular Ca reabsorption, and stimulate $1,25(OH)_2D_3$ synthesis in the proximal tubule which increase absorption of dietary Ca in the small intestine while enhancing renal tubular Ca reabsorption and bone resorption [19]. Hence, PTH is functioning to increase serum Ca, while elevated serum Ca, in turn, inhibits PTH secretion by activating CaSR which keeps serum Ca level within the normal ranges. However, this mechanism may work differently in the presence of other factors modulating CaSR function as in hypomagnesemia; in which hyperparathyroidism and its phosphaturic effect persists in spite of normocalcemia and leads to hypophosphatemia and rickets.

On the other hand, hypercalcemia was the first presentation of our Pt #1 and misled to the diagnosis of primary hyperparathyroidism initially. We postulate that PTH elevation secondary to mild hypomagnesemia initiated the calcium retaining machinery and led to hypercalcemia, while mildly elevated Ca levels could not overcome stimulatory effect of hypomagnesemia on PTH secretion. It is well known that Ca is a more potent regulator on PTH secretion than Mg in physiological concentrations [22, 23]. However, in vitro model shows high Mg concentrations could inhibit PTH secretion in low Ca media from rat parathyroid gland [24]. Thus, under circumstances in which serum Mg deviates from normal, Mg may exert more significant effect on PTH secretion [22]. Experimental models testing reciprocal changes of Ca and Mg on CaSR and PTH secretion are required for further characterization of this issue. Additionally, 1.25(OH)D level was high in Pt #1 which is also reported in *CLDN16* knockdown mice. Serum Ca levels were mildly elevated in these mice model of the disease similar to our observation [15, 25]. Although elevated 1.25(OH) D level in this disease was attributed to be compensatory to renal Ca loss [15, 25, 26], we think that elevated PTH levels secondary to hypomagnesemia is a more likely mechanism. Moreover, similar to Pt #1, a more recent case presenting with nephrocalcinosis and amylogenesis imperfecta was initially thought to have primary hyperparathyroidism [27]. Elevated PTH levels with high normal Ca level (10.3 mg/dl) and hyperplastic parathyroid glands had been detected on parathyroid scintigraphy as in our Pt #1 [27]. Detection of hypomagnesemia led to the diagnosis of FHHNC in that case. It is yet unknown, why some of the patients with *CLDN16* mutations have hyperparathyroidism with hypocalcemia while some have hypercalcemia mimicking primary hyperparathyroidism. It is possible that vitamin D status and/or Ca intake of the patients or related genetic variations and/or degree of functional loss of CLDN16 determine the phenotype. It was shown that dietary calcium restriction causes decrease in bone mineral density in *CLDN16* knockdown mice [25]. In Pt #2, dietary deficiencies seemed to cause rickets, since our patient was not on vitamin D prophylaxis.

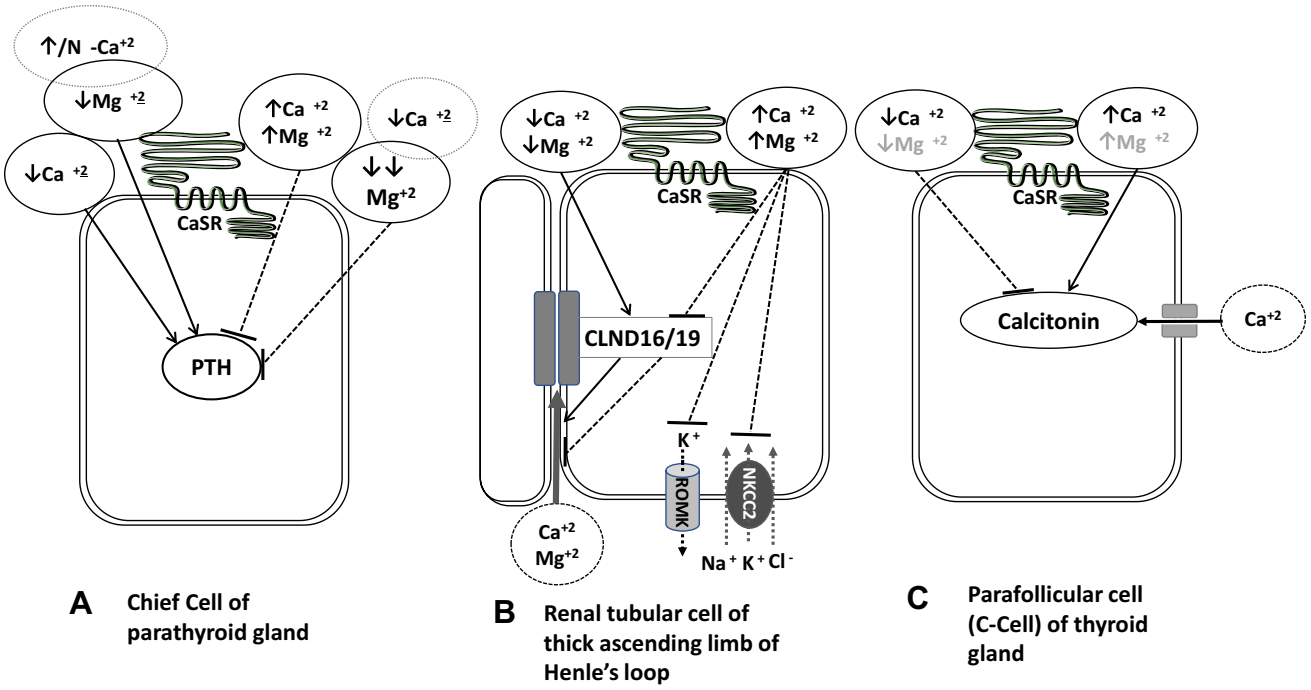

Fig. 3 Schematic representation of Ca^{+2} and Mg^{+2} regulation through CaSR: A Chief cell of parathyroid gland: High plasma Ca^{+2} and Mg^{+2} levels inhibit PTH secretion by activating CaSR and low levels stimulate PTH secretion. Mild hypomagnesemia with mild hypercalcemia still might have stimulatory on PTH secretion, while severe hypomagnesemia disrupts PTH response to hypocalcemia. **B** TALH cells of kidneys: Paracellular transport of Ca^{+2} and Mg^{+2} controlled by the activation of CaSR in basolateral membrane. Plasma Ca^{+2} and/or Mg^{+2} concentrations control their own reabsorption by regulating CLDN16/19 activity and the electrolyte transport handled by NKCC2 and ROMK (Reduction in the lumen-positive electrical potential increases urinary excretion of Ca^{+2} and Mg^{+2}). **C** C-Cells of thyroid gland: In contrast to PTH release, calcitonin secretion stimulated by elevated Ca^{+2} with subsequent activation of that voltage-dependent L-type Ca^{+2} channels. Mg^{+2} has little or no effect on calcitonin secretion. Solid arrow: stimulation; dashed line with blunt end: Inhibition. *CaSR* calcium-sensing receptor, *TALH* the thick ascending limb of Henle's loop, *CLDN16* claudin-16, *CLDN19* claudin-19, *NKCC2* the apical Na^+–K^+–$2Cl^-$, *ROMK* cotransporter the renal outer medullary potassium K^+ channel

Another unexpected finding in Pt #1 is elevated calcitonin levels, misleading the diagnosis of MEN2A. The regulation of calcitonin is also through the CaSR. In physiologic conditions, increased ionized calcium levels stimulate calcitonin secretion and inhibit PTH secretion [20, 28]. Thus, elevated calcitonin levels would not be an unexpected finding in hypercalcemia, if CaSR signaling had not been disrupted by hypomagnesemia. However, it was demonstrated that Mg had little or no effect on calcitonin secretion [29]. So far, elevated calcitonin level in our patient was an indirect measure of hypercalcemia through CaSR. Postulated mechanisms and interaction of Ca and Mg with CaSR are given in Fig. 3.

Therapeutic success is poor in FHHNC and progressive CKD is inevitable. Serum Mg levels usually remain low, despite continuous administration and high doses of Mg supplementations as in Pt #2. The reason of persistent hypomagnesemia is related to the increased urinary Mg excretion proportionally to the serum Mg due to the disease-specific alterations in the renal Mg threshold [30]. Additionally, thiazide, which also increase urinary Mg excretion, have been administered to the majority of patients for hypercalciuria and nephrocalcinosis may led to the worsening of hypomagnesemia as in Pt #2. However, no sustained reduction of hypercalciuria has been achieved with thiazide treatment as seen in Pt #2 [2, 14]. Furthermore, even after a long untreated period, Pt #2 did not show worsening of biochemical parameters. Hypocitraturia was also found in both of our patients and the majority of the patients as well. Hypocitraturia is an important risk factor for urolithiasis and nephrocalcinosis, since citrate inhibits crystallization of calcium oxalate and calcium phosphate salts in urine [31]. Therefore, citrate supplementation (given as Shohl's solution in our cases) help to reduce progression of nephrocalcinosis and CKD. Proteinuria is not a constant finding in FHHNC and can be tubular and glomerular in origin [32, 33]. Additionally, vitamin D deficiency per se could induce proteinuria, yet, Pt #2 continued to have proteinuria after vitamin D replacement. Hypercalciuria and even nephrolithiasis has been described in heterozygous family members of index cases with FHHNC [34, 35]. The clinical, laboratory, and radiological examinations of the genetically heterozygous parents of both patients were normal in our study.

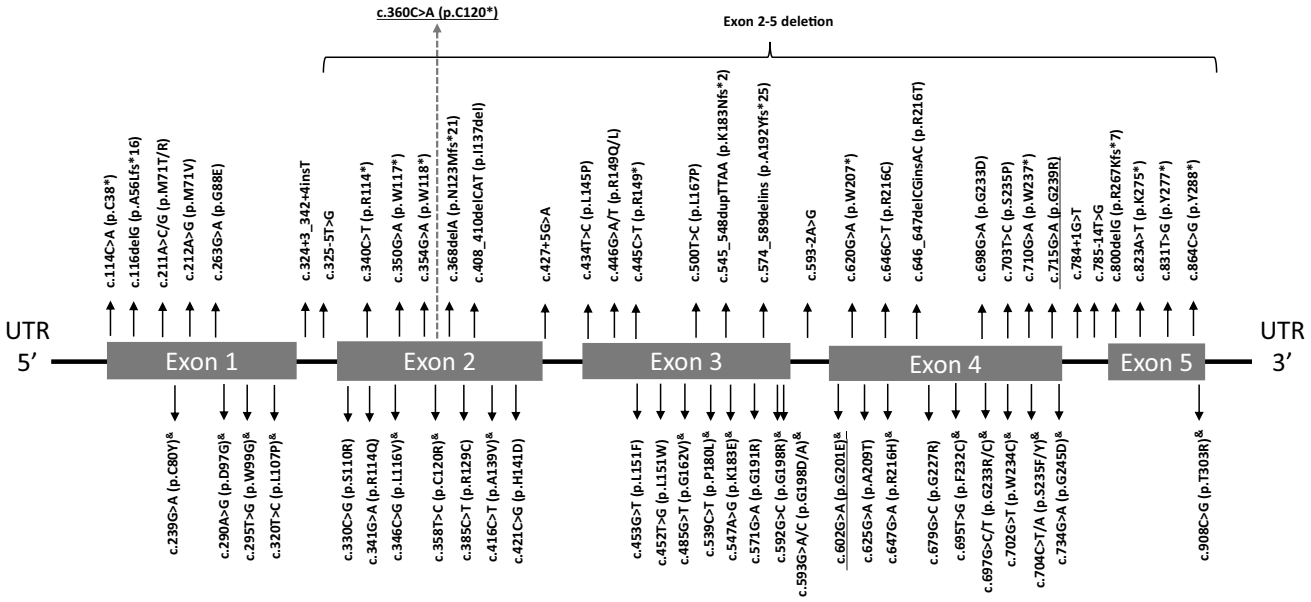

Fig. 4 Previously reported *CLDN16* gene mutations and a novel mutation detected in our study. The boxes indicate the exons of *CLDN16* gene. Upper and lower panels show mutations causing complete and partial functional loss of CLDN16, respectively. The mutations detected in Pt #1 and Pt #2 are shown as underlined and a novel c.360C>A (p.C120*) variant is indicated with dashed arrow. [&]Missense mutations plausibly cause partial loss of function (No functional analysis)

In addition to medullary nephrocalcinosis and associated inflammation, abnormalities during kidney development caused by *claudin* mutations are responsible mechanisms for progressive renal failure [1]. While the nonsense, truncating, splice-site, and missense mutations with resulting in a complete loss of function (CLOF), missense mutations displaying substantial residual function > 40% as compared with wild-type claudin-16 function (PLOF). It has been shown that patients with PLOF on at least one allele have a slower progression to CKD (mean GFR decrease in CLOF and PLOF; 7.3 (5.0–9.6) and 2.9 (1.6–4.9) mL/min/1.73 m^2/year, respectively) [11]. Heterozygous missense p.G239R mutation in Pt #1 was previously described as resulting CLOF [11]. The heterozygous *novel* p.C120* mutation in the other allele is a nonsense variant and it was predicted to be causing CLOF. The decrease in GFR in Pt #1, 9.6 mL/min/1.73m^2/year during follow-up was also supporting finding for CLOF. It was also reported that the symptoms started at an earlier age in patients with CLOF mutations compared to PLOF (mean age at onset 2.2 years (95% CI 1.1 to 3.3 years) and 5.6 years (95% CI 3.6 to 7.0 years, respectively) [11]. Early onset of symptoms in Pt #2 may also be explained with having the homozygous CLOF mutations (p.G201E), yet, the GFR reduction was inconsistent with CLOF (3.5 mL/min/1.73m^2/year). Although, the nature of the p.G201E mutation was not characterized with functional analyses as CLOF or PLOF, the early onset of symptoms in our patient, and in previously described case with severe hypovolemic shock and seizures at 2 months of age suggest that this mutation leads to CLOF [36]. Previously reported *CLDN16* gene mutations and a novel mutation detected in our study were demonstrated in Fig. 4.

In conclusion, we report two cases with FHHNC-1 with distinct clinical features, progressively deteriorating renal function and one with a *novel* nonsense mutation in the *CLDN16* gene. Although hypomagnesemia is an important diagnostic clue, it should be remembered that serum Mg concentrations can be normal or mildly decreased in FHHNC. However, after initiation of thiazides for hypercalciuria, hypomagnesemia can be more pronounced. Additionally, hypomagnesemia cause hyperparathyroidism through the CaSR and hence lead to mild hypercalcemia. In contrast, hypocalcemia can be a part of the clinic picture at the initial phase, depending on the Ca and vitamin D status of the patient. Molecular analyses enable early and accurate diagnosis of FHHNC whereby prevent unnecessary interventions and might help delay the progression to chronic kidney disease.

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Data Availability Primers and protocols are available upon request from the authors.

Declarations

Conflict of interest Mehmet Eltan, Zehra Yavas Abali, Ayberk Turkyilmaz, Ibrahim Gokce, Saygın Abalı, Ceren Alavanda, Ahmet Arman, Tarik Kirkgoz, Tulay Guran, Sukru Hatun, Abdullah Bereket, and Serap Turan declare that they have no conflict of interests.

Human and Animal Rights All procedures performed in the study involving human participants were in accordance with the ethical standards of the National Research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Consent to Participate The patients and their healthy family members signed a written informed consent for the participation to the study.

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