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Claudin-16 and claudin-19 function in the thick ascending limb

Jianghui Hou^a and Daniel A. Goodenough^b

^aWashington University School of Medicine, Division of Renal Diseases, St Louis, Missouri

^bDepartment of Cell Biology, Harvard Medical School, Boston, Massachusetts, USA

Abstract

Purpose of review—Claudin-16 and claudin-19 play a major role in the regulation of magnesium reabsorption in the thick ascending limb (TAL). This review describes recent findings of the physiological function of claudin-16 and claudin-19 underlying normal transport function for magnesium reabsorption in the TAL.

Recent findings—Mutations in the genes encoding the tight junction proteins claudin-16 and claudin-19 cause the inherited human renal disorder familial hypomagnesemia with hypercalciuria and nephrocalcinosis. The cation selectivity of the tight junction is vital for generating the lumen positive transepithelial potential in the TAL, which drives paracellular absorption of magnesium. Claudin-16 and claudin-19 require each other for assembly into tight junctions in the TAL. Heteromeric claudin-16 and claudin-19 interaction forms a cation selective tight junction paracellular channel. 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.

Summary—Epithelial paracellular channels are increasingly understood to be formed from claudin oligomeric complexes. In the mouse TAL, claudin-16 and claudin-19 cooperate to form cation-selective paracellular channels required for normal levels of magnesium reabsorption. Different subsets of the claudin family of tight junction proteins are found distributed throughout the nephron, and understanding their roles in paracellular ion transport will be fundamental to understanding renal ion homeostasis.

Keywords

claudin; hypomagnesemia; thick ascending limb; tight junction; transepithelial voltage

Introduction

The thick ascending limb (TAL) of the loop of Henle is responsible for reabsorbing 25–40% of filtered Na^+ [1], 50–60% of filtered Mg^{2+} [2] and 30–35% of filtered Ca^{2+} [3]. The dissociation of salt and water reabsorption in the TAL serves both to dilute the urine and to establish the corticomedullary osmolality gradient [4]. Active transcellular salt reabsorption results in a lumen-positive transepithelial voltage that drives passive paracellular reabsorption of divalent cations [5,6]. Claudins are the key components of the paracellular channel. The paracellular channels in the tight junction have properties of ion selectivity, pH dependence and anomalous mole fraction effects, similar to conventional transmembrane channels [7]. Genetic mutations in claudin-16 [8] and claudin-19 [9] cause an inherited

human renal disorder, familial hypomagnesemia with hypercalciuria and nephrocalcinosis (FHHNC).

Transcellular and paracellular reabsorption of Na⁺

In the TAL of Henle's loop, the epithelial cells form a water-impermeable barrier, actively transport Na⁺ and Cl⁻ via the transcellular route, and provide a paracellular pathway for the selective absorption of cations [5,6]. Na⁺, K⁺ and Cl⁻ enter the cell through the Na-K-2Cl cotransporter (NKCC2) in the luminal membrane. Na⁺ exits the cell through the Na⁺/K⁺-ATPase, in exchange for K⁺ entry. K⁺ is secreted into the lumen through the renal outer medullary potassium channel. Cl⁻ leaves the cell through the basolateral Cl⁻ channel, made up of two subunits, ClCKb and barttin. The polarized distribution of luminal K⁺ versus basolateral Cl⁻ conductance generates a spontaneous voltage source (V_{sp}) of +7–8mV [10], depending on active transcellular NaCl reabsorption (Fig. 1a). With continuous NaCl reabsorption along the axis of the TAL segment, the luminal fluid is diluted to 30–60mmol/l [11] and a large NaCl transepithelial chemical gradient develops at the end of the TAL. Because the paracellular permeability of the TAL is cation-selective (with a P_{Na}/P_{Cl} value between 2 and 4) [12,13], the diffusion voltage (V_{di}) is superimposed onto the active transport voltage (V_{sp}) and becomes the major source of the transepithelial voltage (V_{te}), which now increases up to +30mV [11–13] (Fig. 1b).

Mechanism of Mg²⁺ transport

Early in-vivo micropuncture studies have shown that approximately 50–60% of the filtered Mg²⁺ is reabsorbed in the TAL [2]. The flux–voltage relationship indicates that Mg²⁺ is passively reabsorbed from the lumen to the peritubular space through the paracellular pathway in this segment, driven by a lumen positive V_{te} [14,15]. V_{te} is made of the sum of V_{sp} and V_{di} . There are two prerequisites required for the paracellular Mg²⁺ reabsorption in the TAL: the lumen-positive V_{te} as the driving force and the paracellular permeability for the divalent cation Mg²⁺.

Claudin-16 and claudin-19 underlie familial hypercalciuric hypomagnesemia with nephrocalcinosis

FHHNC, OMIM #248250, is a rare autosomal recessive tubular disorder. As a consequence of excessive renal Mg²⁺ and Ca²⁺ wasting, patients develop the characteristic triad of hypomagnesemia, hypercalciuria and nephrocalcinosis [16–18]. Recurrent urinary tract infections and polyuria/polydipsia are frequent initial symptoms. Other clinical symptoms include nephrolithiasis, abdominal pain, convulsions, muscular tetany, and failure to thrive (Table 1). Additional laboratory findings include elevated serum parathyroid hormone levels before the onset of chronic renal failure, incomplete distal tubular acidosis, hypocitraturia, and hyperuricemia (Table 1). In contrast to hypomagnesemia and secondary hypocalcemia (HSH, OMIM #602014), FHHNC is generally complicated by end-stage renal failure in early childhood or adolescence.

Simon *et al.* [8] used the positional cloning strategy and identified claudin-16 (formerly known as paracellin-1), which is mutated in patients with FHHNC. Most mutations reported to date in claudin-16 are missense mutations clustering in the first extracellular loop composing the putative ion selectivity filter [19•]. Konrad *et al.* [9] have found mutations in another tight junction gene encoding claudin-19 from new cohorts of FHHNC patients (OMIM #248190). The renal tubular phenotypes are indistinguishable between patients with mutations in claudin-16 and those with mutations in claudin-19. Although claudin-16 and claudin-19 underlie FHHNC and paracellular Mg²⁺ reabsorption in the TAL, the transient

receptor potential channel melastatin 6 (TRPM6) regulates the apical entry of Mg^{2+} into the distal convoluted tubule epithelia [20]. Mutations in TRPM6 cause the HSH syndrome [21].

Claudin-16 is a cation channel

These above data suggested the hypothesis that claudin-16 and/or claudin-19 forms a selective paracellular Mg^{2+}/Ca^{2+} channel, which was tested in a number of in-vitro studies. Ikari *et al.* [22] transfected low-resistance Madin-Darby canine kidney (MDCK) cells with claudin-16 and reported that the Ca^{2+} flux in these cells was increased in the apical to basolateral direction, whereas the Ca^{2+} flux in the opposite direction remained unchanged. The Mg^{2+} flux was without any noticeable change. Kausalya *et al.* [23] transfected the high-resistance MDCK-C7 cell line and found that claudin-16 only moderately increased Mg^{2+} permeability without any directional preference. The effects of claudin-16 on Mg^{2+}/Ca^{2+} permeation appeared so small (<50%) that the Mg^{2+}/Ca^{2+} channel theory incompletely explains the dramatic effect of mutations in claudin-16 on Mg^{2+} and Ca^{2+} homeostasis in FHHNC patients.

In contrast to these studies, Hou *et al.* [24] transfected the anion-selective LLC-PK1 cell line with claudin-16 and found a large increase in Na^+ permeability (P_{Na}) accompanied by a moderately enhanced Mg^{2+} permeability (P_{Mg}). The permeability of claudin-16 to other alkali metal cations was found to be: $K^+ > Rb^+ > Na^+ \approx Li^+$. This sequence is quite different from the sequence of their free-solution mobilities and resembles the Eisenman selectivity sequence V–VIII. The Eisenman selectivity sequence reflects the electrostatic interaction strength (the Coulomb forces) in the claudin-16 paracellular channel and suggests that permeating cations have to dehydrate to enter the channel where negatively charged interaction sites stabilize the permeating cation [25,26]. The increase in P_{Na} was not affected by a Na^+/K^+ -ATPase inhibitor (1mmol/l ouabain) but was partially reduced or completely lost in all FHHNC relevant claudin-16 mutants (Table 2). In an elegant linkage study, Konrad *et al.* [27] had correlated the P_{Na} levels of claudin-16 mutations identified in FHHNC to the phenotypes of FHHNC patients. They found that the patients who had mutations resulting in complete loss of P_{Na} of both alleles were significantly younger at the onset of FHHNC symptoms than the patients who had one wild-type allele providing partial function. In addition, those patients with complete loss of P_{Na} had a more rapid decline in the glomerular filtration rate.

In general, functional studies of paracellular permeability have shown that there are at least two types of paracellular channels: a high-capacity pathway with an estimated pore radius of about 4 Å and a low-capacity pathway with a pore radius of at least 7 Å. The low-capacity pathway permits paracellular permeation on a slower timescale (minutes to hours) and may involve dynamic rearrangements of the tight junction structure [28]. In studies of claudin-2 expression in MDCK cells, paracellular permeability of the two types of paracellular channels was observed [29,30••]. The rapid, high-capacity paracellular channels show claudin-specific selectivity. The selectivity is mediated by the electrostatic interaction of partially dehydrated permeating ions with charged sites within the channel. It is generally accepted that the charges on the extracellular loops (ECL) of claudins line the channel and electrostatically influence passage of dehydrated ions [31,32]. The first ECL of claudin-16 is enriched with 10 negatively charged amino acids. Hou *et al.* [24] have systematically mutated each of the negatively charged amino acids to serine or threonine to study the effects of charge upon the function of claudin-16 in LLC-PK1 cells. A summary of the physiological changes resulting from these mutations is shown in Table 2. Mutational analysis identified a locus of acidic amino acids (D104S, D105S, E119T, D126S and E140T) that affected its cation selectivity and were interspersed with other acidic residues that had no effect. Mutation of each of the five functionally important residues had a modest effect (11–33% reduction in P_{Na}), and combining these mutations appeared to be additive

[24]. However, as emphasized by Yu *et al.* [30••], these residue replacements can influence protein structures that may have impacts on ion permeability independent of amino acid charge.

Heteromeric claudin-16 and claudin-19 interaction generates cation selectivity of the tight junction

Claudins interact with each other both intracellularly and intercellularly: they copolymerize linearly within the plasma membrane of the cell, together with the integral protein occludin, to form the classical intramembrane fibrils or strands visible in freeze-fracture replicas. These intramembrane interactions (side-to-side) can involve one claudin protein (homomeric or homopolymeric) or different claudins (heteromeric or heteropolymeric) [33]. In the formation of the intercellular junction, claudins may interact head-to-head with claudins in an adjacent cell, generating both homotypic and heterotypic claudin–claudin interactions [33]. Using the split-ubiquitin yeast 2-hybrid assay, Hou *et al.* [34] found strong claudin-16 and claudin-19 heteromeric interaction. The point mutations in claudin-16 (L145P, L151F, G191R, A209T, and F232C) or claudin-19 (L90P and G123R) that are known to cause human FHHNC disrupted the claudin-16 and claudin-19 heteromeric interaction. In mammalian cells such as the human embryonic kidney 293 cells, claudin-16 can be coimmunoprecipitated with claudin-19 [34]. Freeze-fracture replicas revealed the assembly of tight junction strands in L cells coexpressing claudin-16 and claudin-19, supporting their heteromeric interaction [34].

Coexpression of claudin-16 and claudin-19 in LLC-PK1 cells resulted in a dramatic upregulation of P_{Na} and down-regulation of P_{Cl} , generating a highly cation-selective paracellular pathway [34]. Certain FHHNC mutations in claudin-16 (L145P, L151F, G191R, A209T, and F232C) or claudin-19 (L90P and G123R) that disrupted their heteromeric interaction abolished this physiological change [34]. As claudin-16 colocalizes with claudin-19 in the TAL epithelia of the kidney [9], claudin-16 and claudin-19 association through heteromeric interactions confers cation selectivity to the tight junction in the TAL. Human FHHNC mutations in claudin-16 or claudin-19 that abolish the cation selectivity diminish the lumen-positive V_{di} as the driving force for Mg^{2+} and Ca^{2+} reabsorption, readily explaining the devastating phenotypes in FHHNC patients.

Claudin-16 is required for renal Mg^{2+} reabsorption

Hou *et al.* [35] generated claudin-16 deficient mouse models using lentiviral transgenesis of siRNA to knock down claudin-16 expression by more than 99% in mouse kidneys. Claudin-16 knockdown mice show significantly reduced plasma Mg^{2+} levels and excessive urinary excretions (approximately four-fold) of Mg^{2+} and Ca^{2+} . Calcium deposits are observed in the basement membranes of the medullary tubules and the interstitium in the kidney of claudin-16 knockdown mice. These phenotypes of claudin-16 knockdown mice recapitulate the symptoms in human FHHNC patients.

Claudin-16 is required for paracellular cation selectivity in the thick ascending limb

The paracellular reabsorption of Mg^{2+} and Ca^{2+} is driven by a lumen-positive V_{te} made up of two components: V_{sp} and V_{di} . When isolated TAL segments were perfused *ex vivo* with symmetrical NaCl solutions, there was no difference in V_{sp} between claudin-16 knockdown and wild-type mice, indicating V_{sp} was normal in claudin-16 knockdown [35]. Blocking the NKCC2 channel with furosemide (thus dissipating V_{sp}), the cation selectivity (P_{Na}/P_{Cl}) was significantly reduced from 3.1 ± 0.3 in wild type to 1.5 ± 0.1 in claudin-16 knockdown, resulting in the loss of V_{di} . When perfused with a NaCl gradient of 145mmol/l (bath) versus 30mmol/l (lumen), the resulting V_{di} was +18mV in wild type, but only +6.6mV in

claudin-16 knockdown [35]. Thus, the reduction in V_{di} accounted for a substantive loss of the driving force for Mg^{2+} and Ca^{2+} reabsorption.

Claudin-16 is required for paracellular Na^+ absorption in the thick ascending limb

Renal handling of Na^+ in claudin-16 knockdown mice is more complex. In the early TAL segment, the transcellular and paracellular pathways form a current loop in which the currents traversing the two pathways are of equal size but opposite direction [5–6]. Net luminal K^+ secretion and basolateral Cl^- absorption polarize the TAL epithelium and generate V_{sp} . As the paracellular pathway is cation selective ($P_{Na^+}/P_{Cl^-}=2-4$ [12,13]), the majority of the current driven by V_{sp} through the paracellular pathway is carried by Na^+ moving from the lumen to the interstitium (Fig. 1a). Hebert *et al.* [36] estimated that, for each Na^+ absorbed through the trans-cellular pathway, one Na^+ is absorbed through the paracellular pathway. With the loss of claudin-16 and the concomitant loss of paracellular cation selectivity, Na^+ absorption through the paracellular pathway is reduced.

In the late TAL segment, dilution of NaCl in the luminal space creates an increasing chemical transepithelial gradient; back diffusion of Na^+ through the cation-selective tight junction generates a lumen-positive V_{di} across the epithelium (Fig. 1b). The paracellular absorption of Na^+ will be diminished when V_{di} equals V_{sp} , and reversed when V_{di} exceeds V_{sp} . As an equilibrium potential, V_{di} blocks further Na^+ backleak into the lumen. Without claudin-16, V_{di} will be markedly reduced well below normal, providing a driving force for substantial Na^+ secretion. Indeed, claudin-16 knockdown mice had increased fractional excretion of Na^+ (FE_{Na}) and developed hypotension and secondary hyperaldosteronism [35]. The observed Na^+ and volume loss are consistent with human FHHNC phenotypes. For example, polyuria and polydipsia are the most frequently reported symptoms from FHHNC patients [16].

Claudin-19 deficient animals phenocopy claudin-16 deficient animals

By using the same transgenic siRNA strategies to developing the claudin-16 knockdown mouse lines, Hou *et al.* [37••] generated claudin-19 deficient knockdown mice. Claudin-19 knockdown mice also develop the FHHNC symptoms of reduced plasma Mg^{2+} levels and excessive renal wasting of Mg^{2+} and Ca^{2+} . Secondary hyperaldosteronism in claudin-19 knockdown indicates increases in TAL luminal NaCl concentration, similar to the findings of claudin-16 knockdown mice.

Claudin-16 and claudin-19 interaction is required for their assembly into tight junctions of the thick ascending limb

In vitro, claudin-16 and claudin-19 interact and form a cation-selective paracellular channel [34]. *In vivo*, either claudin knockdown animal model phenocopies the other, suggesting claudin-16 and claudin-19 interaction is a critical component of normal TAL tight junction function. Hou *et al.* [37••] have shown that siRNA knockdown of claudin-19 causes a loss of claudin-16 from tight junctions in the TAL without a decrease in claudin-16 expression level, whereas siRNA knockdown of claudin-16 produces a similar effect on claudin-19. Thus, knockdown of either claudin results in the loss of both claudins from the junctional structure, explaining the similarity between the phenotypes. In both mouse lines, claudin-10, claudin-18, occludin, and ZO-1, normal constituents of TAL tight junctions, remain correctly localized. Based on these data, Hou *et al.* [37••] proposed a three-stage hypothesis of claudin assembly in the TAL tight junction. First, claudins 16 and 19 *cis* associate within the plane of the membrane into dimers, or higher oligomeric states prior to junctional assembly. If this association does not occur, neither claudin can traffic correctly to the cell junction. This requirement is unique to the cells of the TAL, as the claudins will traffic to junctional structures in cultured transfected cell lines. Second, *trans* interactions between

claudins in adjacent cells take place. Third, additional *cis* interactions occur elaborating the tight junction strands. The intracellular location of the co-oligomerization of claudin-16 and claudin-19 is currently not known.

Conclusion

The knockdown of claudin-16 and claudin-19 in the mouse has highlighted a role of the paracellular pathway in kidney function. Accumulating evidence from both in-vitro and in-vivo studies indicates that the primary role of claudin-16 and claudin-19 is to cogenerate cation selectivity of the tight junction in the TAL underlying normal transport function for Mg^{2+} reabsorption in the TAL. However, the molecular nature of this pathway is far from clear. First, the oligomeric structure of the claudin-16 and claudin-19 complex is not known. Mitic *et al.* [38] have studied claudin oligomerization on the basis of claudin-4 expression in insect cells using the perfluorooctanoic acid (PFO) as detergent to solubilize claudin-4 oligomers revealing a hexameric quaternary structure for claudin-4. A similar study using PFO solubilization followed by sucrose gradient sedimentation might allow determination of the molecular structure for claudin-16 and claudin-19. Second, the molecular basis for the charge selectivity of the claudin-16 and claudin-19 heteromeric channel is not known. It is generally accepted that the charges on the first extracellular loops of claudins line the channel pores and electrostatically influence passage of soluble ions. In a preliminary study, Hou *et al.* [24] found that charge removal in the first extracellular loop of claudin-16 reduces its cation selectivity. However, claudin-19 shares limited homology with claudin-16 in this domain. In any case, the mutational analysis was performed on cells in culture in the absence of claudin-19 expression. The coordinated interaction of claudin-16 and claudin-19 *in vivo* suggests that meaningful conclusions about the molecular basis of charge selectivity will require studies on cells expressing both claudins. Third, the identity of the paracellular Mg^{2+} channel in the TAL is not known. Hou *et al.* [37••] have shown that the loss of neither claudin-16 nor claudin-19 affected the junctional localization of claudin-10 and claudin-18, normal constituents of TAL tight junctions, suggesting that claudin-10 and claudin-18 continue to participate in the barrier function of TAL tight junctions. The barrier function of claudins has been shown recently in human colon where aldosterone enhances Na^+ uptake by both inducing transcellular passage of sodium while decreasing the paracellular ion flux by increasing claudin-8 expression [39•]. Although cation selectivity is lost in the absence of claudin-16 and claudin-19, the TAL epithelia maintain high levels of paracellular permeability for cations such as Mg^{2+} . A reasonable hypothesis is that claudin-10 and/or claudin-18 constitute the paracellular Mg^{2+} channel.

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Additional references related to this topic can also be found in the Current World Literature section in this issue (p. 509).

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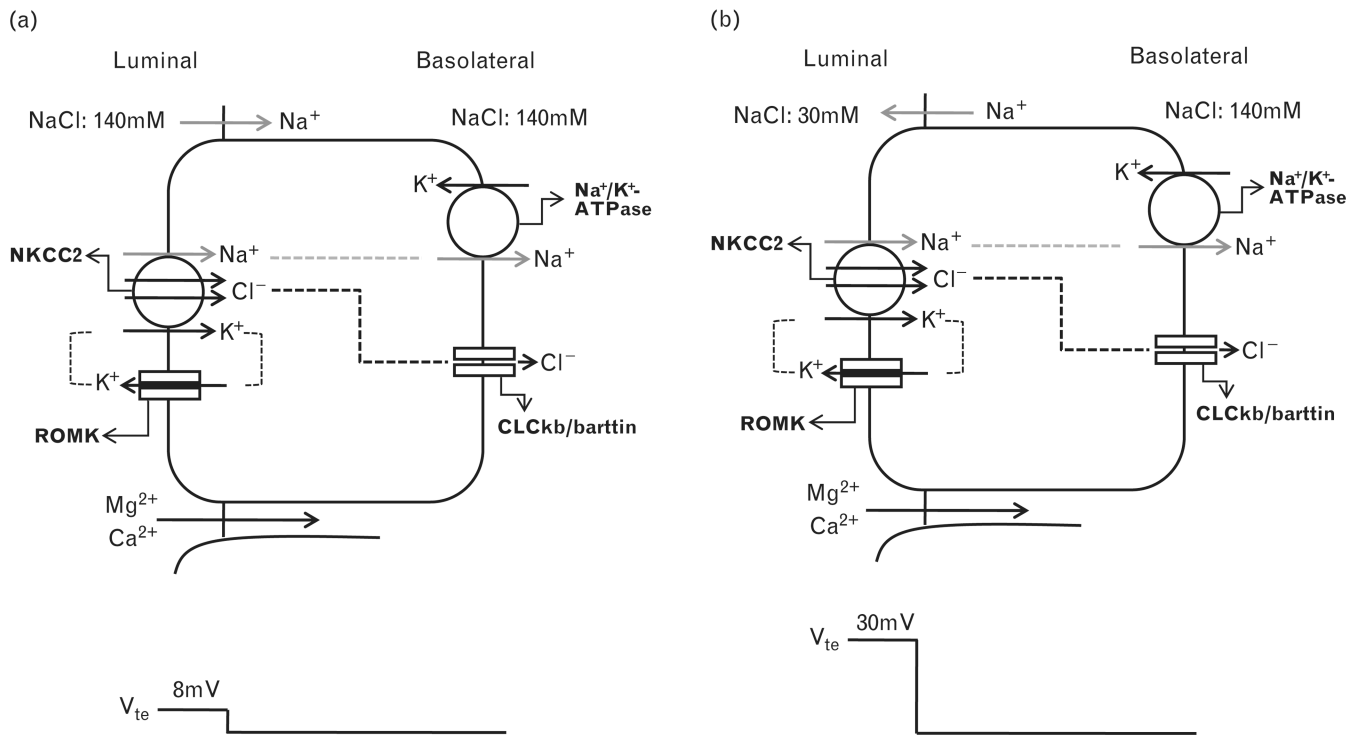


Figure 1. Trans epithelial electrogenic ion transport and generation of lumen-positive potential
 (a) When similar salt concentrations are present at the luminal and basolateral sides, the luminal spontaneous potential V_{sp} is generated by the concerted action of luminal K^+ channels, basolateral Cl^- channels, the $Na^+2Cl^-K^+$ cotransporter, and the Na^+,K^+ -ATPase. V_{sp} drives Na^+ absorption through the paracellular pathway. (b) When a dilute luminal fluid is present after $NaCl$ absorption along the water-tight TAL, the luminal potential is now generated as a diffusion voltage V_{di} by the 'backleak' of Na^+ . The diffusion voltage depends on the permselectivity of the tight junction. NKCC2, Na-K-Cl cotransporter; ROMK, renal outer medullary potassium.

Table 1

Clinical and biochemical abnormalities in familial hypomagnesemia with hypercalciuria and nephrocalcinosis patients

Symptoms	Frequency ^a (%)
Hypomagnesemia	100
Hypercalciuria	100
Nephrocalcinosis	100
Polyuria/polydipsia	90
Increased serum iPTH levels	88
Incomplete dRTA	85
Hypocitraturia	80
Urinary tract infection	69
Hyperuricemia	64
Muscular tetany	33
Nephrolithiasis	32

dRTA, distal renal tubular acidosis; iPTH, intact parathyroid hormone.

^a[16].

Table 2

Mutations affecting the function of claudin-16

Construct	Mutation	Localization	P_{Na} (10-6cm/s)	P_{Cl} (10-6cm/s)	Function
Vector	-	-	6.381 ± 0.107	21.857 ± 0.107	-
WT	-	TJ	25.750 ± 0.092	21.310 ± 0.092	+
D97S	Charge removal	ER	9.065 ± 0.530	34.633 ± 0.530	-
D104S	Charge removal	TJ	19.260 ± 0.206	31.723 ± 0.208	Partial -
D105S	Charge removal	TJ	22.683 ± 0.223	26.917 ± 0.223	Partial -
E108T	Charge removal	TJ	22.937 ± 0.203	21.830 ± 0.200	+
E119T	Charge removal	TJ	22.917 ± 0.101	28.070 ± 0.098	Partial -
D126S	Charge removal	TJ	18.563 ± 0.047	24.120 ± 0.050	Partial -
D132S	Charge removal	TJ	28.983 ± 0.497	22.003 ± 0.500	+
E133T	Charge removal	TJ	23.307 ± 0.103	22.583 ± 0.103	+
D135S	Charge removal	TJ	25.003 ± 0.053	20.880 ± 0.050	+
E140T	Charge removal	TJ	17.063 ± 0.165	24.650 ± 0.162	Partial -
L145P	FHHNC	TJ	13.977 ± 0.174	29.723 ± 0.174	Partial -
R149L	FHHNC	ER	7.830 ± 0.186	26.157 ± 0.187	-
L151F	FHHNC	TJ	17.107 ± 0.093	24.603 ± 0.093	Partial -
L167P	FHHNC	-	7.183 ± 0.038	27.450 ± 0.040	-
G191R	FHHNC	TJ	17.070 ± 0.192	21.980 ± 0.192	Partial -
G198D	FHHNC	-	6.656 ± 0.090	24.453 ± 0.091	-
A209T	FHHNC	TJ	20.513 ± 0.156	31.927 ± 0.156	Partial -
R216T	FHHNC	-	10.023 ± 0.159	29.873 ± 0.156	-
F232C	FHHNC	TJ	31.570 ± 0.070	31.713 ± 0.073	+
G233D	FHHNC	-	8.015 ± 0.106	28.690 ± 0.106	-
S235P	FHHNC	ER	7.273 ± 0.366	26.717 ± 0.364	-
G239R	FHHNC	Golgi	8.906 ± 0.094	23.297 ± 0.094	-

-, abolishing the function of claudin-16; +, showing the function of claudin-16; ECL, extracellular loop; ER, endoplasmic reticulum; FHHNC, familial hypomagnesemia with hypercalciuria and nephrocalcinosis; Golgi, Golgi apparatus; TJ, tight junction; TMD, transmembrane domain; WT, wild type.