

# CLDN16 Genotype Predicts Renal Decline in Familial Hypomagnesemia with Hypercalciuria and Nephrocalcinosis

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## ABSTRACT

Familial hypomagnesemia with hypercalciuria and nephrocalcinosis (FHHNC) is a rare autosomal recessive tubular disorder caused by *CLDN16* mutations. *CLDN16* encodes the renal tight junction protein claudin-16, which is important for the paracellular reabsorption of calcium and magnesium in the thick ascending limb of Henle's loop. That FHHNC is frequently associated with progressive renal failure suggests additional roles for claudin-16 in the maintenance of tight junction integrity. An investigation of 32 patients with FHHNC and 17 different mutations was previously reported; here, the analysis is expanded to 39 additional patients and 12 new mutations. Expression studies revealed that five of the 12 new mutations led to partial loss of claudin-16 function and the remaining seven led to complete loss of function. The 23 patients who had mutations resulting in complete loss of function of both alleles were significantly younger at the onset of symptoms than the 46 patients who had at least one mutant allele providing partial function (2.2 versus 5.6 years;  $P < 0.01$ ). In addition, those with complete loss of function had a more rapid decline in GFR (7.3 versus 2.9 ml/min per 1.72 m<sup>2</sup>/y;  $P < 0.01$ ), leading to 54% requiring renal replacement therapy by age 15 compared with 20% of those with residual function ( $P < 0.05$ ). These data suggest that residual function of claudin-16 may delay the progression of renal failure in FHHNC.

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Familial hypomagnesemia with hypercalciuria and nephrocalcinosis (FHHNC; OMIM \*248250) is a rare autosomal recessive tubular disorder. It is characterized by massive urinary losses of magnesium (Mg) and calcium (Ca) leading to hypomagnesemia and bilateral nephrocalcinosis. The disease usually presents with recurrent urinary tract infections and polyuria/polydipsia. Additional symptoms include rickets, nephrolithiasis, hematuria, muscular tetanias, seizures, failure to thrive, vomiting, and abdominal pain.<sup>1,2</sup> Ocular abnormalities and hearing

impairment have been described in a subset of patients with FHHNC.<sup>3,4</sup> Additional biochemical ab-

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normalities include signs of incomplete distal renal tubular acidosis, hypocitraturia, increased parathyroid hormone (PTH) levels (independent of GFR), and hyperuricemia.<sup>3,5–8</sup> Unlike most other inherited tubular diseases affecting electrolyte homeostasis, FHHNC is generally complicated by progressive renal failure during childhood or adolescence, but the pathogenesis of chronic renal failure remains a matter of debate.

Clearance studies in patients with FHHNC localized the site of disturbed handling of Mg and Ca to the thick ascending limb (TAL) of Henle's loop.<sup>6</sup> The TAL plays an important role in the tubular reabsorption of Mg and Ca, which occurs by paracellular flux, a process driven by the lumen-positive transepithelial potential in this nephron segment.

In 1999, Simon *et al.*<sup>9</sup> identified a new gene (*CLDN16*, formerly *PCLN1*) and characterized mutations in this gene as the underlying molecular defect in FHHNC. Since then, approximately 30 additional families with FHHNC as a result of *CLDN16* defects have been described.<sup>2,10–13</sup> Hypercalciuria and nephrolithiasis have also been observed in heterozygous FHHNC mutation carriers.<sup>2,3</sup> FHHNC is a genetically heterogeneous disease because recently *CLDN19* mutations have been identified in a cohort of patients mainly originating from Spain.<sup>14</sup> The renal phenotype of these patients is very similar to patients exhibiting *CLDN16* mutations; however, patients with *CLDN19* mutations also have severe ocular abnormalities in most cases.

*CLDN16* and *CLDN19* encode the tight junction (TJ) proteins claudin-16 (paracellin-1) and claudin-19, both being members of the claudin multigene family. Claudins are important components of the TJ strands in various tissues.<sup>15</sup> They are part of a complex protein network built up by a variety of different proteins, and there is clear evidence that claudins confer ion selectivity to the paracellular pathway.<sup>16–18</sup>

In the kidney, claudin-16 expression is restricted to the TAL of Henle's loop. It was speculated that claudin-16 constitutes the core of an intercellular pore, allowing paracellular reabsorption of Mg and Ca ions.<sup>9,19</sup> Following the observation that a naturally occurring knockout model in Japanese black cattle shows early-onset renal failure with diffuse interstitial nephritis,<sup>20</sup> it was recently speculated that claudin-16, like other claudins, may also be involved in the regulation of cell growth, proliferation, differentiation, and dedifferentiation.<sup>21</sup> Hou *et al.*<sup>22</sup> described the functional analysis of claudin-16 in polarized cell lines. They demonstrated that in LLC-PK1 cells, claudin-16 modulated the ion selectivity of the TJ by selectively increasing the permeability of Na<sup>+</sup> with no effects on Cl<sup>-</sup>, resulting in a high permeability ratio of Na<sup>+</sup> to Cl<sup>-</sup>. Instead, Mg flux across cell monolayers showed a far less pronounced change after claudin-16 expression. From these data, it might be concluded that claudin-16 defects lead to a loss of cation selectivity with a subsequent decrease in lumen-positive potential that is the driving force for the paracellular flux of cations. This hypothesis of a nonselective paracellular cation channel is supported by a mouse model using transgenic RNAi

depletion of claudin-16. Loss of *CLDN16* in this model caused TJ in TAL to lose the cation selectivity.<sup>23</sup> Hou *et al.*<sup>22</sup> also analyzed the consequences of most of the reported human *CLDN16* mutations by heterologous expression *in vitro*. Whereas most mutations resulted in a complete loss of function, some mutations retained a substantial residual function. One of these mutations (L151F) is by far the most frequent FHHNC mutation, occurring in almost 50% of the patients described so far.<sup>2</sup>

Combining this information with additional functional analysis of new mutations using the same expression system, we present a genotype/phenotype correlation with a special focus on the progression of renal failure in a large cohort of patients with FHHNC. We provide clinical data indicating that homozygous or compound heterozygous patients who carry at least one *CLDN16* mutation with residual function have a much more benign course of the disease than patients with a complete loss of function.

## RESULTS

### Mutation Analysis of *CLDN16*

In our initial study,<sup>2</sup> *CLDN16* mutations were identified in 32 patients with FHHNC, 67% of the mutant alleles exhibited a missense mutation affecting the first extracellular loop of claudin-16, and 48% were affected by an L151F exchange. This mutational hot spot is due to a widespread founder effect (Germany and Eastern European countries). Mutation analysis in families F29 to F88 (with 39 affected individuals) revealed 17 different mutations (Table 1). In addition to the ones already described, 12 novel *CLDN16* mutations were identified, including seven missense mutations, one nonsense mutation truncating the protein in the fourth transmembrane domain; one small internal deletion, one splice-site mutation in exon four, one frameshift mutation truncating the protein before the first extracellular domain, and one mutation resulting in the loss of the translation initiation start site (Figure 1A). Figure 1B depicts the amino acids affected by missense mutations in the claudin-16 protein. All mutations co-segregated with the FHHNC phenotype, and none of the mutations was observed in at least 100 control chromosomes. Both mutant alleles were detected in 34 affected individuals; however, in the remaining five patients from three families (F37, F61, and F73), only one heterozygous mutation could be identified. Families F37 and F61 both have two affected children. Therefore, we performed haplotype analysis to demonstrate identical haplotypes also for the second mutant allele. In both families, the affected siblings share the same haplotypes compatible with linkage to the *CLDN16* locus. Thus, it is most likely that the second mutation was missed because it is not located in the coding sequence. Family F73 could not be studied by haplotype analysis because there is only one affected child.

**Table 1.** Molecular analysis of the new patients with FHHNC

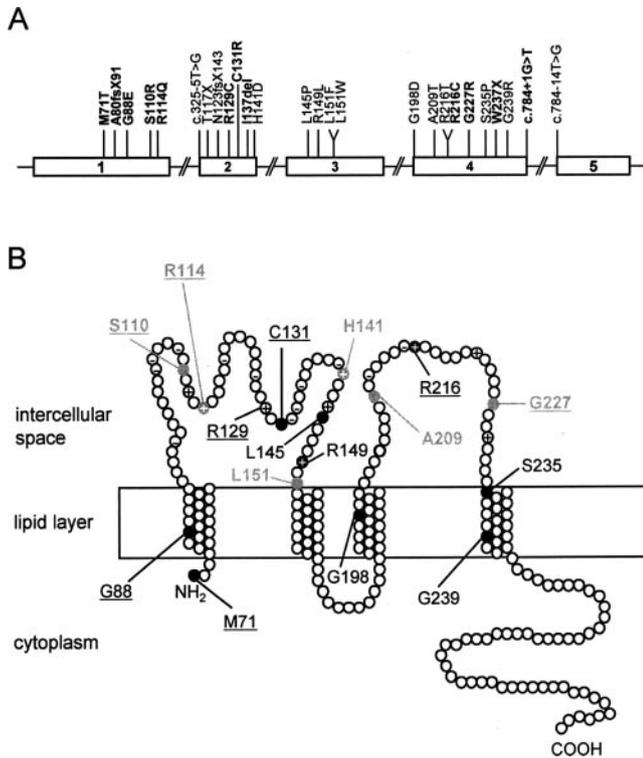
| Patient | Gender | Origin         | Consanguinity | Zygoty                | Nucleotide Change          | Predicted Protein Change           |
|---------|--------|----------------|---------------|-----------------------|----------------------------|------------------------------------|
| F29     | F      | German         | –             | Homozygous            | 453(TTG→TTT)               | p.L151F                            |
| F30     | F      | German         | –             | Homozygous            | 453(TTG→TTT)               | p.L151F                            |
| F32-1   | M      | Turkish        | +             | Homozygous            | 715(GGA→AGA)               | p.G239R                            |
| F32-2   | M      | Turkish        | +             | Homozygous            | 715(GGA→AGA)               | p.G239R                            |
| F33     | M      | German         | –             | Homozygous            | 453(TTG→TTT)               | p.L151F                            |
| F34     | F      | German         | –             | Compound heterozygous | 385(CGC→TGC)-453(TTG→TTT)  | p.R129C <sup>a</sup> -p.L151F      |
| F37-1   | M      | German         | –             | Heterozygous          | 453(TTG→TTT)               | p.L151F                            |
| F37-2   | M      | German         | –             | Heterozygous          | 453(TTG→TTT)               | p.L151F                            |
| F56     | F      | ?              | +             | Homozygous            | 341(CGA→AGA)               | p.R114Q <sup>a</sup>               |
| F60     | F      | German         | –             | Compound heterozygous | 453(TTG→TTT)-625(GCT→ACT)  | p.L151F-p.A209T                    |
| F61-1   | M      | German         | –             | Heterozygous          | 453(TTG→TTT)               | p.L151F                            |
| F61-2   | F      | German         | –             | Heterozygous          | 453(TTG→TTT)               | p.L151F                            |
| F62     | M      | Arab           | +             | Homozygous            | 236delG                    | p.A80fsX91 <sup>a</sup>            |
| F64     | F      | Polish         | –             | Compound heterozygous | 329(AGC→AGG)-453(TTG→TTT)  | p.L151F-p.S110R <sup>a</sup>       |
| F66     | M      | Turkish        | +             | Homozygous            | 710(TGG→TAG)               | p.W237X <sup>a</sup>               |
| F63-1   | M      | British        | –             | Compound heterozygous | 646(CGT→TGT)-784 + 1(G→T)  | p.R216C-splice site <sup>a</sup>   |
| F69-2   | F      | British        | –             | Compound heterozygous | 646(CGT→TGT)-784 + 1(G→T)  | p.R216C-splice site <sup>a</sup>   |
| F70     | F      | Turkish        | +             | Homozygous            | 679(GGA→CGA)               | p.G227R <sup>a</sup>               |
| F71     | M      | Bulgarian      | –             | Compound heterozygous | 434(CTG→CCG)- 453(TTG→TTT) | p.L145P-p.L151F                    |
| F72     | F      | English/German | –             | Compound heterozygous | 408–410delCAT-453(TTG→TTT) | p.I137del <sup>a</sup> -p.L151F    |
| F73     | F      | German         | –             | Heterozygous          | 385(CGC→TGC)               | p.R129C <sup>a</sup> -?            |
| F74-1   | M      | Serbian        | –             | Homozygous            | 453(TTG→TTT)               | p.L151F                            |
| F74-2   | F      | Serbian        | –             | Homozygous            | 453(TTG→TTT)               | p.L151F                            |
| F74-3   | M      | Serbian        | –             | Homozygous            | 453(TTG→TTT)               | p.L151F                            |
| F77     | M      | German         | –             | Compound heterozygous | 453(TTG→TTT)-263(GGG→GAG)  | p.L151F-p.G88E                     |
| F78-1   | M      | Arab           | +             | Homozygous            | 212(ATG→ACG)               | p.M71T, <sup>a</sup> loss of start |
| F79-1   | F      | Arab           | +             | Homozygous            | 212(ATG→ACG)               | p.M71T, <sup>a</sup> loss of start |
| F80-1   | F      | Arab           | +             | Homozygous            | 212(ATG→ACG)               | p.M71T, <sup>a</sup> loss of start |
| F80-2   | F      | Arab           | +             | Homozygous            | 212(ATG→ACG)               | p.M71T, <sup>a</sup> loss of start |
| F80-3   | M      | Arab           | +             | Homozygous            | 212(ATG→ACG)               | p.M71T, <sup>a</sup> loss of start |
| F81-1   | F      | Arab           | +             | Homozygous            | 646(CGT→TGT)               | p.R216C <sup>a</sup>               |
| F81-2   | M      | Arab           | +             | Homozygous            | 646(CGT→TGT)               | p.R216C <sup>a</sup>               |
| F83-1   | F      | Macedonian     | +             | Homozygous            | 453(TTG→TTT)               | p.L151F                            |
| F83-2   | F      | Macedonian     | +             | Homozygous            | 453(TTG→TTT)               | p.L151F                            |
| F83-3   | M      | Macedonian     | +             | Homozygous            | 453(TTG→TTT)               | p.L151F                            |
| F87-1   | M      | Turkish        | +             | Homozygous            | 453(TTG→TTT)               | p.L151F                            |
| F87-2   | M      | Turkish        | +             | Homozygous            | 453(TTG→TTT)               | p.L151F                            |
| F87-3   | M      | Turkish        | +             | Homozygous            | 453(TTG→TTT)               | p.L151F                            |
| F88-1   | M      | Turkish        | +             | Homozygous            | 385(CGC→TGC)               | p.C131R <sup>a</sup>               |

<sup>a</sup>New mutation.

### Functional Analysis of the New Mutations

The functional consequences of the new *CLDN16* missense mutations were analyzed after heterologous expression in MDCK and LLC-PK1 cells. In addition, we studied H141D and L151W, which have been described previously<sup>2</sup> but not functionally characterized by Hou *et al.*<sup>22</sup> The profile of expression and localization of the mutant proteins are summarized in Table 2. Most of the mutant proteins display a normal trafficking to the cell membrane. Only two mutations (M71T and C131R) were retained inside the cell; the M71T mutation is localized to lysosomes, whereas C131R is retained in the endoplasmic reticulum (Figure 2). The mutants that were properly targeted to TJ showed a significant loss of function compared with wild-type claudin-16 when

expressed in LLC-PK1 cells. We observed a significant decrease of the dilution potential and the ratio of permeability of Na<sup>+</sup> over Cl<sup>–</sup>. This indicates a disturbance of the cation selectivity of LLC-PK1 cells expressing wild-type claudin-16. As in the previous study, not all mutations showed a complete loss of function (CL; Figure 3). Several mutants retain a significant residual claudin-16 function. To compare the results obtained in this study with the data from the initial expression study,<sup>22</sup> we calculated the residual function as percentage relative to wild-type claudin-16 function. Mutants with a residual function >40% were considered “partial” loss of function (PL); the remaining mutants were considered CL. These categories were also used for the genotype/phenotype analysis (see next section).



**Figure 1.** (A) Genomic organization of *CLDN16*. Mutations are depicted above the schematic presentation of *CLDN16* (novel mutations in bold). (B) Claudin-16 protein model deduced from hydrophilicity plots. Amino acid residues affected by novel missense mutations are underlined. CL mutations are depicted in black; PL mutations are depicted in gray.

**Genotype/Phenotype Correlation**

*Assignment of Mutation Category*

To analyze a possible genotype/phenotype correlation in FHHNC, we assigned *CLDN16* mutations to three different categories: (1) Complete loss of function (CL) mutations including missense mutations resulting in a complete loss of function after heterologous expression (Figure 3). In addition,

nonsense, truncating and splice-site mutations were attributed to this category. Bioinformatic analysis of the three splice site mutations clearly indicated significant disturbance of *CLDN16* mRNA splicing. (2) Missense mutations that displayed substantial residual function >40% as compared with wild-type claudin-16 function were considered as mutations with partial loss of function (PL) (Figure 3). (3) Missense mutations that could not be assigned in categories CL or PL are given as X. This assignment was done separately for both mutant alleles.

Following these criteria, 23 patients had a CL/CL genotype, 29 patients had PL/PL, 12 patients had PL/CL, five patients had PL/X, one patient had X/X, and one patient had CL/X. Because of the lack of information in X/X and CL/X patients, these two patients were completely excluded from the genotype/phenotype analysis (Table 3).

*Confirmation of the Recessive Nature of FHHNC*

In a first step of the genotype/phenotype analysis, only the groups with classified mutations on both mutant alleles were analyzed (CL/CL, PL/CL, and PL/PL). PL/X patients were excluded from this first statistical analysis because no information is available with respect to the severity of the second allele (for comparison, the data are shown in Figure 4). In theory, given the recessive nature of the disease, one might expect that the effect of a PL mutation can also be observed if this mutation occurs in compound heterozygosity with a severe mutation (CL). This has been previously shown for other recessive diseases, for example, with respect to the preservation of pancreatic sufficiency in cystic fibrosis<sup>24</sup>: as long as a loss of function mutant was in compound heterozygosity with a mutant with residual function, no severe pancreatic phenotype was observed.

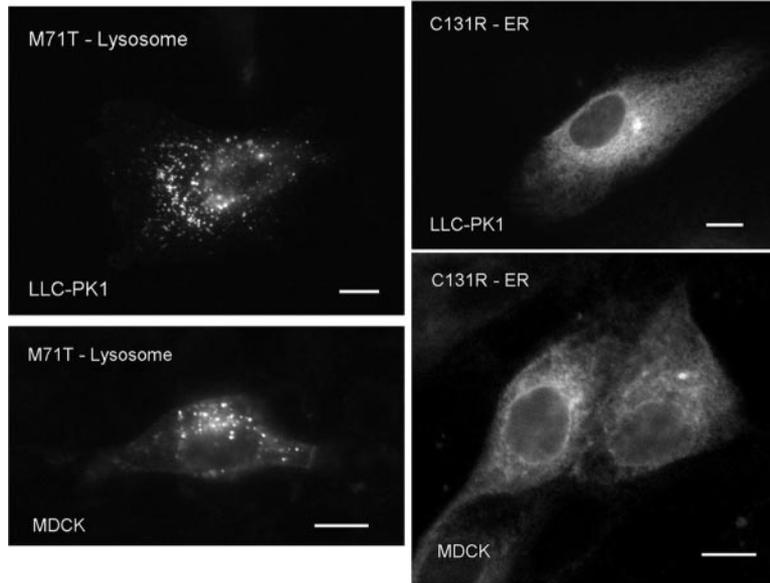
Analysis of the three groups with categorized mutations on both alleles (CL/CL, PL/CL, and PL/PL) revealed a significant correlation between the genotype and age at onset ( $P < 0.05$ ) and decline of renal function ( $P < 0.01$ ). CL/CL patients were

**Table 2.** Functional analysis of *CLDN16* mutations (expressed in LLC-PK1 cells)<sup>a</sup>

| Mutation | Position   | TER        | Dilution Potential        | $P_{Na}/P_{Cl}$            | $P_{Na}$ ( $10^{-6}$ cm/s)  | $P_{Cl}$ ( $10^{-6}$ cm/s) | Localization | Function |
|----------|------------|------------|---------------------------|----------------------------|-----------------------------|----------------------------|--------------|----------|
| Vector   | —          | 61.0 ± 2.1 | -8.13 ± 0.19 <sup>b</sup> | 0.296 ± 0.010 <sup>b</sup> | 6.866 ± 0.184 <sup>b</sup>  | 23.220 ± 0.182             | —            | —        |
| WT       | —          | 33.0 ± 1.5 | 2.97 ± 0.19               | 1.500 ± 0.038              | 33.363 ± 0.346              | 22.257 ± 0.346             | TJ           | +        |
| M71T     | N terminus | 60.0 ± 2.5 | -7.83 ± 0.12 <sup>b</sup> | 0.313 ± 0.007 <sup>b</sup> | 7.282 ± 0.121 <sup>b</sup>  | 23.307 ± 0.123             | Lysosome     | CL       |
| G88E     | First TMD  | 50.7 ± 2.9 | -7.13 ± 0.09 <sup>b</sup> | 0.354 ± 0.005 <sup>b</sup> | 9.398 ± 0.105 <sup>b</sup>  | 26.590 ± 0.106             | TJ           | CL       |
| S110R    | First ECL  | 38.3 ± 0.9 | -3.30 ± 0.12 <sup>b</sup> | 0.637 ± 0.010 <sup>b</sup> | 18.787 ± 0.188 <sup>b</sup> | 29.513 ± 0.188             | TJ           | PL       |
| R114Q    | First ECL  | 34.7 ± 3.7 | -2.33 ± 0.09 <sup>b</sup> | 0.728 ± 0.009 <sup>b</sup> | 22.100 ± 0.153 <sup>b</sup> | 30.340 ± 0.153             | TJ           | PL       |
| C131R    | First ECL  | 60.7 ± 3.0 | -7.50 ± 0.06 <sup>b</sup> | 0.332 ± 0.003 <sup>b</sup> | 7.493 ± 0.057 <sup>b</sup>  | 22.597 ± 0.055             | ER           | CL       |
| I137del  | First ECL  | 41.7 ± 2.3 | -4.57 ± 0.09 <sup>b</sup> | 0.530 ± 0.007 <sup>b</sup> | 15.143 ± 0.130 <sup>b</sup> | 28.557 ± 0.130             | TJ           | PL       |
| H141D    | First ECL  | 39.3 ± 2.6 | -3.17 ± 0.09 <sup>b</sup> | 0.649 ± 0.008 <sup>b</sup> | 18.513 ± 0.141 <sup>b</sup> | 28.547 ± 0.141             | TJ           | PL       |
| L151W    | First ECL  | 39.7 ± 0.9 | -1.97 ± 0.18 <sup>b</sup> | 0.766 ± 0.018 <sup>b</sup> | 19.903 ± 0.271 <sup>b</sup> | 25.983 ± 0.273             | TJ           | PL       |
| R216C    | Second ECL | 44.3 ± 2.7 | -5.10 ± 0.20 <sup>b</sup> | 0.490 ± 0.015 <sup>b</sup> | 13.710 ± 0.280 <sup>b</sup> | 28.003 ± 0.277             | TJ           | CL       |
| G227R    | Second ECL | 39.0 ± 1.0 | -3.20 ± 0.06 <sup>b</sup> | 0.646 ± 0.005 <sup>b</sup> | 18.460 ± 0.092 <sup>b</sup> | 28.600 ± 0.092             | TJ           | PL       |

<sup>a</sup>Number of independent monolayers,  $n = 3$ . TER, transepithelial resistance; P, permeability; TMD, transmembrane domain; ECL, extracellular loop; ER, endoplasmic reticulum.

<sup>b</sup> $P < 0.05$ , mutant versus WT.



**Figure 2.** CLDN16 mutations resulting in trafficking defects. Epifluorescence images showing mistargeted localization of claudin-16 mutants in LLC-PK1 and MDCK cells. M71T is in the lysosome with a punctuate distribution toward periphery of the cell. C131R shows a reticular cytoplasmic and perinuclear distribution indicating retention in the endoplasmic reticulum. Bar = 10  $\mu$ m.

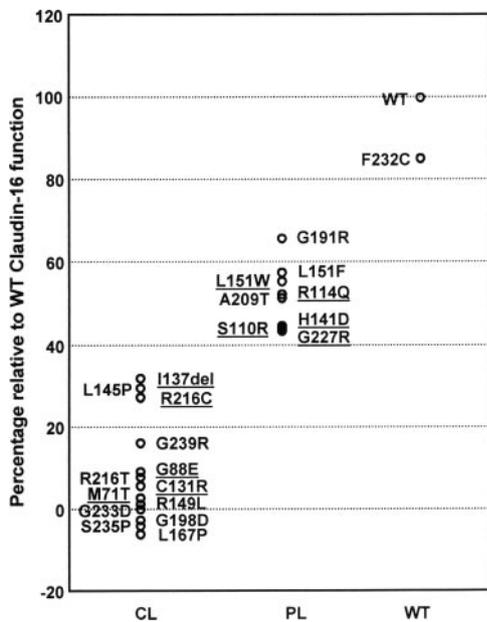
significantly younger at presentation of first symptoms (2.2 yr; 95% confidence interval [CI] 1.1 to 3.3 yr) compared with PL/PL patients (5.7 yr; 95% CI 3.3 to 8.2 yr), whereas age at onset in PL/CL patients (4.9 yr; 95% CI 2.3 to 7.6 yr) was similar to that of PL/PL patients, nevertheless not reaching statistical significance (Table 4, Figure 4A). The progression of renal failure expressed as loss of GFR/yr was significantly faster

in the CL/CL patients (7.3 ml/min per 1.73 m<sup>2</sup>/yr; 95% CI 5.0 to 9.6) as compared with either PL/PL (3.3 ml/yr; 95% CI 1.6 to 4.9) or PL/CL patients (3.0 ml/yr; 95% CI 1.1 to 4.9), whereas no difference between PL/PL and PL/CL patients could be observed (Table 4, Figure 4B). The decline of GFR in the various groups during the study period is shown in Figure 5.

The similar clinical courses of the PL/PL and PL/CL groups, which is in sharp contrast to the CL/CL group, indicates that in FHHNC, the nature of the second allele in combination with a defined PL mutation is not important for the clinical course/phenotype. The PL/X group, which was excluded from this first analysis, further supports these findings (Table 4, Figures 4 and 5). For all criteria, the PL/X group is very similar to either the PL/PL or the PL/CL group. We therefore combined PL/PL, PL/CL, but also PL/X patients into one large group: “PL”. This group was then compared with the “CL” group of CL/CL patients.

*Age at Onset and Loss of Renal Function Differ Between CL and PL Patients*

Age at onset in the CL group (complete loss of function of both alleles) was significantly lower than in the PL group (partial loss of function of at least one allele) with 2.2 yr (95% CI 1.1 to 3.3 yr) versus 5.6 yr (95% CI 3.6 to 7.0 yr; *P* < 0.01; Table 4). During follow-up, CL patients showed a faster decline of GFR than PL patients, as expressed by a loss of GFR of 7.3 versus 2.9 ml/min per 1.73 m<sup>2</sup>/yr (*P* < 0.01). As a consequence, at the time of the last observation, the renal function differed significantly between both groups (*P* < 0.01): Fewer CL patients still had a GFR >60 ml/min per 1.73 m<sup>2</sup> compared with PL patients (9 versus 54%), and more CL than PL patients had a GFR <60 ml/min per 1.73 m<sup>2</sup> (50 versus 20%) or even needed renal re-



**Figure 3.** Dilution potential of individual CLDN16 missense mutations after heterologous expression in LLC-PK1 cells. Values are given as percentage relative to wild-type claudin-16 function. New mutations are underlined; the remaining mutations were taken from Hou et al.<sup>22</sup> WT, wild-type.

**Table 3.** Functional consequences of the *CLDN16* mutations

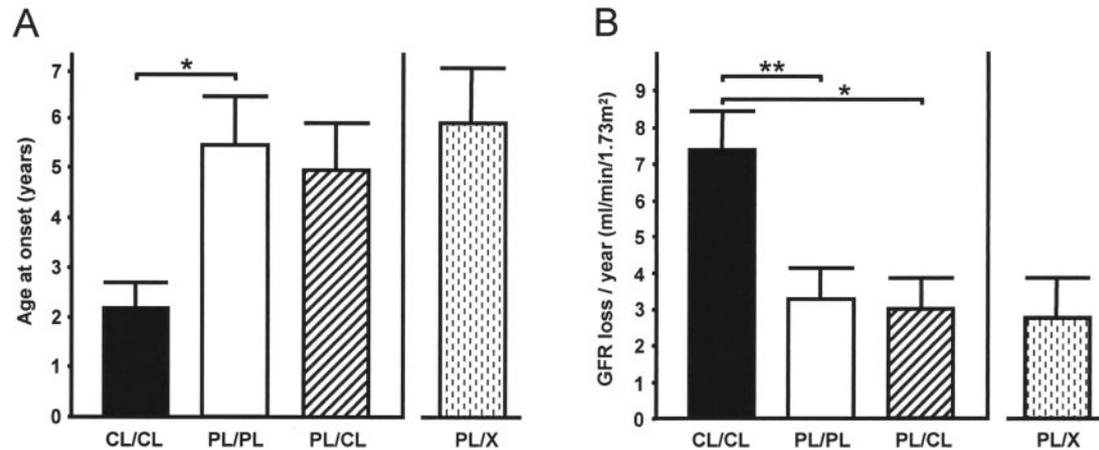
| Patient | Gender | Predicted Change    |                     | Predicted Function | Patient | Gender | Predicted Change |          | Predicted Function |
|---------|--------|---------------------|---------------------|--------------------|---------|--------|------------------|----------|--------------------|
|         |        | Allele 1            | Allele 2            |                    |         |        | Allele 1         | Allele 2 |                    |
| F03     | F      | p.G198D             | p.G198D             | CL/CL              | F02-1   | F      | p.L151F          | p.L151W  | PL/PL              |
| F04     | F      | p.G239R             | p.G239R             | CL/CL              | F02-2   | M      | p.L151F          | p.L151W  | PL/PL              |
| F09-1   | F      | p.R216T             | p.R216T             | CL/CL              | F07-1   | M      | p.L151F          | p.L151F  | PL/PL              |
| F09-2   | M      | p.R216T             | p.R216T             | CL/CL              | F07-2   | M      | p.L151F          | p.L151F  | PL/PL              |
| F10     | F      | p.L145P             | p.L145P             | CL/CL              | F08-1   | M      | p.H141D          | p.H141D  | PL/PL              |
| F11     | F      | p.G239R             | p.G239R             | CL/CL              | F08-2   | F      | p.H141D          | p.H141D  | PL/PL              |
| F15     | F      | p.R149L             | p.R149L             | CL/CL              | F13     | M      | p.L151F          | p.L151F  | PL/PL              |
| F21-1   | M      | p.L145P             | p.D123fs            | CL/CL              | F14-1   | F      | p.L151F          | p.L151F  | PL/PL              |
| F21-2   | F      | p.L145P             | p.D123fs            | CL/CL              | F14-2   | F      | p.L151F          | p.L151F  | PL/PL              |
| F32-1   | M      | p.G239R             | p.G239R             | CL/CL              | F17     | F      | p.L151F          | p.L151F  | PL/PL              |
| F32-2   | M      | p.G239R             | p.G239R             | CL/CL              | F18     | F      | p.L151F          | p.L151F  | PL/PL              |
| F62     | M      | p.A80fsX91          | p.A80fsX91          | CL/CL              | F22     | F      | p.L151F          | p.L151F  | PL/PL              |
| F66     | M      | p.W237X             | p.W237X             | CL/CL              | F25     | M      | p.L151F          | p.L151F  | PL/PL              |
| F69-1   | M      | p.R216C             | Splice site         | CL/CL              | F29     | F      | p.L151F          | p.L151F  | PL/PL              |
| F69-2   | F      | p.R216C             | Splice site         | CL/CL              | F30     | F      | p.L151F          | p.L151F  | PL/PL              |
| F78-1   | M      | p.M71T <sup>a</sup> | p.M71T <sup>a</sup> | CL/CL              | F33     | M      | p.L151F          | p.L151F  | PL/PL              |
| F79-1   | F      | p.M71T <sup>a</sup> | p.M71T <sup>a</sup> | CL/CL              | F56     | F      | p.R114Q          | p.R114Q  | PL/PL              |
| F80-1   | F      | p.M71T <sup>a</sup> | p.M71T <sup>a</sup> | CL/CL              | F60     | F      | p.L151F          | p.A209T  | PL/PL              |
| F80-2   | F      | p.M71T <sup>a</sup> | p.M71T <sup>a</sup> | CL/CL              | F64     | F      | p.L151F          | p.S110R  | PL/PL              |
| F80-3   | M      | p.M71T <sup>a</sup> | p.M71T <sup>a</sup> | CL/CL              | F70     | F      | p.G227R          | p.G227R  | PL/PL              |
| F81-1   | F      | p.M71T <sup>a</sup> | p.M71T <sup>a</sup> | CL/CL              | F74-1   | M      | p.L151F          | p.L151F  | PL/PL              |
| F81-2   | M      | p.M71T <sup>a</sup> | p.M71T <sup>a</sup> | CL/CL              | F74-2   | F      | p.L151F          | p.L151F  | PL/PL              |
| F88-1   | M      | p.C131R             | p.C131R             | CL/CL              | F74-3   | M      | p.L151F          | p.L151F  | PL/PL              |
| F01-1   | F      | p.L151F             | p.W117X             | PL/CL              | F83-1   | F      | p.L151F          | p.L151F  | PL/PL              |
| F01-2   | F      | p.L151F             | p.W117X             | PL/CL              | F83-2   | F      | p.L151F          | p.L151F  | PL/PL              |
| F05-1   | F      | p.L151F             | p.L145P             | PL/CL              | F83-3   | M      | p.L151F          | p.L151F  | PL/PL              |
| F05-2   | F      | p.L151F             | p.L145P             | PL/CL              | F87-1   | M      | p.L151F          | p.L151F  | PL/PL              |
| F06     | M      | p.L151F             | p.G239R             | PL/CL              | F87-2   | M      | p.L151F          | p.L151F  | PL/PL              |
| F12     | M      | p.A209T             | p.L145P             | PL/CL              | F87-3   | M      | p.L151F          | p.L151F  | PL/PL              |
| F16     | F      | p.L151F             | Splice site         | PL/CL              | F34     | F      | p.R129C          | p.L151F  | PL/X               |
| F20     | M      | p.L151F             | p.L145P             | PL/CL              | F37-1   | M      | p.L151F          | ?        | PL/X               |
| F23     | M      | p.L151F             | p.S235P             | PL/CL              | F37-2   | M      | p.L151F          | ?        | PL/X               |
| F71     | M      | p.L151F             | p.L145P             | PL/CL              | F61-1   | M      | p.L151F          | ?        | PL/X               |
| F72     | F      | p.L151F             | p.I137del           | PL/CL              | F61-2   | F      | p.L151F          | ?        | PL/X               |
| F77     | M      | p.L151F             | p.G88Q              | PL/CL              | F19     | M      | Splice site      | ?        | CL/X               |

<sup>a</sup>Loss of translation start site.

placement therapy (41 *versus* 26%; Table 5). CL patients also reached ESRD earlier in life than PL patients. At the age of 15 yr, 46% of CL patients needed renal replacement therapy *versus* 20% of PL patients ( $P < 0.05$ ); these percentages further increased to 70% in CL and 25% in PL by the age of 20 yr (NS). We also analyzed the Mg and Ca metabolism, but it has to be kept in mind that varying amounts of supplementation with Mg and Ca salts at different stages of chronic renal failure may influence these results. The lowest serum Mg and Ca levels were low in both groups but did not differ significantly between the CL and PL groups. Mg excretion was high in both groups but without a significant difference. In contrast, the urinary Ca excretion was significantly higher in the CL group *versus* the PL group, both in 24-h urine collections and in spot urine samples. PTH levels were inadequately high in both groups when compared with control patients with chronic renal failure of other origin (Figure 6).

**DISCUSSION**

In contrast to most other tubular disorders, children affected by FHHNC are at high risk to develop progressive renal failure. Approximately one third of the patients reach ESRD already during adolescence, requiring renal replacement therapy. The pathogenesis of chronic renal failure in FHHNC still remains a matter of debate. The progressive tubulointerstitial nephropathy in FHHNC has been attributed to the concomitant hypercalciuria and nephrocalcinosis; however, other authors have questioned this explanation because many other disease states associated with early-onset nephrocalcinosis are not followed by a severe deterioration of renal function.<sup>25,26</sup> All patients with FHHNC are affected by marked medullary nephrocalcinosis, but their degree of renal insufficiency is varying to a great extent. The reason for this variation has been unknown. No obvious genotype/phenotype correlation could be established in



**Figure 4.** (A) Age at manifestation of first clinical symptoms. (B) Progression of renal failure expressed as loss of GFR (ml/min per 1.73 m<sup>2</sup>/yr). Data are means  $\pm$  SEM, ANOVA was performed only between the groups with categorized mutations on both alleles. \* $P < 0.05$ ; \*\* $P < 0.01$ .

the patient cohorts that had first been analyzed for *CLDN16* mutations<sup>2,9</sup>; however, the work of Hirano *et al.*<sup>20,27</sup> in 2000 describing two different large deletion mutations of *CLDN16* in Japanese Black cattle already pointed to a specific function of claudin-16 for tubular integrity: These animals present with chronic interstitial nephritis, diffuse zonal fibrosis, and death with renal failure.<sup>20</sup> Detailed pathoanatomic studies in these cattle characterized an abnormal nephron development with immature glomeruli and tubules.<sup>28</sup> This bovine knockout model suggests that the complete loss of claudin-16 is associated with a severe deterioration of renal function, whereas the heterozygous deletion remains asymptomatic. In humans, no large *CLDN16* gene deletions have been identified; however, the recently published work of Hou *et al.* demonstrated for the first time functional differences *in vitro* among the human *CLDN16* missense mutations identified so far. Twelve human missense mutations were transfected in a cellular system, and TJ localization and ion conductance were evaluated.<sup>22</sup> Seven of the mutants showed a CL (no TJ localization), four mutants showed a partial loss of function (TJ localization but reduced ion fluxes), and one mutant displayed a normal function comparable to the wild-type protein (F232C, not identified in our patient cohort). Mutants with residual function were correctly

expressed and targeted to TJ but showed a significant (but not complete) loss of function compared with wild-type claudin-16. In this study, we performed mutational analysis of *CLDN16* in 39 new patients with FHHNC and analyzed the time course of renal insufficiency in this cohort together with the 32 patients of our initial report.<sup>2</sup> After completing the expression studies by functional data for the new mutations, all 71 patients were stratified according to their mutation status for genotype/phenotype analysis.

The clinical course in patients bearing two mutations with CL (including also stop, frameshift, and splice-site mutations) is significantly worse compared with patients with at least one mutation with residual function (PL). Patients with CL present symptoms earlier in life and show a substantially faster decline of GFR. As a consequence, significantly more patients with two CL mutations had a GFR  $< 60$  ml/min per 1.73 m<sup>2</sup> at the end of the study or even needed renal replacement therapy, and ESRD was reached earlier in life than in the patients with PL of at least one allele.

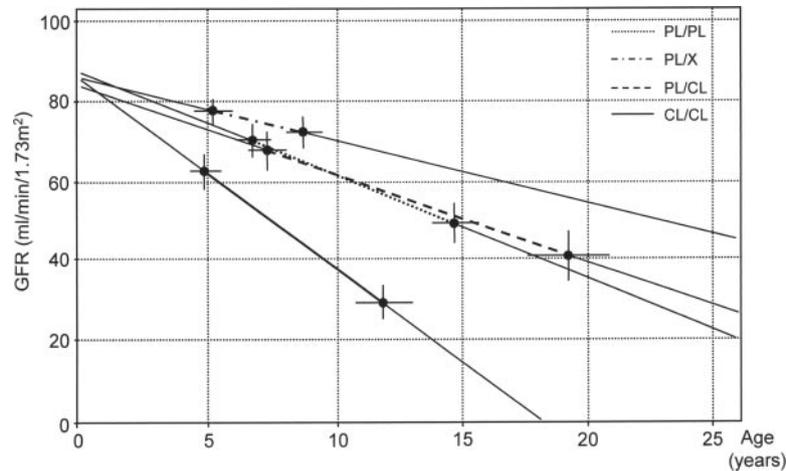
Whereas in the CL group most of the families exhibit a private *CLDN16* mutation, in the PL group, the majority of patients carry the L151F founder mutation on at least one mutant allele. Only four families have other mutations with PL on both mutant al-

**Table 4.** Genotype/phenotype correlation in FHHNC, subgroups separately

| Genotype  | CL/CL<br>(n = 23) | PL/PL<br>(n = 29)             | PL/CL<br>(n = 12)             | PL/X<br>(n = 5)  |
|---|-------------------|-------------------------------|-------------------------------|------------------|
| Age at onset (yr; mean [95% CI])                                | 2.2 (1.1 to 3.3)  | 5.7 (3.3 to 8.2) <sup>a</sup> | 4.9 (2.3 to 7.6)              | 5.9 (2.6 to 9.2) |
| Loss of GFR/yr (ml/min per 1.73 m <sup>2</sup> ; mean [95% CI]) | 7.3 (5.0 to 9.6)  | 3.3 (1.6 to 4.9) <sup>b</sup> | 3.0 (1.1 to 4.9) <sup>a</sup> | 2.9 (1.6 to 4.1) |
| Renal function at end of study                                  |                   |                               |                               |                  |
| GFR $> 60$ ml/min per 1.73 m <sup>2</sup>                       | 9% (2/22)         | 55% (16/29) <sup>b</sup>      | 5/12                          | 4/5              |
| GFR $< 60$ ml/min per 1.73 m <sup>2</sup>                       | 50% (11/22)       | 17% (5/29) <sup>b</sup>       | 3/12                          | 1/5              |
| ESRD  | 41% (9/22)        | 18% (8/29) <sup>b</sup>       | 4/12                          | 0/5              |
| ESRD at 15 yr   | 54% (7/13)        | 25% (5/20)                    | 1/10                          |                  |
| ESRD at 20 yr   | 70% (7/10)        | 36% (5/14)                    | 3/6                           |                  |

<sup>a</sup> $P < 0.05$  versus CL/CL.

<sup>b</sup> $P < 0.01$  versus CL/CL (ANOVA with Bonferroni multiple comparison test).



**Figure 5.** Progression of renal failure over time in FHHNC according to the mutation category. Data are means  $\pm$  SEM.

les. This raises the possibility that the less severe phenotype in the PL group depends on the L151F mutation. Even if this cannot be excluded, it seems unlikely because the expression studies did not reveal any functional peculiarity for L151F. Furthermore, the individual courses of loss of GFR in the four families without L151F in the PL group are comparable to the patients with L151F mutations with the two oldest patients having a normal GFR at young adult age (18 and 20 yr). The previously made observation of a close intrafamilial concordance with respect to the clinical course in the majority of the multiplex families supports the important role of distinct *CLDN16* genotypes for the progression of renal insufficiency.<sup>2</sup>

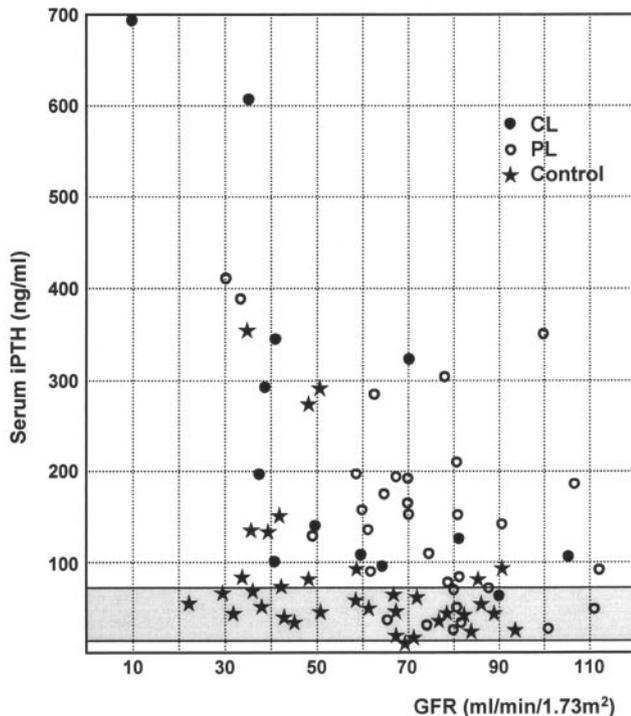
The handling of Mg and Ca in FHHNC was also analyzed in this study. As expected, we found profound hypomagnesemia and a tendency toward hypocalcemia, a phenomenon that is widely known in FHHNC. We also could confirm previous results showing increased PTH levels already before the onset of chronic renal failure<sup>3</sup>; however, we were unable to identify differences between patients with CL and PL. Increased PTH levels before chronic renal failure may be explained by the excessive loss of Ca with subsequent mobilization from bone. In

addition, hypomagnesemia is known to stimulate PTH secretion<sup>29</sup> except in cases of very severe hypomagnesemia, in which it rather seems to have an inhibitory effect. This phenomenon is often observed in patients with hypomagnesemia with secondary hypocalcemia. In these patients, PTH is frequently very low despite hypocalcemia,<sup>30</sup> but the Mg levels in hypomagnesemia with secondary hypocalcemia are far below the levels in FHHNC.

In contrast to serum Mg, Ca, and PTH, urinary Ca excretion in FHHNC seems to depend on the *CLDN16* genotype. CL patients excrete more Ca in the urine than PL patients. Whether this is a direct effect of the residual claudin-16 function is difficult to analyze. Assuming a residual claudin-16 function, as shown in the expression system, also in humans affected by these PL mutations, one would expect higher reabsorption rates for both Ca and Mg; however, Mg excretion did not differ between the groups, but we have to admit that either Mg excretion rates or Mg/creatinine ratios in the context of varying degrees of renal failure are questionable. Unfortunately, we could not calculate fractional excretion rates for Mg. The increased Ca excretion rates in CL patients also may be

**Table 5.** Genotype/phenotype correlation in FHHNC

| Genotype  | CL (n = 23)         | PL (n = 46)         | P     |
|---|---------------------|---------------------|-------|
| Age at onset (yr; mean [95% CI])                                | 2.2 (1.1 to 3.3)    | 5.6 (3.6 to 7.0)    | <0.01 |
| Loss of GFR/yr (ml/min per 1.73 m <sup>2</sup> ; mean [95% CI]) | 7.3 (5.0 to 9.6)    | 2.9 (1.8 to 4.3)    | <0.01 |
| Renal function at end of study                                  |                     |                     | <0.01 |
| GFR >60 ml/min per 1.73 m <sup>2</sup>                          | 9% (2/22)           | 54% (25/46)         |       |
| GFR <60 ml/min per 1.73 m <sup>2</sup>                          | 50% (11/22)         | 20% (9/46)          |       |
| ESRD  | 41% (9/22)          | 26% (12/46)         |       |
| ESRD at 15 yr   | 54% (7/13)          | 20% (6/30)          | <0.05 |
| ESRD at 20 yr   | 70% (7/10)          | 25% (8/20)          | NS    |
| Lowest serum Mg (mmol/L; mean [95% CI])                         | 0.40 (0.35 to 0.45) | 0.41 (0.38 to 0.45) | NS    |
| Urinary Mg excretion ( $\mu$ mol/kg per d; mean [95% CI])       | 188 (92 to 283)     | 153 (123 to 183)    | NS    |
| Lowest serum Ca (mmol/L; mean [95% CI])                         | 2.11 (1.96 to 2.27) | 2.17 (2.1 to 2.25)  | NS    |
| Urinary Ca excretion ( $\mu$ mol/kg per d; mean [95% CI])       | 314 (181 to 446)    | 200 (168 to 233)    | <0.01 |
| Urinary Ca/creatinine (mol/mol; mean [95% CI])                  | 2.46 (1.37 to 3.55) | 1.52 (1.26 to 1.79) | <0.05 |
| Intact PTH (pg/ml; mean [95% CI])                               | 249 (122 to 376)    | 169 (117 to 220)    | NS    |
| Follow-up time (yr; mean [95% CI])                              | 13.4 (9.2 to 17.6)  | 11.9 (9.2 to 14.7)  | NS    |



**Figure 6.** Serum intact PTH levels with corresponding GFR in patients with FHHNC and control subjects. Normal range is indicated in gray.

related to thiazide therapy, which has been demonstrated to reduce effectively Ca excretion in patients with FHHNC.<sup>31</sup> It might be possible that patients with FHHNC and PL mutations (who have better renal function) receive more thiazide than patients with advanced renal failure, which is more frequent in the CL group. The issue of tubular handling of Ca and Mg certainly deserves to be addressed in much more detail in further studies.

No phenotypical differences (including progression of renal failure) could be found between patients bearing two mutations with residual function and patients affected by one mutation with residual function and one mutation with CL on the second allele. These results are in accordance with the recessive nature of the disease: One mutation with residual function is sufficient to predict a milder clinical course. However, we cannot completely rule out that distinct mutations may exert different effects dependent on the genotype of the second allele (e.g., a dominant negative effect).

How can we explain ESRD in patients affected by mutations in *CLDN16*? Tubulointerstitial fibrosis, characterized by glomerulosclerosis, interstitial fibrosis, and tubular atrophy, is the final common pathway to ESRD.<sup>32–34</sup> Renal histology documented for 11 patients with FHHNC of our initial report demonstrated Ca deposits, glomerular sclerosis, immature glomeruli, tubular atrophy, and interstitial fibrosis to a variable extent. In bovine *CLDN16*-associated interstitial nephritis, tubular epithelial cells were reported as “immature” with loss of both polarization and attachment to the basement membrane.

A close association between fibrosis and abnormal tubules was noted, and the term “renal tubular dysplasia” was used to underline the lesion of the epithelial cells of the renal tubules.<sup>35</sup> These features are consistent with epithelial-to-mesenchymal transformation (EMT), a process of transforming tubular epithelial cells into new fibroblasts that are considered to play a major role in the progression of renal fibrosis. EMT is characterized by the disruption of epithelial junction complexes and the loss of cell polarity, transforming stationary epithelial cells into migratory mesenchymal fibroblast-like cells. These observations suggest that TJ disassembly could be an early event in EMT. Ikenouchi *et al.*<sup>36</sup> demonstrated that induction of EMT *in vitro* is associated with downregulation of claudins and occludin, major proteins of the TJ complex. Therefore, one might speculate that defective claudin-16 function could be an early event in disruption of the tubular TJ complex, followed by alterations of cell polarity and an induction of tubular dysplasia. Patients who have FHHNC and are affected by two loss-of-function mutations will therefore have an early onset of chronic renal failure as demonstrated in this study. Conversely, patients with at least one mutation with residual function (and localization of the protein to the TJ complex) are protected from rapid loss of renal function.

Recently, a second study related to the consequences of human *CLDN16* mutations was published.<sup>37</sup> That study focused on intracellular trafficking, and the comparison with the results of Hou *et al.*<sup>22</sup> in general nicely fit together; however, with respect to membrane localization, there were some differences. Most important, two of the mutations with residual function (L151F and G191R) could not be detected at the cell surface by Kausalya *et al.*<sup>37</sup> Moreover, one mutation (R216T), which was characterized as a trafficking mutant by Hou *et al.*,<sup>22</sup> was found to be targeted to TJ by Kausalya *et al.* With respect to electrophysiology, the two studies are not fully comparable because different cell lines were used. It seems that the residual function described by Hou *et al.* as an intermediate phenotype between control and wild-type claudin-16 with respect to the permeability ratio of Na<sup>+</sup> over Cl<sup>-</sup> and diffusion potential can be determined only in LLC-PK1 cells and not in MDCK-7 cells, which were used by Kausalya *et al.*<sup>37</sup> These differences are related to different transepithelial resistances: In LLC-PK1 cells, paracellular conductance greatly exceeds transcellular conductance as a result of the low transepithelial resistance.

We also analyzed the key criteria of this study (age at onset and loss of GFR) in our patients with respect to the various types of mutations defined by Kausalya *et al.*<sup>37</sup> and, accordingly, attributed the patients either to “normal expression at the cell surface” (indicated as “defective Mg transport” by Kausalya *et al.*<sup>37</sup>) or to “trafficking defects.” Age at onset and loss of GFR were nearly identical in both groups (4.3 versus 4.4 yr; loss of GFR 4.61 versus 4.63 ml/min per 1.73 m<sup>2</sup>/yr), suggesting that the stratification solely based on trafficking without electrophysiologic evidence of residual claudin-16 function is not useful for a genotype/phenotype correlation. This is further supported by the observation of additional mutations

that are correctly targeted to TJ but still display a CL (Table 2). Taken together, our *in vivo* data suggest that residual function of claudin-16 may considerably delay the progression of renal failure in FHHNC; therefore, genotyping in patients with FHHNC will allow the prediction of the clinical course to some extent and improve genetic counseling of affected families.

## CONCISE METHODS

### Patients and Families

Seventy-one patients who had FHHNC from 50 families were included in this study (40 females and 31 males). Clinical findings and molecular results of families F1 to F25 (25 families with 33 affected individuals) were reported previously.<sup>2</sup> The remaining patients (F29 to F88, 39 affected individuals from 26 families) were recruited since 2001 applying the same criteria as in our initial study: Hypomagnesemia (<0.65 mmol/L), hypercalciuria (as defined in the Clinical and Laboratory Data section), bilateral nephrocalcinosis, and the absence of hypokalemic metabolic alkalosis. Clinical data of F78, F80, and F81 were reported previously.<sup>38</sup>

Parental consanguinity was documented in 15 families; 17 families were multiplex with two or more affected individuals. The study was approved by the local ethics committee, and informed consent was obtained from the patients (whenever appropriate) and/or their parents.

### Clinical and Laboratory Data

Serum levels of Mg, Ca, creatinine, and PTH were analyzed using standard methods. Additional parameters evaluated included urinary excretion of Ca and Mg. Urinary Ca excretion of >100  $\mu\text{mol/kg}$  per 24 h was considered hypercalciuric for children who were older than 2 yr. The age-dependent upper reference values for Ca/creatinine and Mg/creatinine ratios in spot urine samples in younger children and infants were based on the reference values by Matos *et al.*<sup>39</sup> The control group for PTH levels with respect to GFR consisted of 35 individuals (aged 2.0 to 15.2 yr) with chronic renal failure without tubular disorder. The estimated GFR was calculated using the Schwartz formula.<sup>40</sup> The loss of GFR was calculated from first and last GFR:  $(\text{GFR}_{\text{first}} - \text{GFR}_{\text{last}}) / (\text{Age}_{\text{last}}\text{GFR}_{\text{first}} - \text{Age}_{\text{first}}\text{GFR}_{\text{last}})$ . For loss of GFR, only patients with a follow-up >1 yr were included.

### Mutation Analysis

*CLDN16* mutation analysis was performed in families F29 through F81 by single-strand conformation analysis and direct sequencing as described previously.<sup>41</sup> In all affected individuals in whom single-strand conformation analysis did not reveal both mutant alleles, *CLDN16* was completely sequenced (F37, F61, and F73). For all new mutations, at least 100 control chromosomes were analyzed. In families F37 and F61, haplotype analysis was performed as described previously.<sup>2</sup>

### Expression Studies

The full-length human claudin-16 (GenBank accession no. AF152101) was cloned into the retroviral vector pLNCX2. The site-

directed mutagenesis was performed with a PCR-based mutagenesis method (Stratagene, La Jolla, CA). Molecular clones for each mutant were verified by DNA sequencing. VSV-G-pseudotyped retroviruses were produced by transfection of the 293T cell line with pLNCX2-based constructs as described previously.<sup>22</sup> The retrovirus-containing supernatants were used for infection of MDCK and LLC-PK1 cells. The expression and localization of claudin-16 mutant proteins were verified by immunoblotting and immunostaining with anti-claudin-16 antibody (Zymed, San Francisco, CA). Electrophysiologic studies were performed on cell monolayers grown on porous filters (Transwell, Cambridge, MA) as described previously.<sup>22</sup>

### Statistical Analyses

The significance of difference between groups was determined by one-way ANOVA with Bonferroni multiple comparisons post test and by *t* test as appropriate; proportion of different groups (categorical variables) was compared by Cochran-Armitage exact trend test and Fisher exact test, respectively.<sup>42</sup> If not specified otherwise, results are expressed as mean with corresponding 95% CI. Two-sided tests were used throughout.

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## DISCLOSURES

None.

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