

Expression Profiles of CLDN2, CLDN14, and CaSR Genes in Idiopathic Calcium Oxalate Kidney Stones Patients of South Punjab, Pakistan

Asya¹, Tahir Maqbool^{2*}, Ovais Murid³, Zahra Haq⁴, Muhammad Umair Rafiq⁵, Momna Tariq⁵, Sami ur Rehman⁶, Maria Muddassir⁷, Awais Altaf²

¹ Department of Anatomy, Quaid-e-Azam Medical College, Bahawalpur

² Institute of Molecular Biology and Biotechnology, The University of Lahore

³ Department of Urology, Kidney Centre, Bahawalpur

⁴ Department of Physiology, Shahida Islam Medical and Dental College, Lodhran

⁵ Gujranwala Medical College/GMC Gujranwala, Pakistan

⁶ Sir Ganga Ram Hospital, Fatima Jinnah Medical University Lahore

⁷ M Islam Medical & Dental College, Gujranwala

ABSTRACT

Background: Kidney stone disease is a prevalent and multifactorial condition with mechanisms that remain poorly understood. Idiopathic calcium oxalate urolithiasis (ICOU) is characterized by calcium stone formation despite normal serum calcium levels. This study investigated the expression profiles of CLDN14, CLDN2, and CaSR genes in patients with ICOU from South Punjab, Pakistan. **Methods:** A case-control study was conducted involving 80 participants: 40 patients diagnosed with idiopathic calcium oxalate stones and 40 healthy controls selected through non-probability sampling. Gene expression was analyzed using polymerase chain reaction (PCR) techniques, and data were statistically evaluated using GraphPad Prism 8, with $p < 0.05$ considered significant. **Results:** The mean age of participants was 39 years, with males comprising 72% and females 27.5% of the cohort. A positive family history of nephrolithiasis was observed in 23.7% of cases. Significant differences were noted in biochemical parameters, including serum creatinine and 24-hour urinary calcium, between cases and controls. Molecular analysis revealed markedly increased expression of CLDN14 and CaSR in patients compared to healthy individuals, indicating their involvement in stone pathogenesis. **Conclusion:** The altered expression of CaSR, CLDN14, and CLDN2 genes in patients with idiopathic calcium oxalate urolithiasis suggests their potential role in the genetic susceptibility and molecular mechanisms underlying calcium-based nephrolithiasis in this population.

Keywords: Claudin 14, Casr, Idiopathic Calcium Oxalate Urolithiasis (ICOU), Kidney Stones

INTRODUCTION

The prevalence of kidney stone disease varies across regions, affecting approximately 7–13% of individuals in the United States, 5–9% in Europe, 1–5% in Asia, and reaching 14.8% in Turkey, according to a multicenter study [1]. A key characteristic of this condition globally is its high recurrence rate, with around 50% of patients developing stones again, and future projections suggest this could rise to 80–90% due to environmental factors such as global warming, humidity, and socioeconomic conditions [2]. Over the past five decades, both the incidence and prevalence of kidney stones have increased in Pakistan. Besides causing symptoms like pain, hematuria, and fever, untreated cases may progress to chronic kidney disease [3]. The disease often recurs and may also reflect underlying systemic disorders such as diabetes mellitus, hypertension, and obesity. Both genetic predisposition and environmental influences contribute to its pathogenesis [4]. Kidney stones frequently form as a result of idiopathic hypercalciuria, a metabolic abnormality characterized by elevated calcium excretion in the urine [5]. This calcium loss is influenced by a person's age and dietary habits, making it difficult to define a universal threshold. However, calcium excretion above 4 mg/kg or 0.1 mmol/kg over 24 hours is commonly considered excessive [6]. More specifically, hypercalciuria is diagnosed when urinary calcium excretion exceeds 6.2 mmol/day in women and 7.5 mmol/day in men [7]. Typically, the kidneys reabsorb about 98% of filtered calcium through both transcellular and paracellular

mechanisms. Claudins (CLDNs) are transmembrane proteins, 21–28 kDa in size, that serve both as barriers and pores in the paracellular pathways of epithelial cells [8]. They contribute to salt and water reabsorption in the proximal tubules, regulate calcium and magnesium transport in the thick ascending limb, and form cation barriers in distal nephron segments. Claudin-2, in particular, is abundantly expressed in proximal renal tubular epithelial cells (RTECs) and facilitates paracellular calcium transport. Research indicates that deletion of the CLDN2 gene results in hypercalciuria [9]. Another key protein, the calcium-sensing receptor (CaSR), located in the membranes of renal tubule cells, detects elevated extracellular calcium levels [10]. Upon stimulation by increased calcium concentration in the interstitial fluid, CaSR suppresses calcium reabsorption in the ascending limb of the loop of Henle [11]. Renal regulation of calcium excretion is crucial for maintaining stable serum calcium levels. Disruptions in this process can cause hypercalciuria and promote calcium-containing stone formation [12]. Increased expression of CLDN14 in the cortical thick ascending limb (TAL) impairs calcium reabsorption by reducing pore permeability and limiting sodium backflow, thereby diminishing the electrochemical gradient necessary for calcium transport [13]. Tight junctions are fundamental in modulating paracellular permeability, and the diverse expression patterns of their components likely reflect structural adaptations essential for nephron function in fluid and

electrolyte regulation [14]. Despite the known roles of claudins and CaSR, large-scale studies examining their expression in patients with idiopathic calcium oxalate stones are limited. This study aims to assess and compare the expression of CLDN gene family members (CLDN2, CLDN14) and CaSR in patients with idiopathic calcium oxalate kidney stones and in controls presenting to our clinic with complaints other than kidney stones.

MATERIAL AND METHODS

A case-control study was conducted at the Institute of Molecular Biology and Biotechnology, University of Lahore, with ethical approval number UOL/IREB/24/03/0032

Sampling Criteria

Sample size was calculated by using the OpenEpi calculator. A total of 80 patients were enrolled in the study divided into a diagnosed with idiopathic calcium oxalate stones group of 40 patients (D) and 40 healthy individuals as a control group (C) (1:1). A convenient non-probability sampling technique was employed to select the study participants. The inclusion criteria for the patient group were aged >18 years, indication for surgery for calcium oxalate calculi, while the inclusion criteria for the control group were aged >18 years, no history of urinary system calculi and indication for surgery for reasons other than renal calculi. Exclusion criteria were pregnant patients, patients with incomplete medical records or missing crucial information necessary for the study, patients with renal stones due to secondary causes such as metabolic disorders or anatomical abnormalities or taking diuretics and steroids or not willing to participate.

Sample Collection

Venous blood samples were taken aseptically from all study subjects. For this, a five (5) ml blood sample was collected through venipuncture and distributed equally in two (02) customized blood collecting tubes, with and without anti-coagulant such as ethylenediaminetetraacetic acid (EDTA). These samples were transported under cold conditions to the laboratory at IMBB, The University of Lahore. The tubes containing the coagulated blood sample were centrifuged at 5000 rpm for 10-15 minutes. Supernatant plasma was pipetted out and stored at -20°C for later use.

Demographic Data Collection

Anthropometric measurements (height and weight), Gender, age, body mass index (BMI), comorbidities, presence or absence of a history of stones, and sociodemographic data of the individuals in the patient and control groups were collected by a self-made Performa. Plain-film kidney, ureter, and bladder radiography (KUB) and abdominal ultrasonography were performed.

Biochemical Parameters

Blood was collected in sterile vacutainers from each patient using EDTA and/or heparin as preservative. Blood urea was estimated by urease (calorimetric), and serum creatinine was estimated by an enzymatic method (IFCC-IDMS). Serum calcium was estimated by Direct-ISE using VITROS® 5600 Integrated System (Ortho-Clinical Diagnostics, NJ, US).

Enzyme-Linked Immunosorbent Assay

Serum samples were added to microplate wells containing specific antibodies. Incubation is done to allow protein binding. Unbound

components were washed off. An enzyme-linked protein-specific detection antibody was added, followed by a chromogenic substrate. Protein levels were quantified by spectrophotometric measurement of color change.

DNA Extraction

DNA was extracted using a blood genomic DNA extraction kit (QIAAMP DNA mini kit, Qiagen, Batch no. 166030545, Biogene pvt. Ltd., Germany) according to the manufacturer's guidelines. DNA was extracted from five milliliters of anticoagulated whole blood and stored at 20°C.

PCR Amplification

Conventional PCR was performed with 100 ng purified genomic DNA, 0.5 µM of CLDN14 P1 and CLDN2 P2 primers; P1 Forward sense (5'AACAAGTACCCCGGTGGCG 3'), and P2 Antisense (5' ATGGCGCTGAGGCGCGCTC 3'), and P3 forward sense 5'CCACCTGCGCAAGCT 3'. The reaction mixture contained (200 µM of dNTP mixture, 1 M betaine, 2mM MgCl₂, 2.5 U Taq DNA polymerase (Bio Quest), and reaction buffer in a 50-µl reaction volume. Each PCR reaction mixture comprised of 100ng (almost 2 µL) of DNA, 0.8 µL of both primers (10 pmol/µL each), 8 µL master mix, and 5 µL of distilled water. The amplification was initiated at 95°C for 2 minutes, followed by 45 cycles of 95°C for 30 seconds, 68°C for 30 seconds, and 72°C for 30 seconds, and then a final extension at 72°C for 7 minutes [13].

Statistical Analysis

Statistical analysis was performed using the GraphPad Prism 8 software. The numerical variables were presented as means with standard deviation, while categorical variables were presented as frequency and percentage. An independent t-test was used to find out the association of variables in cases and controls. $p < 0.05$ was considered statistically significant.

Results

Demographic and Biochemical Analysis

Current study enrolled 80 participants with an equal distribution of 40 diseased and 40 control individuals. The mean age with standard deviation of study participants was 39.93 ± 11.37 years (range 18–65 years) with no significant difference between the cases and the controls. A slightly higher proportion of males 58 (72%) compared to females 22 (27.5%) was noted, with most participants (78.8%) residing in rural areas of Bahawalpur Division. Family history patterns revealed that 23.7% of the subjects had a family history of kidney stones. Lifestyle data showed that 11.5 % of the male participants were smokers, while alcohol consumption was absent in all subjects. In the control group (C) BMI was between 18 - 22.9 in males (normal) and 19.5-22.9 (normal) in females. The group (D) BMI was between 27.5-32.0 (pre-obese/ obese class 1) in males and 30.0 and 33.7 (obese class 1) in females. Of the biochemical variables mean hemoglobin level was 10.8 ± 1.87 g/dL, indicating mild anemia likely due to chronic inflammation or early renal insufficiency. Serum creatinine and 24 hours urinary calcium excretion showed significant difference between case and control groups although the serum calcium had no significant difference between two groups. The graph shows a significant increase in CRP levels in the diseased groups, as compare to the control group. The characteristics of study participants are mentioned in Table 1.

Table 1: Characteristics of Study Participants

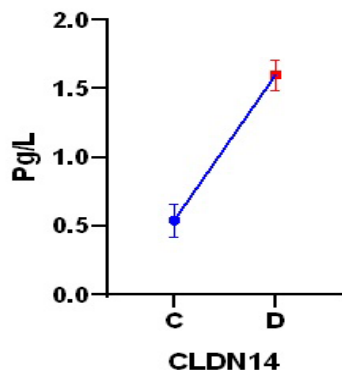
Variables	Patients (N=40)	Controls (N=40)	P-Value
Age (years)	39.9+ ₋ 11.3	38.13+ ₋ 16	0.1249
Gender			
Male	28	12	
Female	30	10	
BMI > 25kg/m ² Above	29.3 ± 1.78	19.63 ± 0.8	***
Hb	10.80 ± 1.87	14.76 ± 0.53	**
Sr calcium (mmol/L)	9.42+ ₋ 0.32	9.46+ ₋ 0.238	NS
Ur ca+ (mmol/24h)	7.87+ ₋ 0.59	4.04+ ₋ 0.68	**
Ur RBC	++++	negative	NS
Urea	55.4 ± 2.5	26.2 ± 4.7	**
Creatinine (mg/dL)	1.41 ± 0.42	0.62 ± 0.23	**
Spot urine PH	5.72+ ₋ 0.31	5.79+ ₋ 0.55	NS
CRP (mg/L)	5.54 ± 0.4	7.56 ± 1.6	

Protein Expression Analysis

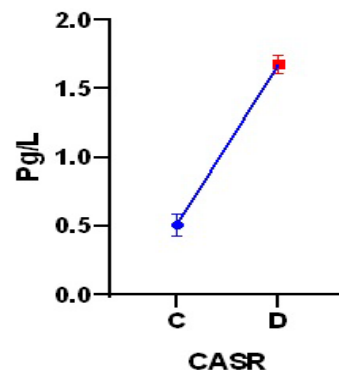
Protein quantification via ELISA demonstrated markedly higher levels in patients, as the mean with standard deviation of CLDN14 in cases was 1.714 ± 1.936 while in controls it was 0.6 ± 1.71 , likewise, CaSR in cases were 1.72 ± 0.1492 while in control were $0.538 \pm$

0.9365 , similarly CLDN 2 in cases were 1.658 ± 0.2425 and 0.602 ± 0.1307 . CLDN 2 was reduced to 100 pg/dL (versus 200 pc/dL in controls), CLDN 14 increased to 230 pg/dL (versus 100 pg/dL), and CaSR to 200 pc/dL (versus 110 pg/dL). The results were strongly significant as presented in Figure 1.

Protein Expression Analysis



Protein Expression Analysis



Protein Expression Analysis

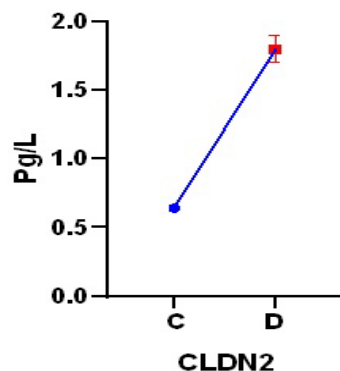


Figure 1: CLDN14, CaSR, and CLDN2 Protein Expression in the Study Groups. Representative Images or Quantitative Analysis Showing the Expression Levels of Calcium-Sensing Receptor (CaSR), Claudin-14 (CLDN14), and Claudin-2 (CLDN2) Proteins in Control (C) And Diseased (D) Groups. The Control Group (C) Represents Healthy Individuals without Kidney Stones, while the Diseased Group (D) Includes Subjects Diagnosed with Nephrolithiasis. Differences in Protein Expression Indicate the Potential Involvement of CaSR And CLDN2 in the Pathophysiology of Kidney Stone Formation.

Gene Expression Analysis

Molecular assessments of gene expression and protein quantification presented compelling evidence supporting claudin genes as a pivotal mechanism in idiopathic calcium oxalate

urolithiasis. Real-time PCR results revealed significantly increased gene expression of CLDN 14, CLDN 2, and CaSR in diseased individuals compared to healthy controls. Graphical presentation of gene expression is shown in Figure 2.

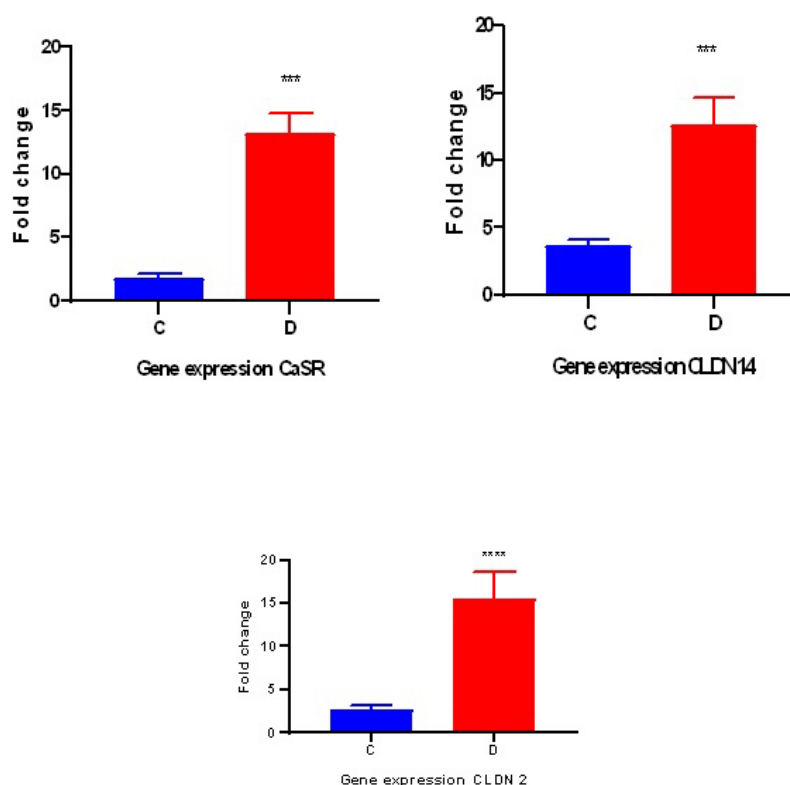


FIGURE 2: Relative Gene Expression Levels of CaSR, CLDN14, and CLDN2 in the Study Groups. The Figure Illustrates the Mrna Expression Patterns of Calcium-Sensing Receptor (CaSR), Claudin-14 (CLDN14), and Claudin-2 (CLDN2) Genes in Control (C) and Diseased (D) Groups. The Control Group (C) Represents Healthy Individuals Without Kidney Stones, Whereas the Diseased Group (D) Includes Subjects Diagnosed with Nephrolithiasis. Variations in Gene Expression Suggest the Possible Regulatory Role of these Genes in Calcium Homeostasis and Kidney Stone Formation.

Discussion

The present study reveals a higher occurrence of kidney stones in males (72%) compared to females (27.5%), which aligns with findings from various regions in Pakistan, such as Rawalpindi (56.65%), Lahore (54%), and Karachi (62.3%) [15, 16]. Additionally, 23.7% of individuals with a family history of kidney stones were found to have the condition. Previous studies conducted in Pakistan also observed a strong link between family history and the development of kidney stones [15, 17, 18]. It is postulated that obesity may increase the risk of kidney stones by increasing the excretion of urinary calcium, uric acid, and other components of the urine, which in turn increases the risk of kidney stones [19]. The BMI in the current study participants was high in 92.7% cases ($\geq 25 \text{ kg/m}^2$). Out of them, 34.3% were males and 27.4% were females, but this was not significant among cases and controls, while the mean with standard deviation was $22.11 \pm 1.19 \text{ kg/m}^2$, which is favored by Guha et al. [20] ($22.11 \pm 1.19 \text{ kg/m}^2$). The current study revealed an increased incidence of renal stones among individuals who were classified as overweight, as well as among those with a lower socioeconomic and educational status and residing in rural areas. These trends are consistent with observations in other studies [16, 21]. In the current study population, renal stones were found to be more prevalent among individuals who were normotensive, non-diabetic, and non-smokers. This aligns with findings from previous studies conducted in other regions of Pakistan and India [15, 16, 21]. Kidney stone formation is a multifactorial disorder influenced by the interaction of genetic predispositions with dietary and environmental elements. Advances in genetic testing have made it possible to identify monogenic causes of nephrolithiasis and nephrocalcinosis in patients presenting with severe phenotypes [22].

Genetic mutations affecting renal tight junction proteins, such as CLDN2, CLDN14, and the calcium-sensing receptor (CaSR), have been implicated in the development of kidney stones [23]. Members of the claudin gene family are critical for paracellular ion transport in renal tubular epithelium, with specific isoforms determining ion selectivity and permeability [10]. Notably, recent studies indicate that CLDN expression plays a key role in $\text{TNF}\alpha$ -induced early transepithelial resistance changes, suggesting its involvement in the pathogenesis of idiopathic calcium oxalate stones [24]. Claudin-2 is predominantly located in the proximal tubule and descending limb of Henle's loop, facilitating the paracellular reabsorption of sodium and calcium [25]. Claudin-14, expressed in the thick ascending limb, restricts Claudin-16-mediated calcium transport [26]. The CaSR is integral to calcium homeostasis and is mainly found on the basolateral membrane of the thick ascending limb. It reduces calcium reabsorption and promotes urinary excretion during calcium excess [27]. According to Guha et al., [20] CLDN14 is also expressed in the collecting ducts and acts as a barrier for cations. Activation of CaSR significantly increases CLDN14 expression, and disruptions in the CaSR-CLDN14 regulatory pathway may contribute to stone formation. Research by Curry et al. [28] suggested a specific defect in calcium reabsorption within the proximal tubule of patients with idiopathic calcium oxalate stones, largely due to impaired CLDN2 function. This defect could hinder calcium reabsorption and favor stone formation. However, a study by Uysal et al. [29] found no statistically significant direct correlation between CLDN2 expression and stone formation. They did note, however, a parallel correlation between the expression of CLDN1, CLDN2, and CLDN8, highlighting their collective influence on transepithelial resistance and their potential role in recurrent stone disease pathogenesis. Although no significant association was found

for CLDN14 expression in that study, the authors pointed out the lack of data regarding its profile in recurrent calcium oxalate stone cases and emphasized the need for further investigation [29]. The renal localization of CLDN14 has been inconsistently reported, but recent research using Cldn14-deficient mice and β -galactosidase reporters confirmed its expression in the thick ascending limb through colocalization and RT-PCR analyses [30]. Kidney stone disease is a multifactorial disorder that demands a comprehensive and

multidisciplinary approach to uncover its molecular basis. The present findings highlight the significant roles of CaSR, CLDN14, and CLDN2 genes in the pathogenesis of nephrolithiasis. Alterations in their expression may disrupt calcium homeostasis and tubular transport mechanisms, contributing to stone formation. A deeper understanding of these genetic interactions could pave the way for the identification of reliable molecular biomarkers and the development of targeted therapeutic strategies for the effective management of kidney stone disease.

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