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Original Article

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Evaluation of Phenotypes and *CLDN16* Variants in 2 Different Familial Hypomagnesemia with Hypercalciuria and Nephrocalcinosis Families: Phenotypic Differences in Siblings and Phenotypic Similarity in Monozygotic Twins

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ABSTRACT

Objective: Familial hypomagnesemia with hypercalciuria and nephrocalcinosis is a rare disease characterized by the kidney loss of magnesium and calcium and by bilateral medullary nephrocalcinosis. It is caused by mutations in the *CLDN16* and *CLDN19* genes. In this study, we aimed to present the clinical and laboratory findings of 5 patients with 2 different pathogenic variations.

Methods: The clinical features and the detected variants in the *CLDN16* gene of 5 children with familial hypomagnesemia with hypercalciuria and nephrocalcinosis from 2 different families are presented.

Results: The median age of the 5 female patients included in the study was 11.2 years. The monoamniotic monochorionic twins from the first family had similar clinical and laboratory findings. In these patients, a previously defined pathogenic variant (a homozygous variant of c.710G>A (p.W237*)) in the *CLDN16* gene was detected. The 3 sisters from the second family had variable estimated glomerular filtration rates, height and weight severe short stature values, serum calcium, magnesium, uric acid, parathyroid hormone values, as well as variable 24-hour urine calcium, magnesium, citrate, protein, and fractional excretion of magnesium ($FEMg^{2+}$ %). A novel c.646C>A (p.R216S) homozygous, likely pathogenic variant was detected in the *CLDN16* gene of the 3 sisters.

Conclusions: Our findings showed that, despite the same mutation, the clinical features of the siblings with familial hypomagnesemia with hypercalciuria and nephrocalcinosis may differ significantly while monozygotic twins with the same mutation had similar phenotypes. This suggests that some other genetic factors are playing a role in the kidney failure process of patients with familial hypomagnesemia with hypercalciuria and nephrocalcinosis who have mutations in the *CLDN16* gene.

Keywords: A novel CLDN16 gene mutation, chronic kidney disease, hypercalciuria, hypomagnesemia, medullary nephrocalcinosis

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INTRODUCTION

Familial hypomagnesemia with hypercalciuria and nephrocalcinosis (FHHNC) is a rare autosomal recessive genetic disease characterized by the kidney loss of magnesium (Mg^{2+}) and calcium (Ca^{2+}) and bilateral medullary nephrocalcinosis. Familial hypomagnesemia with hypercalciuria and nephrocalcinosis occurs as a result of mutations in the *CLDN16* or *CLDN19* genes, which encode proteins (claudin16 and claudin19) that play an important role in the passage of cations in the tight junction of the thick ascending limb of Henle's loop.[1,2] In FHHNC, calcium oxalate/phosphate/urate accumulates in the kidney parenchyma and tubules, causing nephrocalcinosis.[1] It has been suggested that kidney l failure in FHHNC may be related to nephrocalcinosis.[3]

The main symptoms of FHHNC are polyuria, polydipsia, urinary tract infection, feeding difficulties, vomiting,

failure to thrive, abdominal pain, enuresis, rickets, and seizures. The most common laboratory/radiological findings include hypomagnesemia, elevated serum creatinine, high parathyroid hormone (PTH), hyperuricemia, hypermagnesuria, hypercalciuria, hypocitraturia, sterile leukocyturia, hematuria, nephrolithiasis, and medullary nephrocalcinosis.[1,4-8]

The differential diagnosis of FHHNC should include distal renal tubular acidosis; hypomagnesemia with secondary hypocalcemia; isolated dominant hypomagnesemia; isolated recessive hypomagnesemia; autosomal dominant hypocalcemic hypercalciuria; Bartter syndrome types 1, 2, and 3; Gitelman syndrome; primary hyperparathyroidism; hypervitaminosis D; Williams–Beuren syndrome; Dent's disease; Lowe syndrome; primary hyperoxaluria types 1, 2, and 3; cystinuria; and certain medication use (proton-pump inhibitors, diuretics, calcineurin inhibitors).[1,4,6,9,10] Among these diseases, only hyperoxaluria, Dent's disease, and FHHNC develop end-stage kidney disease (ESKD).[4] In FHHNC, deterioration in kidney function is more rapid than in other diseases presenting with nephrocalcinosis.[8] Patients usually develop ESKD in young adulthood.[1,4] Progression to chronic kidney disease (CKD) in patients with a *CLDN19* mutation is faster than those with a *CLDN16* mutation.[8,11]

Currently, there are no published guidelines for the management of patients with FHHNC.[5] Although there is no specific treatment for FHHNC, high fluid intake, salt restriction, and protein and potassium intake adjusted for CKD are recommended. In addition, magnesium citrate or carbonate, hydrochlorothiazide (HCT), amiloride, and potassium citrate are prescribed for treatment.[5,6] Patients with kidney failure are treated with kidney replacement therapy, both hemodialysis and its variants, and peritoneal dialysis. Kidney transplant is the only curative therapy for FHHNC patients.[6]

Today, different clinical spectrums related to mutations in the *CLDN16* and *CLDN19* genes continue to be defined. In the literature, patients who are non-twin siblings with the same genetic mutation have been reported.[1,4,5,12,13] However, in our literature search, we have found no twin siblings diagnosed with FHHNC.

MAIN POINTS

- A novel likely pathogenic variant of the *CLDN16* gene, which has not been previously described in the literature, is reported in 3 siblings with familial hypomagnesemia with hypercalciuria and nephrocalcinosis.
- The non-twin siblings with the same genetic mutation had different clinical features and rates of progression to end-stage kidney disease (ESKD).
- The phenotypic, clinical, and laboratory findings and the rate of progression to ESKD were similar between the monoamniotic monochorionic twins with the *CLDN16* gene mutation.
- This indicates the importance of genetic factors that have not yet been identified in the development of ESKD. It should be considered that modifying genes may have an effect on this disease phenotype.

This study presents the phenotypic, clinical, and laboratory results of identical twin sisters with a homozygous pathogenic variant in the *CLDN16* gene, and another 3 sisters with a novel, homozygous, likely pathogenic variant in the *CLDN16* gene. In addition, the phenotype similarities of monozygotic twins and the phenotypic differences of siblings were emphasized.

METHODS

The medical data of the 5 patients who were followed up in the pediatric nephrology department with the diagnosis of FHHNC (*CLDN16* mutation) between 2012 and 2021 were retrospectively evaluated. Serum blood samples were analyzed using standard techniques. Medullary nephrocalcinosis was detected using ultrasound and classified according to Hoyer's grading.[14] The growth parameters of the patients were evaluated using the height and weight percentile charts developed for Turkish children.[15] Serum creatinine was measured by the Jaffe method. The estimated glomerular filtration rate (eGFR) was calculated using the Schwartz formula.[16] Patients with CKD were graded according to the guidelines presented in the Kidney Disease: Improving Global Outcomes study.[17]

Variant Analysis

The genomic DNA isolation of the patients was performed according to the manufacturer's protocol (Anadolu Magnesia Blood Kit, Anatolia Geneworks, Türkiye). For a total of 5 coding exons, 5 pairs of primers were used. Amplicon sizes were 457-809 bp. Polymerase chain reaction (PCR) products were purified with NucleoFast® 96 PCR kit (MACHEREY-NAGEL GmbH). The amount of DNA was standardized to 0.2 ng/µL (Nanodrop N1000, Thermo Inc.). All coding exons and adjacent regions of *CLDN16* were amplified. The *CLDN16* gene sequence analyses of the patients were performed using the MiSeq (Illumina(CA, USA) next-generation sequencing (NGS) system. Sequenced data were aligned to human reference genome, hg19 by MiSeq Reporter (Illumina). The sequence analysis data were viewed using Integrative Genomics Viewer (IGV 2.3 (Broad Institute)) software. The in silico analysis of the variants was performed with VarSome software.[18] Pathogenicity was evaluated according to the criteria of the American College of Medical Genetics and Genomics (ACMG). In this study, variants are stated according to the NM_006580.3 transcript, and the nomenclature for the currently used NM_006580.4 transcript is presented in the following table.

The nomenclature of the variants according to 2 transcripts

NM_006580.3	NM_006580.4
c.710G>A (p.(W237*))	c.500G>A (p.(W167*))
c.646C>A (p.(R216S))	c.436C>A (p.(R146S))
c.416C>T (p. (A139V))	c.206C>T (p. (A69V))
c.715G>A (p. (G239R))	c.505G>A (p. (G169R))
c.295T>G (p. (W99G))	c.85T>G (p. (W29G))
c.453G>T (p.(L151F))	c.243G>T (p.(L81F))
c.211A>G (p.(M71V))	c.1A>G (p.(M1?))

RESULTS

Demographic Findings

The median age of the 5 female patients included in the study was 11.2 years (range, 39-207 months). Cases 1 and 2 were monochorionic monoamniotic twin sisters. These 2 patients had no other siblings, and their parents were first cousins and third-degree relatives. Cases 3, 4, and 5 were sisters of different ages, who had a healthy sister and a healthy brother, and their parents were also first cousins (Table 1). Pedigrees are shown in Figure 1.

Clinical, Laboratory, and Genetic Findings

Cases 1 and 2 were delivered at 29 weeks of gestation by cesarean section in another hospital. While the patients were being followed up in the neonatal clinic due to seizures, FHHNC was diagnosed upon the detection of hypomagnesemia, hypercalciuria, and medullary nephrocalcinosis. Cases 1 and 2 were referred to our pediatric nephrology outpatient clinic at the age of 7 months with complaints of polyuria, polydipsia, and not gaining weight. Bilateral medullary nephrocalcinosis was found in the ultrasonography (USG) of the urinary system (Figure 2). The laboratory and kidney ultrasound findings of both sisters indicated FHHNC. The NGS analysis revealed a homozygous, nonsense pathogenic variant of c.710G>A (p.(W237*)) (NM_006580.3) in the *CLDN16* gene in the monozygotic twins. This variant was interpreted as a pathogenic variant in VarSome software according to the ACMG criteria (PVS1, PM2, PP3) and was first reported in 2006.[19] The parents were heterozygous carriers of the mutation.

Case 3 had been previously followed up in another health center with the diagnosis of CKD and hypomagnesemia. When this patient was 16 years, she was admitted to our pediatric nephrology outpatient clinic with complaints of polyuria, polydipsia, and not gaining weight. Bilateral medullary nephrocalcinosis was detected on urinary USG. Her laboratory and radiological findings indicated the presence of FHHNC. The NGS analysis revealed a novel, homozygous, missense variant of c.646C>A (p.(R216S)) (NM_006580.3) in the *CLDN16* gene. This variant was interpreted as likely pathogenic in VarSome software according to the ACMG criteria (PM5, PM1, PM2, PP2, PP3).

Case 4 was the sister of cases 3 and 5. She was evaluated in our clinic at the age of 14.4 years due to complaints of drinking excessive water and frequent urination. Her laboratory and radiological findings suggested FHHNC.

Case 5 was the sister of cases 3 and 4. The patient was evaluated in our clinic at the age of 3 years. The patient did not have short stature. The laboratory tests and renal USG indicated FHHNC. Cases 4 and 5 were screened for the variant identified in their sister (case 3), and the same variant was present in both. The remaining 2 siblings and parents were also screened for this variant, and they were all heterozygous carriers. The genetic variants of the cases are shown in Figure 3. Tables 1 and 2 present the clinical and laboratory findings of the 5 patients.

Case 3 had hypertension, and therefore, she was started on an angiotensin-converting enzyme inhibitor. In the urinary USG of the patients, there was a grade 2-3 increase in bilateral kidney parenchyma echoes and bilateral grade 3 medullary nephrocalcinosis (Figure 2).

Treatment Choices and Follow-Up

Cases 1 and 2 had been receiving HCT, potassium sodium hydrogen citrate, and magnesium oxide treatments since the neonatal period. Case 3 was on oral magnesium and hydrochlorothiazide therapy. High fluid intake and salt restriction were recommended for all patients. In medical treatment, 20 mg/kg of magnesium oxide was administered twice a day, HCT at 2 mg/kg/day in 2 doses, and potassium sodium hydrogen citrate at a dose of 100 mg/kg twice a day. The patients with severe hyperparathyroidism or kidney failure were also given 1,25-dihydroxycholecalciferol (0.25-1 µg/day). Calcium lactate (50 mg/kg/day elemental calcium) was administered to cases 3 and 4 with hypocalcemia. It was observed that these treatments could not fix the serum magnesium level and prevent hypercalciuria. Case 4 was referred to our clinic again due to renal colic within the first year of follow-up because she did not use her medications regularly. Ultrasonography revealed an increase in the size of the left kidney and a stone in the distal right ureter, upon which the urology clinic performed extracorporeal shock wave lithotripsy (Figure 4).

DISCUSSION

Familial hypomagnesemia with hypercalciuria and nephrocalcinosis was first reported in 1972 by Michelis et al.[20] Later, Simon et al[21] defined FHHNC type 1 caused by the mutations in the *CLDN16* gene (OMIM #248250) in 1999 and in 2006, and Konrad et al[22] defined FHHNC type 2 caused by the mutations in the *CLDN19* gene (OMIM #248190).

The *CLDN19* gene plays an important role in the organization and development of the retina. Horizontal nystagmus, pigmentary retinitis, strabismus, myopia manga, macular coloboma, macular degeneration, astigmatism, or vision loss can be seen in patients with *CLDN19* gene mutations.[1,4,6,8,11] All 5 patients in this study had homozygous variants in the *CLDN16* gene, and their ophthalmological examinations were normal.

Early development of CKD in FHHNC is attributed to different risk factors, such as nephrocalcinosis, chronic dehydration due to polyuria, recurrent urinary tract infection (UTI), and gender.[6] Unlike most other inherited tubular diseases, FHHNC is generally complicated by progressive kidney failure during childhood or adolescence, but the pathogenesis of CKD remains a matter of debate.[2] In animal experiments, it has been reported that the histopathological examination of the kidneys shows a decrease in the number of glomeruli, compensatory hypertrophy of the

Table 1. Clinical and Genetic Data of the Patients

	Twins		Siblings		
Presenting Features	**Case 1**	**Case 2**	**Case 3**	**Case 4**	**Case 5**
Gender	F	F	F	F	F
Age (month)	123	123	207	185	39
Age at diagnosis (month)	1	1	192	172	36
Age at first admission to our clinic (month)	7	7	192	172	36
Follow-up (month)	116	116	15	13	3
Affected gene	*CLDN16*	*CLDN16*	*CLDN16*	*CLDN16*	*CLDN16*
Zygosity	Homozygote	Homozygote	Homozygote	Homozygote	Homozygote
Nucleotide	c.710G>A	c.710G>A	c.646C>A	c.646C>A	c.646C>A
Protein	(p.W237*)	(p.W237*)	(p.R216S)	(p.R216S)	(p.R216S)
RefSeq	NM_006580.3	NM_006580.3	NM_006580.3	NM_006580.3	NM_006580.3
Novel mutation	–	–	+	+	+
Height (cm, percentile, SDS value)					
At first admission	58, <3rd, −2.81	57, <3rd, −3.17	149, <3rd, −3.7	149, <3rd, −2.34	92, 10th-25th, −1.21
Last follow-up	134, 10th-25th, −0.85	133, 10th-25th, −1.02	150, <3rd, −3.95	151, <3rd, −2.98	93, 10th-25th, −1.02
Weight (kg, percentile, SDS value)					
At first admission	6.2, 3rd-10th, −1.36	6, 3rd-10th, −1.57	40, <3rd, −3.45	40, 3rd, −2.03	11.8, 3rd-10th, −1.87
Last follow-up	27.9, 10th-25th, −1.05	29, 10th-25th, −0.82	40, <3rd, −4.28	41, <3rd, −2.79	12.2, 3rd-10th, −1.44
Last follow-up puberty (according to the Tanner classification)					
Axillary hair	+	+	+	+	–
Thelarche stage	3	3	5	4	1
Pubic hair stage	3	3	5	4	1
Treatment	HCT	HCT	HCT	HCT	HCT
	PSHS	PSHS	PSHS	PSHS	PSHS
	MS	MS	MS	MS	MS
	Calcitriol	Calcitriol	CL	CL	
			Calcitriol Enalapril	Calcitriol	
Initial clinical presentation					
Polyuria/polydipsia	(+)	(+)	(+)	(+)	(+)
Stomach ache	(–)	(–)	(+)	(–)	(–)
Sterile leukocyturia	(–)	(–)	(+)	(+)	(–)
Short stature	(–)	(–)	(+)	(+)	(–)
Nephrocalcinosis	(+)	(+)	(+)	(+)	(+)
Convulsion	(+)	(+)	(–)	(–)	(–)

(*Continued*)

Table 1. Clinical and Genetic Data of the Patients (*Continued*)

Presenting Features	Twins		Siblings		
	Case 1	Case 2	Case 3	Case 4	Case 5
Other clinical presentation					
Hypertension	(–)	(–)	(+)	(–)	(–)
Hearing loss	(–)	(–)	(–)	(–)	(–)
Eye findings	(–)	(–)	(–)	(–)	(–)
DEXA: Z-score	3.8 (3 years old) 1 (9 years old)	3.1 (3 years old) 0.8 (9 years old)	1.5 (17 years old)		

CKD, chronic kidney disease; CL, calcium lactate; DEXA, dual-energy x-ray absorptiometry; eGFR, estimated glomerular filtration rate; F, female; HCT, hydrochlorothiazide; MS, magnesium citrate; PSHS, potassium sodium hydrogen citrate.

glomeruli and tubules, glomerular and tubular atrophy accompanied by interstitial fibrosis, and lymphocytic infiltration.[23] It has been suggested that the variability in the progression rate of kidney failure in siblings of different ages diagnosed with FHHNC may be related to the degree of nephrocalcinosis.[3] Contrary to this view, although the age and eGFR levels of the patients evaluated in the current study were different, stage 3 medullary nephrocalcinosis was detected in all the patients according to Hoyer's grading[14] (Figure 2). The presence of medullary nephrocalcinosis even in the neonatal period in 2 patients (cases 1 and 2) suggests that this condition may have started in the intrauterine period. The appearance of nephrocalcinosis in areas matching the kidney lodge in the abdominal radiograph taken for case 4 suggests that the deposits in the medulla were radiopaque (Figure 4B).

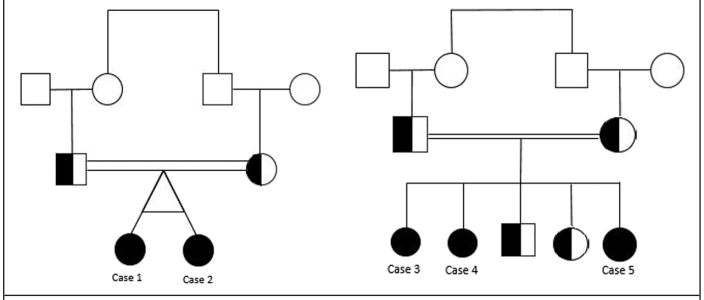


Figure 1. Pedigrees of families with familial hypomagnesemia with hypercalciuria and nephrocalcinosis.

It has been reported that blood calcium levels are in the normal range in most patients with FHHNC.[1,3,24] There are studies reporting that some patients with *CLDN16* gene mutations have severe hypocalcemia.[4] All patients diagnosed with FHHNC have hypercalciuria, which was also the case in our study. At the time of diagnosis and during the follow-ups, the serum calcium level was normal in cases 1, 2, and 5, while it remained below normal in cases 3 and 4 despite calcium lactate supplementation.

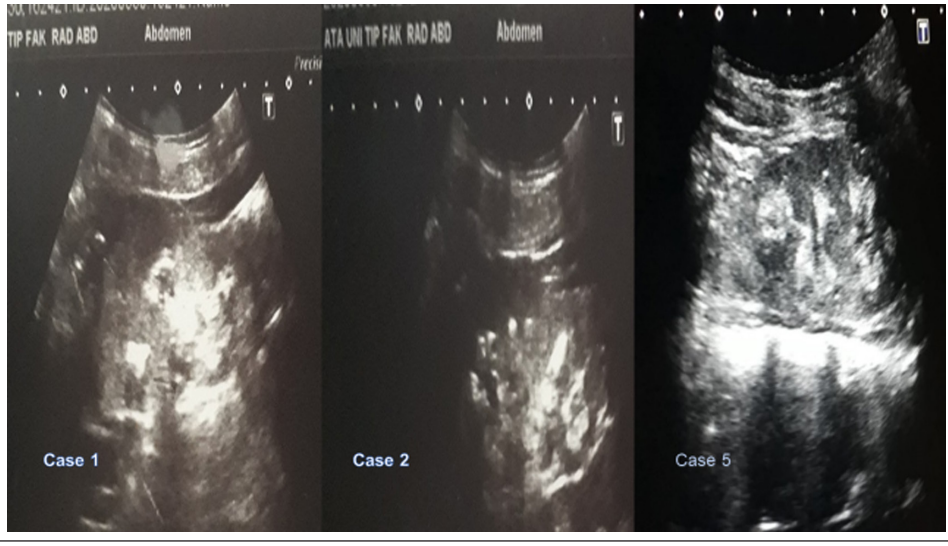
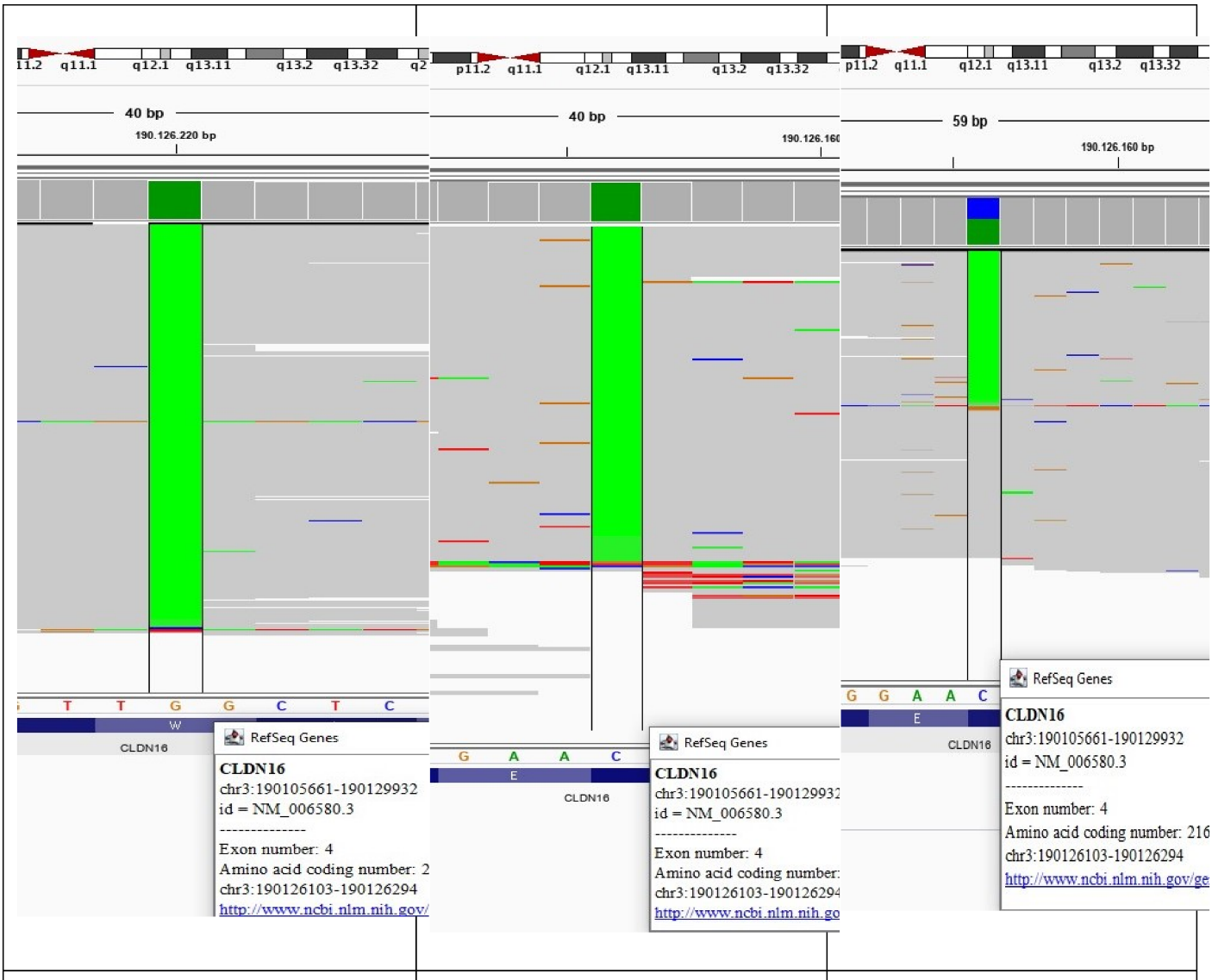


Figure 2. Bilaterally medullary nephrocalcinosis in kidney ultrasonography in cases 1, 2, and 5, showing an increase in bilateral parenchymal echoes and an intense increase in echogenicity in bilateral medullary pyramidal structures (grade 3 medullary nephrocalcinosis).



A. IGV image of Cases 1 and 2: a homozygote c.710G>A (p.W237*) variant (NM_006580.3)	B. IGV image of Cases 3,4 and 5: a homozygote c.646C>A (p.R216S) variant (NM_006580.3)	C. IGV image of carriers of the heterozygote c.646C>A (p.R216S) variant (NM_006580.3)

IGV: Integrative Genomics Viewer

Figure 3. Genetic mutations of the cases.

Hyperparathyroidism (76%-88%) develops secondary to hypercalciuria in FHHNC.[5,7] Hyperparathyroidism is more severe in patients with FHHNC than in chronic kidney disease due to other causes.[6] Sikora et al[5] reported that symptomatic treatment did not significantly decrease the mean serum PTH level, and the serum PTH level was higher than expected for the CKD stage. All the patients in the current study had high serum PTH levels. Despite active vitamin D and magnesium supplementation, their PTH levels remained elevated. In such patients with hypercalciuria, elevated PTH may be constantly high in order to keep the serum calcium level at a normal level.

Short stature has been reported in some cases diagnosed with FHHNC.[1,5,13] Peco et al[25] reported severe short stature (SDS: −2.75)

Table 2. Blood and Urine Data and CKD Values of the Patients (At First Admission/Last Follow-Up)

	Twins		Siblings		
Parameters (Normal Ranges)	**Case 1**	**Case 2**	**Case 3**	**Case 4**	**Case 5**
eGFR (mL/min/1.73 m^2)-(>90)	**36.8/35.9**	**30.8/35.3**	**36.1/28.4**	**106/105**	**99.2/92**
CKD phase (<1)	**3/3**	**3/3**	**3/4**	**1/1**	**1/1**
Blood tests					
BUN (mg/dL)	14.9/34.4	16.8/50.2	25/51	9.8/11	16.1/18
Serum creatinine (mg/dL)	**0.52/2.05**	**0.61/2.07**	**2.27/2.9**	**0.77/0.79**	**0.51/0.55**
Calcium (mg/dL)	**9.8/9.24**	**9.79/9.67**	**7/7.6**	**8.2/8.56**	**10/10.2**
Magnesium (mg/dL)	**1.62/1.49**	**1.59/1.53**	**1.6/1.13**	**1.3/1.2**	**1.69/1.58**
Parathyroid hormone (pg/mL)	**205/468**	**384/547**	**368/161**	**407/217**	**105/173**
Uric acid (mg/dL)	**5.3/7.4**	**5.1/7.3**	**7.6/10.5**	**8/8.2**	**7.9/8**
25/OH/Vitamin D (ng/mL)	51.9/40.1	46.4/34	5.8/19.8	15/19	19/16
Glucose (mg/dL)	47/74.4	96/77.7	93/78	87/79	63/82
Sodium (mEq/L)	141/145	144/145	141/138	137/139	136/141
Potassium (mEq/L)	4.65/4.12	4.48/4.81	4.2/3.9	4.59/4.35	5.0/5.2
Chloride (mEq/L)	106/107	106/103	104/102	100/104	101/105
Phosphorus (mg/dl)	5.6/4.8	5.65/4.7	3.9/3.7	4.7/4.5	5.8/5.1
Alkaline phosphatase (U/L)	434/380	439/338	180/131	281/232	740/240
pH (7.35-7.45)	7.38/7.35	7.4/7.36	7.37/7.34	7.34/7.37	7.39/7.38
Bicarbonate (mmol/L)-(22-26)	22.1/23.2	22.4/21	19.9/22.5	22/22.3	22.3/21.4
Beta 2 microglobulin (mg/L)-(1.8–2.6)	**-/11.29**	**-/7.94**	**-/5.36**	**-/2.9**	**-/3.17**
Spot urine tests					
pH-(4.5/7.5)	5/6.5	6.5/7	6.5/6.5	7/7	7.5/6
Density-(1010/1020)	1004/1006	1005/1,007	1006/1009	1009/1006	1005/1010
Glucose	Neg/Neg	Neg/Neg	Neg/Neg	Neg/Neg	Neg/Neg
Protein	Neg/**Trace**	Neg/Neg	**Trace/Trace**	Neg/**Trace**	Neg/Neg
White blood cells (HPF)-(<5)	**2/2**	**7/4**	**23/25**	**28/13**	**2/1**
Red blood cells (HPF)-(<5)	**3/2**	**4/2**	**8/4**	**4/12**	**7/2**
24-hour urine tests					
Calcium (mg/kg/24 h)-(<4[28,29])	**-/5.5**	**-/5.3**	**6.2/5.7**	**6.1/6.0**	**5.9/8.2**
Magnesium (mg/1.73 m^2/24 h)-(<88[29])	**-/406**	**-/420**	**109/98**	**90.5/118**	**97/114**
Uric acid (mg/1.73 m^2/24 h)-(<815[28])	-/278	-/217	259/271	225/252	241/347
Oxalate (mg/1.73 m^2/24 h)-(<52[28])	-/38	-/25.4	14.5/19.7	10.9/43	21.8/-
Citrate (mg/1.73 m^2/24 h)-(>310[29])	**-/181**	**-/221**	**220/283**	**208/222**	**281/-**
Protein (mg/m^2/h)-(<4[28])	**-/17.3**	**-/15.8**	**31/25**	**4.55/6.3**	**5.5/5**
$FEMg^{2+}$ (%)-(<5[30])	**-/52.3**	**-/51**	**-/24**	**-/11.7**	**17.5/13.3**

BUN, blood urea nitrogen; CKD, chronic kidney disease; eGFR, estimated glomerular filtration rate; FEMg, fractional excretion of magnesium; Neg, negative. Values outside the reference values are given in bold.

in a 9-year-old girl with a homozygous c.453G>T (p.(L151F)) pathogenic variant in the *CLDN16* gene. In the current study, 3 patients (cases 1, 2, and 5) did not have short stature, while the other 2 siblings (cases 3 and 4) had short stature. Although cases 3, 4, and 5 carried the same c.646C>A (p.(R216S)) variant, case 5 did not have short stature. The causes of short stature

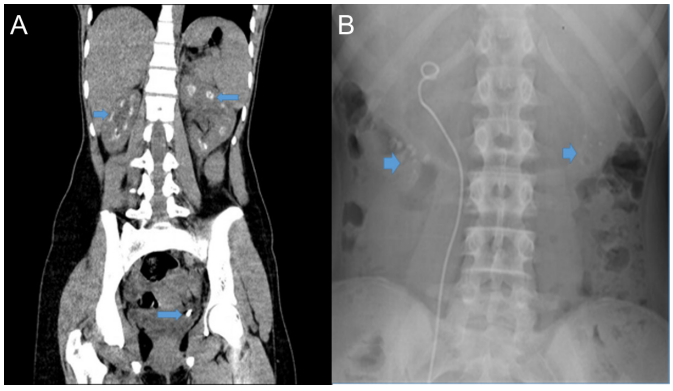


Figure 4. Abdominal radiographs of case 4, showing a diffuse density increase in the pyramidal structures of the bilateral kidneys and an appearance consistent with 2 cm stones in the lower poles (nephrocalcinosis), grade 3 hydronephrosis in the left kidney, and a 7 mm stone in the left ureter distal (A), and an appearance consistent with nephrocalcinosis in areas matching the kidney lodge (B).

in FHHNC patients may be CKD complications, mineral loss, or other primary genetic factors. Interestingly, although case 4 had a normal eGFR, she still suffered from short stature.

It has been reported that hypomagnesemia is one of the main features in patients with FHHNC, and their urinary fractional excretion of magnesium (FEMg) also increases and hypocitraturia is detected in most patients.[7,24] It was determined that oral magnesium supplementation administered to patients did not cause a significant increase in their serum magnesium levels and was not able to compensate for their urinary magnesium losses. In addition, it was noted that the efficacy of HCT treatment on the natural history of FHHNC and the rate of decrease in eGFR could not be demonstrated, but it was reported to reduce hypercalciuria.[5] The 5 patients evaluated in the current study had high levels of magnesium in urine at the time of diagnosis and at the last examination. While hypermagnesuria was prominent in cases 1 and 2, hypercalciuria was prominent in cases 3, 4, and 5. Despite magnesium treatment (25 mg/kg/day magnesium oxide supplementation), the serum magnesium levels could not be maintained in a normal range in cases 1, 2, 3, and 4. Failure to increase serum magnesium levels despite oral magnesium treatment may indicate that the treatment did not compensate for the renal loss.

In sibling cases reported by Seeley et al.[13] FHHNC was diagnosed at a younger age, and therefore, the patients were started on potassium citrate, hydrochlorothiazide, and amiloride treatments at an earlier stage; however, their eGFR values were lower than those obtained in our study. This supports the idea that symptomatic treatments are not effective in preventing the progression of ESRD.

Some *CLDN16* variants are known to be more common in certain populations. The c.453G>T (p.(L151F)) variant has been reported to be more common in Eastern Europe and Germany; the c.416C>T (p.(A139V)) variant in North Africa[6]; and the c.453G>T (p.(L151F)), c.715G>A (p.(G239R)), and c.295T>G (p.(W99G)) variants in Türkiye.[2,4,12] To date, more than 70 mutations in the *CLDN16* gene have been reported for FHHNC, with most being missense variants. These mutations are mostly associated with the first and second extracellular segments of the protein, respectively, and are less frequently related to the transmembrane domains and cytoplasmic regions. *CLDN16* gene variants may have a partial or complete loss-of-function effect.[6]

It is suggested that the c.453G>T (p.(L151F)) variant has a partial loss-of-function effect; therefore, the decrease in the rate of eGFR is slower than that in other mutations.[5] Some studies have suggested that patients with a complete loss-of-function mutation in the *CLDN16* gene had a more rapid decrease in eGFR, with this value being determined as 60 mL/min per 1.73 m^2.[2] Contrary to this view, Seeley et al[13] reported that the phenotypic findings, laboratory findings, and the rate of progression of kidney failure in 3 siblings of different ages with homozygous c.211A>G (p.(M71V)) variants in the *CLDN16* gene differed from each other, which could be due to genes or environmental factors that have not yet been identified. On the other hand, in a study reporting 3 Turkish siblings diagnosed with FHHNC, Peru et al[12] found that urea nitrogen, creatinine, uric acid, 24-h urine calcium, and eGFR decreased, and height and weight percentiles were not the same.

Both variants found in the current study, c.710G>A (p.(W237*)) and c.646C>A (p.(R216S)), were located in the fourth exon of the *CLDN16* gene, affecting the extracellular segment 2 domain. The c.710G>A (p.(W237*)) variant seen in cases 1 and 2 (monozygotic twins) has been previously reported in non-twin cases in the literature.[19] This is a nonsense pathogenic variant that creates a stop codon and has a complete loss-of-function effect.[2] The c.646C>A (p.(R216S)) variant seen in our cases 3, 4, and 5 is a novel, missense, and likely pathogenic variant which to the best of our knowledge has not been previously reported in the literature. In the literature, 2 affected siblings with the c.646 C>T (p.(R216C)) missense variant in one allele and the 784 +1 G>T variant in the other allele (compound heterozygote), and other c.646 C>T (p.(R216C)) homozygote 2 siblings also have been reported; both mutations had a complete loss-of-function effect.[2] The c.646 C>T (p.(R216C)) variant was located in the same region, similar to our cases. However, in our patients, arginine was replaced by serine (p.(R216S)) instead of cysteine (p.(R216C)). Therefore, we consider that the variant in our patients also had a complete loss-of-function effect. It is reported that the complete loss-of-function effect of *CLDN16* mutations leads to ESKD in the early period.[2] All of our cases had mutations with complete loss-of-function effect. However, the eGFRs of the siblings carrying the same variant were variable. Only the monozygotic twins had similar eGFRs and clinical findings. Based on the clinical severity being more similar between monozygotic twins than between dizygotic twins, it

has been suggested that modifier genes may be responsible for this diversity.[26]

The effects of modifier genes are being investigated in many diseases, particularly in cystic fibrosis. The variability of the severity of lung involvement has been found to be associated with modifier genes. In a genome-wide association meta-analysis involving 6365 patients with cystic fibrosis, genomic variants strongly associated with clinical differences were found. These variants were reported to be potential targets for new therapies.[27]

The onset of clinical findings of FHHNC in families 1 and 2 was at different ages. Family 1 (cases 1 and 2) was diagnosed at an age before 1 year, while family 2 (cases 3, 4, and 5) was diagnosed at the average age of 11 years. Family 1 had a nonsense pathogenic variant. This variant creates a stop codon and, as a result, an inactive protein is formed. Family 2 had a missense variant, which creates an amino acid change in the protein. Therefore, the FHHNC phenotype may have been seen at an earlier stage in family 1. The different onset of the phenotype between families may be associated with the function of the variants.

Our results showed that among siblings with FHHNC, despite the same mutation, the clinical and laboratory features may be different. Although the monozygotic twins with FHHNC with the same mutation had similar phenotypes, it is suggested that various other genetic modifier factors are playing a role in the clinical presentation of FHHNC due to mutations in the *CLDN16* gene.

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