Mutations in *CLDN2* Are Not a Common Cause of Pediatric Idiopathic Hypercalciuria in Canada

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Abstract

Background: Hypercalciuria is the most common risk factor for kidney stone formation, including in pediatric patients. However, the etiology is often unknown and children are frequently diagnosed with idiopathic hypercalciuria. Nearly 50% of children with hypercalciuria have a first-degree relative with kidney stones, suggesting a strong genetic basis for this disease. A failure of calcium reabsorption from the proximal nephron is implicated in the pathogenesis of hypercalciuria. Claudin-2 is a tight junction protein abundantly expressed in the proximal tubule. It confers paracellular permeability to calcium that is essential for transport across the proximal tubule where the majority of filtered calcium is reabsorbed.
Objective: Our objective was to examine the frequency of coding variations in *CLDN2* in a cohort of children with idiopathic hypercalciuria.
Design: Mixed method including retrospective chart review and patient interview, followed by genetic sequencing.
Setting: Three tertiary care centers in Canada.
Patients: Children (age 1-18 years) with idiopathic hypercalciuria. Patients with other causes of hypercalciuria were excluded.
Methods: Data were collected from 40 patients with idiopathic hypercalciuria. Informed consent to collect DNA was obtained from 13 patients, and the final and only coding exon of *CLDN2* was sequenced.
Results: The majority of patients were male, white, and had a positive family history of kidney stones. Parathyroid hormone levels were significantly lower than the reference range ($P < .001$). The levels of 1,25-dihydroxyvitamin D were also significantly higher in our patient cohort, relative to the reference range ($P < .001$). Sequence analysis of *CLDN2* did not identify any coding variations.
Limitations: Sequencing analysis was limited to the final coding exon and small sample size.
Conclusions: *CLDN2* coding variations are not a common cause of idiopathic hypercalciuria in Canadian children. Further study is needed to determine the causes of hypercalciuria in pediatric patients and develop targeted therapies.

Abrégé

Contexte: L'hypercalciurie est le facteur de risque le plus courant pour la formation de calculs rénaux, y compris chez les patients pédiatriques. Son étiologie est cependant souvent inconnue et les enfants sont fréquemment diagnostiqués avec une hypercalciurie idiopathique. Près de 50 % des enfants atteints d'hypercalciurie ont un parent de premier degré souffrant de calculs rénaux, ce qui suggère une importante contribution génétique à cette maladie. Une atteinte de la réabsorption du calcium au niveau du néphron proximal est impliquée dans la pathogenèse de l'hypercalciurie. La claudine-2, une protéine de jonction abondamment exprimée dans le tubule proximal, confère une perméabilité paracellulaire au calcium, laquelle est essentielle pour le transport à travers le tubule proximal, où la majorité du calcium filtré est réabsorbée.
Objectif: Étudier la fréquence des variations dans le codage de *CLDN2* dans une cohorte d'enfants atteints d'hypercalciurie idiopathique.
Conception de l'étude: Une méthode mixte, comprenant un examen rétrospectif des dossiers médicaux et un entretien avec les patients, suivie d'un séquençage génétique.
Cadre: Trois centres de soins tertiaires au Canada.
Sujets: Des enfants (1 à 18 ans) atteints d'hypercalciurie idiopathique. Les patients dont l'hypercalciurie avait une autre cause ont été exclus.
Méthodologie: Les données proviennent de 40 patients atteints d'hypercalciurie idiopathique. Le consentement éclairé à la collecte d'ADN a été obtenu pour treize patients. L'exon final et le seul exon codant pour *CLDN2*, a été séquencé.

Résultats: La majorité des sujets étaient des garçons d'origine caucasienne et avaient des antécédents familiaux de calculs rénaux. Les taux d'hormone parathyroïdienne étaient significativement plus faibles que les valeurs de référence (p < 0,001). Les taux de 1,25 dihydroxyvitamine D étaient significativement plus élevés dans notre cohorte de patients, par rapport à l'intervalle de référence (p < 0,001). Le séquençage de *CLDN2* n'a pas révélé de variations dans le codage.
Limites: L'étude porte sur un faible échantillon de patients et le séquençage s'est limité à l'exon final du gène.
Conclusion: Les mutations du gène *CLDN2* ne sont pas une cause fréquente d'hypercalciurie idiopathique chez les enfants canadiens. D'autres études sont nécessaires pour préciser la ou les causes de l'hypercalciurie chez les patients pédiatriques et développer des traitements ciblés.

Introduction

Kidney stone disease causes significant morbidity with a lifetime incidence of 10% in white males.[1-5] The cost of medical intervention and time lost from work is estimated at $10 billion per year in the United States alone.[2,6-8] Furthermore, the prevalence continues to increase across all age groups, including children.[6,9,10] The majority of renal calculi (85%) are composed primarily of calcium, most frequently coupled to oxalate.[2,5] The greatest risk factor for the development of kidney stones is hypercalciuria, which occurs in 40% to 50% of cases.[2,4]

Pediatric patients with hypercalciuria often present with heterogeneous, nonspecific symptoms, including recurrent episodes of gross hematuria, persistent microscopic hematuria, or abdominal pain, even in the absence of radiographic kidney stones.[2,10-12] Idiopathic hypercalciuria is also associated with lower urinary tract symptoms, including urinary frequency, dysuria, and enuresis, as well as recurrent urinary tract infections (UTIs).[11-15] Microcrystals and epithelial injury may impair clearance of bacteria from the lower genitourinary tract. Hypercalciuria can be caused by a number of conditions, including hypercalcemia, hyperparathyroidism, vitamin D intoxication, distal renal tubular acidosis, other genetic syndromes (e.g., Williams or Bartter syndromes), and by medications, including furosemide or prednisone.[2,10,16] However, the majority of patients do not have one of these diagnoses and instead are labeled as having idiopathic hypercalciuria.

A failure to reabsorb filtered calcium plays a critical role in the pathogenesis of hypercalciuria. Most calcium reabsorption occurs in the proximal tubule via passive paracellular transport. Claudins are tight junction proteins that form paracellular pores and barriers across epithelia of the body.[17-19] Different claudin isoforms exhibit different patterns of expression along the nephron. The localization of many of the claudins in the kidney has largely been determined, although their physiologic roles continue to be described. Their primary role is to regulate paracellular transport of small ions and several are known to be involved in calcium transport. Mutations in claudin-16 and claudin-19 are implicated in familial hypomagnesemia with hypercalciuria and nephrocalcinosis.[18,20-22] Altered claudin-14 expression likely also plays a role in hypercalciuria.[18,23-27]

Claudin-2 is expressed in the proximal nephron and intestine and is preferentially cation permeable.[28-31] Intestinal expression of claudin-2 enables the paracellular absorption of calcium.[30] Patients with idiopathic hypercalciuria have increased postprandial distal tubular delivery of sodium and calcium, consistent with reduced proximal reabsorption.[32] *CLDN2* knockout mice (*Cldn2^{-/-}*) have hypercalciuria,[33] and a family with missense *CLDN2* mutations has hypercalciuria and kidney stones. We thus hypothesized that mutations in *CLDN2* in children could also cause hypercalciuria and kidney stone formation. To determine whether mutations in *CLDN2* might cause hypercalciuria, we examined this gene in a cohort of Canadian children with idiopathic hypercalciuria and kidney stones.

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Methods

Ethics approval was obtained from the University of Alberta (Pro00018459), University of Saskatchewan (Bio 11-126), and University of Toronto (STUDY #: 1000039105). Children with idiopathic hypercalciuria were recruited from outpatient pediatric nephrology clinics at the Stollery Children's Hospital, Edmonton, Alberta; Jim Pattison Children's Hospital, Saskatoon, Saskatchewan; and The Hospital for Sick Children, Toronto, Ontario in Canada. Data were collected using retrospective chart review and patient interview. Hypercalciuria was defined as urine calcium:creatinine (Ca:Cr) ratio (mmol:mmol) >2.24 (<6 months), >1.68 (6-12 months), and >0.56 (1-18 years) with the age of the upper limit of urinary calcium in brackets. Patients with abnormal serum calcium, magnesium, phosphorus, or parathyroid hormone (PTH) were excluded. We also calculated PTH percentile (PTH %ile) based on the reported normal range of local laboratories to account for variations in local assays. We collected vitamin D levels, where available. Children with a genetic diagnosis of Bartter syndrome, Dent disease, distal renal tubular acidosis, or Williams syndrome were also excluded, as were those taking corticosteroids or furosemide. Medication data, including vitamin D supplementation, was collected, where available. The data collection form is available in supplementary data (Supplementary Figure 1).

Sequencing of *CLDN2* was performed with patient consent, as previously described.[23] In order to sequence *CLDN2*, genomic DNA was isolated from whole blood using the DNeasy Blood & Tissue Kit (Qiagen Inc, ON, Canada). We then amplified the final and coding exon of human *CLDN2* with polymerase chain reaction (PCR) promoters 5′-GCA AGA GCT TCA GCC TGA AGA CAA G-3′ and reverse 5′-CTG TTT TTC ACA GAC CCA GCC ACC-3′. After PCR clean up with the QIAquick PCR purification Kit (Qiagen Inc), the PCR product underwent Sanger sequencing at the Applied Genomics Core, Faculty of Medicine and Dentistry, University of Alberta. The sequence data were analyzed using pairwise sequence alignment software at https://www.ebi.ac.uk/Tools/psa/emboss_needle/.

All analyses were planned a priori and statistical analyses conducted using STATA version 12 (College Station, TX, USA). The data are expressed as mean ± standard deviation. Comparisons were made to local laboratory reference ranges using Wilcoxon signed-rank test, as appropriate, and *P* value ≤ .05 was considered statistically significant.

Results

We enrolled 40 children with idiopathic hypercalciuria. Patient characteristics are summarized in Table 1. The majority of patients were male (60.0%) and had a positive family history of kidney stones (51.3%). The average age at symptom onset was 5.6 ± 5.1 years. Ethnicity was not known in 50%; but of those identified, 75% (or 37.5% of the overall cohort) were white. The majority of patients had a history of kidney stones (87.5%), defined as either having previously passed a stone and/or radiographic evidence of stone formation. Stone analysis using urine microscopy was completed in 60% of the cohort. The most common kidney stone isolated was calcium oxalate (65.7%), followed by uric acid (20.0%) and calcium phosphate (14.3%).

The average urine Ca:Cr ratio was higher than the established normal range, stratified by age (Table 1). The majority of patients presented with hematuria (gross or microscopic) (Figure 1). Nearly 40% of the cohort had recurrent episodes of abdominal pain. Children presented with symptoms of dysuria (28%), recurrent UTIs (19%), or urinary frequency (13%).

All patients in our cohort had normal serum calcium, phosphorus, and magnesium levels in order to be diagnosed with idiopathic hypercalciuria. While all children had a normal PTH level, many patients had levels in the lower end of the laboratory-reported normal range. Therefore, we converted PTH levels to PTH %ile using the normal ranges reported by local laboratories. The mean PTH %ile value was 36.1 ± 23.6 (Table 2). The average 25-hydroxyvitamin D level was within normal limits, and no children had elevated 25-hydroxyvitamin D levels (>200 nmol/L). However, median 25-hydroxyvitamin D level was lower than median of the local laboratory range ($P < .001$). The average 1,25-dihydroxyvitamin D level was elevated at 201.6 pmol/L ± 102.7 (Table 2), and median 1,25-dihydroxyvitamin D level was higher than median of the local laboratory range ($P < .001$).

Sequence analysis of the final and only coding exon of *CLDN2* was performed for 13 patients in the cohort and revealed no single nucleotide variations (SNV) different to the reference sequence.

Discussion

We identified a pediatric cohort with isolated idiopathic hypercalciuria followed at three tertiary centers in Canada. The clinical characteristics of our cohort reflect that reported in the literature.[2,3,9] Patients were diagnosed after presenting with highly variable and nonspecific symptoms. The most common presenting feature was hematuria (including gross and persistent microscopic hematuria). A large proportion of patients presented with persistent abdominal pain and nonspecific urinary symptoms, including dysuria, frequency, urgency, and recurrent UTIs. The majority of patients were male and had a positive family history of kidney stones. The majority of patients had kidney stones, which were composed of calcium oxalate.

Claudin-2 plays an important role in paracellular calcium transport in the renal proximal tubule and colon, as demonstrated by genetic studies and mouse models.[30,32,33] *CLDN2* knockout mice have reduced calcium uptake from the colon and reduced renal calcium reabsorption, leading to hypercalciuria and kidney stones. A recent study also identified a *CLDN2* mutation in an Iranian family with hypercalciuria

Table 1. Patient Characteristics.

Age at symptoms onset (years), mean (SD)	5.6 (5.1)
Male, n (%)	24 (60.0%)
Ethnicity, n (%)	
Original peoples	1 (2.5%)
Black or African origin	1 (2.5%)
Asian	1 (2.5%)
White	15 (37.5%)
Middle Eastern	1 (2.5%)
East Indian	1 (2.5%)
Unknown	20 (50%)
History of renal calculi, n (%)	35 (87.5%)
Calcium phosphate	5 (14.3%)
Calcium oxalate	23 (65.7%)
Uric acid	7 (20.0%)
Family history of renal calculi, n (%)	20 (51.3%)
First degree relative	8 (25.0%)
Second degree relative	16 (50.0%)
Investigations at time of diagnosis, mean (SD)	
Serum calcium (mmol/L)	2.46 (0.08)
Normal range: 2.00-2.60 mmol/L	
Serum magnesium (mmol/L)	0.89 (0.17)
Normal range: 0.70-1.00 mmol/L	
Serum phosphate (mmol/L)	1.57 (0.25)
Normal range: 0.80-1.45 mmol/L	
Serum total CO_2 (mmol/L)	24 (3)
Normal range: 23-31 mmol/L	
Urine calcium:creatinine (mmol/mmol) for children >1 year[a]	1.13 (0.40)
Normal range: <0.56 mmol/mmol	
Urine citrate:creatinine (mmol/mmol)	0.23 (0.17)
Normal range: >0.1 mmol/mmol	
Urine magnesium:creatinine (mol/mol)	0.55 (0.42)
Normal range: <0.6 mol/mol	
Urine osmolarity (mmol/kg)	739 (185)
Normal range: 250-900 mmol/kg	
Urine oxalate:creatinine (mmol/mmol)	0.06 (0.04)
Normal range: <0.08 mmol/mmol	
Urine pH	6.5 (0.8)
Urine urate:creatinine (mol/mol)	0.50 (0.38)
Normal range: <0.56 mol/mol	

Note. Normal ranges for serum and urine investigations obtained from local lab ranges; if not available, normal ranges obtained from pediatric literature.[5]
[a]Urine Ca:Cr specifically at the time of first presentation was available for 33/40 children. Age at time of first presentation was >1 year (range: 1-15 years) in 30/33 children.

and kidney stones.[30] Despite its role in the pathogenesis of hypercalciuria, no patients in our cohort had any variation in the coding exon of *CLDN2* different to the reference sequence. This is the likely consequence of *CLDN2* not harboring any common variations, with the most common variation reported in *CLDN2* in the gnomAD browser occurring in less than 0.1% of alleles. It does not appear, therefore, that *CLDN2* mutations are a common cause of idiopathic hypercalciuria in Canadian children.

Targeted genetic studies of kidney stone cohorts from stone clinics have previously been performed in order to identify pathogenic mutations.[34-36] Causative mutations were identified in 10% to 30% of the studied cohorts, providing clear evidence that pathogenic mutations are highly prevalent. Identifying heritable causes of kidney stones is also important for prognostic purposes, tailored therapeutics, and potential screening of asymptomatic family members. Although these studies have examined genes in the claudin family (including *CLDN16* and *CLDN19*), the genes studied have been heterogeneous and did not include *CLDN2*. Therefore, our study, despite being restricted to a single candidate gene and carried out in a smaller cohort, contributes to the existing literature, but should be repeated in larger cohorts.
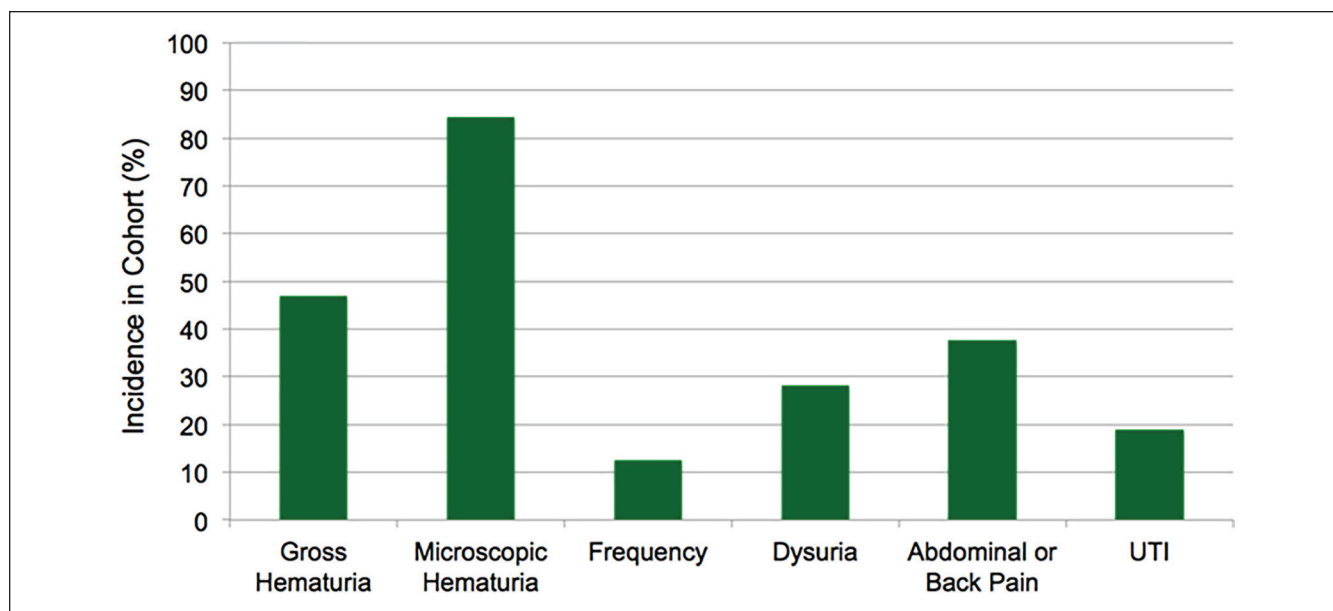
Figure 1. Symptoms at initial presentation.
Note. Incidence of urinary symptoms at initial visit with nephrology for assessment in pediatric cohort. UTI = urinary tract infection.

Table 2. PTH and Vitamin D Levels.

	Hypercalciuric cohort[a]		
	Mean (SD)	Median (IQR)	Normal range
PTH %ile	36.1 (23.6)	29.6 (36.2)*	—
25-hydroxyvitamin D (nmol/L)	80.1 (27.3)	83.0 (36.0)*	80-200
1,25-dihydroxyvitamin D (pmol/L)	201.6 (102.7)	160.0 (90.3)*	43-168

Note. Normal range for local laboratory included for reference. PTH %ile = parathyroid hormone percentile (based on reported normal range by local laboratory); IQR = interquartile range.
[a]Of the hypercalciuric cohort (N = 40), 39 children had available PTH measurement, 33 had 25-hydroxyvitamin D measurement, and 20 had 1,25-dihydroxyvitamin D measurement.
*$P < .001$.

PTH and vitamin D are key regulators of calcium homeostasis.[16] Vitamin D supplementation has been associated with increased calcium uptake from the gastrointestinal tract, leading to hypercalcemia, hypercalciuria, and kidney stones.[37] Meta-analyses have demonstrated a clear association between vitamin D supplementation and hypercalciuria and stone formation in adults and children.[38,39] Several studies have demonstrated elevated 1,25-dihydroxyvitamin D levels in adults with idiopathic hypercalciuria.[40] In children, *CYP24A1* mutation has been shown to cause idiopathic infantile hypercalcemia through deficiency of the enzyme 25-hydroxyvitamin D 24-hydroxylase, which degrades 1,25-dihydroxyvitamin D.[41] In our cohort, PTH levels were within the normal range (as per our inclusion criteria); however, PTH %ile was significantly lower than the median of the local laboratory reference range ($P < .001$). No children had elevated 25-hydroxyvitamin D levels (>200 nmol/L); however, median 25-hydroxyvitamin D levels were significantly lower, relative to the local laboratory reference range. Average 1,25-dihydroxyvitamin D levels were high and median levels were significantly higher, relative to the local laboratory reference range. These finding suggest that abnormal PTH and vitamin D levels may contribute to hypercalciuria and kidney stone formation in children. However, further studies in larger cohorts are needed.

Our study has several limitations. As we only examined the final coding exon, we cannot exclude mutations in the noncoding exons or intronic regions causing hypercalciuria in our patients. Our study population was relatively homogeneous; of the patients where the ethnicity was known, 75% were white. Thus, we cannot conclude that mutations in *CLDN2* may be a cause of hypercalciuria in populations that are not predominantly white. Our study also included patients with clinical criteria of hypercalciuria, but without a first-degree family history of nephrocalcinosis or kidney stones, which likely reduced the sensitivity of screening for

candidate genes causing hypercalciuria. However, we felt that reporting of family history was unlikely to be reliable, as often only one parent accompanied their child to appointments. Furthermore, as confirmed by our study findings, hypercalciuria is often associated with a nonspecific presentation and delayed diagnosis. It is possible that elevated urine Ca:Cr levels could be related to low muscle mass and low urinary creatinine excretion. Although we did not specifically assess the muscle mass of children in our cohort, we inquired as to whether children had significant comorbidities including failure to thrive or short stature, which were not reported in any patients. Most of our cohort also had complications of hypercalciuria, which would reduce the likelihood of false positives. Finally, while our study is fairly large by pediatric standards, we are still limited by the small number of patients and controls. Future studies are needed with larger cohorts to confirm our findings that *CLDN2* mutations are rare in children with idiopathic hypercalciuria.

Conclusions

We described a cohort of children with idiopathic hypercalciuria. Our patients had significant morbidity, including hematuria, urinary symptoms, abdominal pain, and kidney stones (either on history or radiographic evidence). Coding *CLDN2* mutations do not appear to be a common cause of idiopathic hypercalciuria in children in Canada. The role of other claudins as a cause of hypercalciuria is still being examined, with further studies needed.

Ethics Approval and Consent to Participate

Ethics approval was obtained from Research Ethics Board at each of the participating institutions: University of Alberta (Pro00018459), University of Saskatchewan (Bio 11-126), and University of Toronto (STUDY #: 1000039105).

Consent for Publication

Written informed consent for publication was also obtained during study visits (from legal guardian for children <18 years).

Availability of Data and Materials

Raw sequencing data was generated at the University of Alberta. Derived data supporting the findings of this study are available from the corresponding author [RTA] on request.

Declaration of Conflicting Interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: RTA work closely with Dr Hartwig as a member of the KRESCENT leadership team (she is a handling editor).

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This

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Supplemental Material

Supplemental material for this article is available online.

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