Brief report

Haplotype analysis of *CLDN19* single nucleotide polymorphisms in Spanish patients with familial hypomagnesemia with hypercalciuria and nephrocalcinosis

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Background: **Familial hypomagnesemia with hypercalciuria and nephrocalcinosis (FHHNC) is an autosomal recessive tubular disease caused by mutations in the *CLDN16* or *CLDN19* gene. Previous studies using microsatellite markers flanking the *CLDN19* locus estimated that p.G20D (c.59G>A), a recurrent mutation in Spanish families, is a founder mutation. In the present study, we assessed the haplotype of Spanish patients using single nucleotide polymorphisms (SNPs).**

Methods: **Twenty-seven FHHNC patients were included in this study. We analyzed four SNPs located in *CLDN19* introns 3 and 4 by polymerase chain reaction amplification and DNA sequencing.**

Results: **Three new patients with homozygous p.G20D were identified. The SNP genotyping analysis showed that alleles carrying this mutation shared a common SNP haplotype.**

Conclusions: **Our findings suggest the existence of a founder effect responsible for FHHNC in our cohort. Testing for the presence of mutation p.G20D should be the first genetic screening in Spanish patients.**

World J Pediatr 2015;11(3):272-275

Key words: **chronic kidney disease;**
founder effect;
gene mutation;
nephrocalcinosis

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doi: 10.1007/s12519-014-0528-3
Online First November 2014.

Introduction

Familial hypomagnesemia with hypercalciuria and nephrocalcinosis (FHHNC; OMIM #248250) is a rare autosomal recessive tubular disorder characterized by excessive renal magnesium and calcium excretion, nephrocalcinosis and chronic kidney failure.[1,2] Patients typically present during early childhood or before adolescence with recurrent urinary tract infections (UTIs), nephrolithiasis, polyuria, polydipsia, and failure to thrive.[1,3-6] Rodriguez-Soriano et al[3] first found that FHHNC could be caused by a defect in renal tubular reabsorption in the thick ascending limb of Henle's loop (TAL). This disease is due to mutations in the *CLDN16* or *CLDN19* gene, encoding tight junction proteins claudin-16 and claudin-19, respectively.[7,8] Patients with *CLDN19* mutations also present with severe ocular abnormalities.[7,9-11] Claudin-16 and claudin-19 are expressed in the TAL and play a key role in the regulation and selectivity of paracellular calcium and magnesium reabsorption in the kidney.[12,13] Loss of either claudin-16 or claudin-19 in the mouse kidney abolishes the cation selectivity for the TAL paracellular pathway, leading to excessive renal wasting of magnesium.[14] Claudin-19 is also expressed at high levels in the retina[7] although we do not know why *CLDN19* mutations cause ocular defects. The disease caused by *CLDN19* mutations has been named FHHNC with severe ocular involvement (MIM #248190). The identification of several FHHNC patients from Spain carrying missense mutation p.G20D (c.59G>A) in *CLDN19* has raised the question of the origin of this mutation from a common ancestor.[7] Subsequent studies[10,11] showed that this mutation is indeed found at a very high frequency among Spanish families with FHHNC. Recent results obtained with families of Spanish origin using microsatellite markers close to the *CLDN19* locus support the hypothesis of a founder effect for the p.G20D mutation.[10,11]

In the present study, we determined the haplotype of our patients by genotyping four single nucleotide

polymorphisms (SNPs) located in *CLDN19* introns. We also described the clinical data and genotype of three new FHHNC patients with the homozygous p.G20D mutation.

Methods

Samples selected

Our study included 27 FHHNC patients (17 females and 10 males) from 26 families in different regions of Spain and 13 controls. In this series there were 24 patients from a Spanish cohort reported previously (19 homozygous for the p.G20D mutation and 5 heterozygous)[11] and 3 new patients who were subsequently identified [P551 (Family 28), P479 (Family 29) and P590 (Family 30) (Table 1)]. The controls included two patients homozygous for a different *CLDN19* mutation, p.G122R and p.Q57E, respectively. Diagnosis was based on the established clinical criteria and confirmed by the identification of a *CLDN19* mutation.[1,5,11] DNA was isolated from peripheral blood using the QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany). Ethics authorization for DNA analysis was granted from the Ethics Committee of Clinical Research, Hospital Nuestra Señora de Candelaria (Santa Cruz de Tenerife, Spain). All patients or their parents and healthy controls signed an informed consent for the genetic analysis before inclusion.

Table 1. SNPs genotypes for FHHNC patients and controls

Patient	Pedigree	c.59G>A	rs10890211	rs11210708	rs7548008	rs12141833
P396	F1	A/A	A/A	G/G	C/C	G/G
P393	F2	A/A	A/A	G/G	C/C	G/T
P211	F5	A/A	A/A	G/G	C/C	G/G
P316	F6	A/A	A/A	G/G	C/C	G/T
P216	F8	A/A	A/A	G/G	C/C	G/G
P343	F9	A/A	A/A	G/G	C/C	G/G
P298	F11	A/A	A/A	G/G	C/C	G/G
P297	F13	A/A	A/A	G/G	C/C	G/T
P389	F14	A/A	A/A	G/G	C/C	G/G
P388	F15	A/A	A/A	G/G	C/C	G/G
P410	F17	A/A	A/A	G/G	C/C	G/G
P415	F19	A/A	A/A	G/G	C/C	G/G
P416	F20	A/A	A/A	G/G	C/C	G/G
P494	F22	A/A	A/A	G/G	C/C	G/G
P451	F23	A/A	A/A	G/G	C/C	G/T
P462	F24	A/A	A/A	G/G	C/C	G/G
P444	F25	A/A	A/A	G/G	C/C	G/G
P455	F26	A/A	A/A	G/G	C/C	G/G
P463	F27	A/A	A/A	G/G	C/C	G/T
P551	F28	A/A	A/A	G/G	C/C	G/G
P583	F29	A/A	A/A	G/G	C/C	G/G
P590	F30	A/A	A/A	G/G	C/C	G/G
P203	F3	A/G	A/C	G/A	C/T	G/T
P206	F4	A/G	A/A	G/G	C/C	G/G
P291	F12	A/-	A/A	G/G	C/T	G/T
P439	F12	A/-	A/A	G/G	C/T	G/T
P406	F18	A/G	A/C	G/A	C/T	G/T
C243	-	G/G	C/C	A/A	T/T	T/T
C429	-	G/G	C/C	A/A	T/T	T/T
C541	-	G/G	A/C	G/A	C/T	G/T
C542	-	G/G	A/A	G/A	C/T	G/T
C557	-	G/G	C/C	A/A	T/T	T/T
C558	-	G/G	A/C	G/A	C/T	G/G
C579	-	G/G	A/C	G/A	T/T	G/T
C580	-	G/G	C/C	A/A	T/T	G/G
C586	-	G/G	A/A	G/G	C/C	G/G
C588	-	G/G	A/A	G/G	C/T	G/G
C598	-	G/G	C/C	A/A	T/T	G/T
C604	-	G/G	A/C	G/A	C/T	T/T
C606	-	G/G	C/C	A/A	T/T	T/T
C607	-	G/G	C/C	A/A	T/T	T/T
C608	-	G/G	A/C	G/A	T/T	T/T

Nucleotides refer to the non-coding strand. SNPs: single nucleotide polymorphisms; FHHNC: familial hypomagnesemia with hypercalciuria and nephrocalcinosis.

Haplotype analysis

Four SNPs, rs10890211, rs11210708, rs7548008 and rs12141833, located in *CLDN19* introns 3 and 4 (Fig. 1), were selected based on their allelic frequencies calculated from the GenBank database (National Center for Biotechnology Information). Genotyping was performed by polymerase chain reaction (PCR) amplification from genomic DNA and sequencing. Primers pairs used were: C19Int3F (5'-ACACTGATGGCTCTGTCTGC-3') and C19Int3R (5'-TTGGGCTCATTTCCTGATCT-3'), and C19Int4F (5'-CCCAGGCATACACACAGATG-3') and C19Int4R (5'-AGGGTGAAGGTTTCTGCTCA-3'). PCR reactions were carried out using the following conditions: 94ºC for 5 minutes, 30 cycles of 94ºC for 30 seconds, 54ºC for 45 seconds, and 72ºC for 45 seconds, and a final extension at 72ºC for 5 minutes. PCR products were sequenced with the BigDye terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). Sequencing reactions were purified with Performa DTR Gel Filtration Cartridges (EdgeBio, Gaithersburg, MD, USA) and analyzed on a 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Haplotype analysis in new patients using microsatellite markers D1S463, D1S193 and D1S447, flanking the *CLDN19* locus, was performed as described previously.[10,11]

Mutation screening

CLDN19 exons and flanking intronic regions of the three new patients were amplified by PCR using

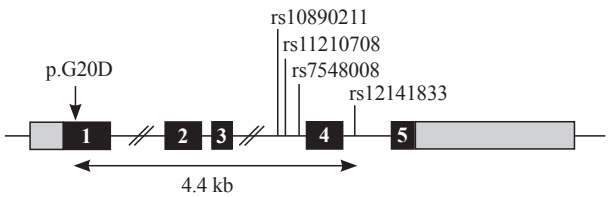

Fig. 1. Schematic representation of the *CLDN19* gene and SNPs location. The four SNPs studied and the p.G20D mutations are indicated. SNPs: single nucleotide polymorphisms.

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primers and conditions described previously.[11] PCR products were sequenced as described above.

Results

The main clinical and biochemical characteristics of the 3 new diagnosed patients are as follows. Patient P551 was a two-year-old boy was referred to the hospital at two months of age because of the ultrasound finding of hyperechogenicity of renal pyramids and cortex. He was diagnosed with nephrocalcinosis, hypercalciuria (2.98 mmol/d, normal range <2.5 mmol/d), hypomagnesemia (0.57 mmol/L, normal range 0.65-1.05 mmol/L) and severe myopia. His glomerular filtration rate (GFR) was 74 mL/min per 1.73 m^2 at 2 months of age. Patient P479 was a 14-year-old girl who was referred to the hospital at age of 7 months because she had two UTIs. Radiological examination revealed bilateral nephrocalcinosis. The patient presented with hypomagnesemia (0.49 mmol/L, normal range 0.65-1.05 mmol/L) and hypercalciuria (5.35 mmol/d, normal range <2.5 mmol/d). At age of 13 months, her GFR was 95 mL/min per 1.73 m^2. Ophthalmologic examination revealed myopia and nystagmus. Patient P590 was a 33-year-old woman who was diagnosed with nephrocalcinosis at 6 years of age. She had recurrent UTIs including pyelonephritis. Eye examination showed horizontal nystagmus and at age of 14 years, she had surgery for a left divergent strabismus. Laboratory data obtained at age of 24 years when the patient was admitted to the hospital for acute pyelonephritis showed hypomagnesemia (0.49 mmol/L, normal range 0.65-1.05 mmol/L) and hypercalciuria (6.4 mmol/d; normal range <6.2 mmol/d). At age of 30 years, her GFR was 30 mL/min/per 1.73 m^2, and at age of 33 years she started hemodialysis and a kidney transplant. We identified homozygous mutation p.G20D in the *CLDN19* gene of these three patients. Their parents were heterozygous carriers. The results of microsatellite analysis demonstrated the presence of a mutation-carrying haplotype (Fig. 2) as had been previously found for the rest of the patients.[11]

An SNP haplotype analysis was performed to check whether our 27 FHHNC patients had a common p.G20D (c.59G>A) linked haplotype. The results revealed that almost every patient shared the same allele sequence in the vicinity of the mutation, **A**-A-G-C-G, where the bold A is the c.59A mutant allele (Table 1). To assess whether this strong association of a specific haplotype was indeed the result of a founder effect, fifteen unrelated Spanish individuals not carrying the p.G20D mutation were genotyped for the four SNPs. The most common alleles in these individuals for the SNPs rs10890211, rs11210708 and rs7548008 were the C (19/30: 63.3%), A (20/30: 66.7%), and T (23/30: 76.7%) alleles, respectively, which were never associated with c.59G>A among the disease chromosomes (Table 1). While the most common allele for SNP rs12141833 (18/12: 60%) in the controls was linked with the mutation in only a few cases (Table 1). A comparison of the allele frequency of each SNP between our controls, global population and Iberian population in Spain, and the FHHNC patients is shown in Table 2. The overall distribution of this haplotype was significantly different between the patients and controls (P<0.0001).

Table 2. Allele frequency of each SNP in patients and controls

SNP	Nucleotide variation	Distance to c.59G>A (bp)	Allele	Frequency		
				Patients*	Controls*	Global/Iberian populations†
rs10890211	C>A	3244	A	0.963	0.366	0.456/0.535
rs11210708	A>G	3298	G	0.963	0.333	0.456/0.535
rs7548008	T>C	3519	C	0.926	0.233	0.434/0.535
rs12141833	T>G	4440	G	0.833	0.400	0.553/0.714

*: patients *vs.* controls, P<0.001, for all Chi^2 test comparisons. †: Global population (2178 chromosomes from 1039 individuals) and Iberian population in Spain (28 chromosomes from 14 individuals) data were taken from 1000 Genome Browser version 3.0. Nucleotides shown refer to the non-coding strand. SNP: single nucleotide polymorphism.

Discussion

In 2006, Konrad et al[7] reported the first *CLDN19* mutations in patients with FHHNC. They identified the same homozygous mutation, p.G20D, in seven unrelated Spanish/Hispanic patients and suggested a founder effect.

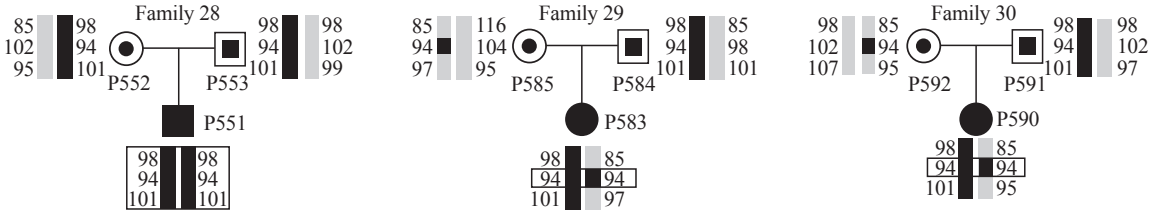

Fig. 2. Analysis of three polymorphic microsatellite markers flanking the *CLDN19* locus in three Spanish families with the common p.G20D mutation. The pedigrees and haplotypes are shown. Vertical bars represent haplotypes for markers D1S463, D1S193 and D1S447 (top to bottom). The disease-associated alleles are shown in black and the others in gray. Numbers indicate the allele size in nucleotides. Black circles and squares represent affected subjects; black circles and squares within open frames represent heterozygous individuals. Squares represent males and circles females. The p.G20D mutation was homozygous in the three patients.

Several studies[7,10,11] showed that most Spanish patients present mutation in the *CLDN19* gene; as far as we know only three patients have mutations in *CLDN16*.[8,15] The p.G20D mutation in a homozygous state results in absence of claudin-19 protein in the cell membrane and a complete loss of function.[7,12] Subsequently, two studies using microsatellite markers with FHHNC families from Spain and from the South of France further supported the founder effect hypothesis.[10,11] It should be noted that a large immigration from Spain to France occurred after the Spanish civil war in 1936-1939.

The results of the present study showed that the SNP haplotype **A**-A-G-C-G was specifically linked to p.G20D, and are therefore consistent with the existence of a founding ancestor in our cohort. Founder effects have been also identified for FHHNC in patients from three North African families harboring the same *CLDN16* p.A139V mutation[10] and in 14 families who originated from Germany or eastern European countries carrying *CLDN16* p.L151F mutation.[5] Mutations that persist following their occurrence in a single founder may reflect selection-favoring heterozygotes, perhaps by providing the carriers with a survival advantage. However, there is no apparent reason why the p.G20D carriers should have a benefit.

In conclusion, the previously described founder effect for p.G20D supported by the conservation of a specific mutation-linked SNP haplotype shown in this study suggests that the c.59A allele arose from one independent ancestral allele. This effect explains the high frequency of the mutation in the Spanish FHHNC families. Our finding is fundamental for the implementation of a protocol for genetic diagnosis, genetic counseling of affected families, and search of new therapies.

Acknowledgements

We are grateful to the patients and their parents for their cooperation in the study. Cordoba-Lanus E, Gonzalez-Acosta H and Claverie-Martin F are members of the Renaltube Group (www.renaltube.org).

Funding: This work was supported by a research grant (PI11/00342) from Fondo de Investigacion Sanitaria (co-funded by the European Regional Development Fund, "A way to build Europe") to F.C.M. This funding was granted as coordinated project Renaltube.

Ethical approval: This study was approved by the Ethics Committee of Clinical Research, Hospital Nuestra Señora de Candelaria, Santa Cruz de Tenerife, Spain.

Competing interest: None of the authors have a financial relationship with a commercial entity that has an interest in the subject of this study.

Contributors: Martin-Nuñez E, Cordoba-Lanus E and Gonzalez-Acosta H performed the experiments, analyzed the data and helped to draft the manuscript. Oliet A and Izquierdo E performed the clinical evaluation and analyzed the clinical data. Claverie-Martín F participated in the design of the experiments, analysis of data and coordination, and wrote the manuscript. All authors contributed to the intellectual content and approved the final version of the manuscript.

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Received January 9, 2014
Accepted after revision March 6, 2014