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Research Article

Mutational analysis of CLDN 19 gene from the patients with nephrolithiasis and comparative analysis of the gene in cats and dogs

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Abstract

Kidney stones (renal calculi) are solid mineral aggregates that develop in the renal system due to a combination of genetic, environmental, and biochemical factors. This study explores the genetic basis of nephrolithiasis with a focus on mutations in the *CLDN 19* and *SCNN1A* genes, which are critical for maintaining magnesium and sodium homeostasis, respectively. Twenty families from different regions of Punjab, Pakistan, with a history of kidney stones, were selected. Clinical diagnosis was confirmed through biochemical testing and radiological imaging. Blood samples were collected from both affected and unaffected individuals, and genomic DNA was extracted using an organic method. DNA quality and concentration were assessed using agarose gel electrophoresis and NanoDrop spectrophotometry. PCR primers were designed for all exons of *SCNN1A* and exons 2 and 4 of *CLDN19*. Amplified products were purified, sequenced using Sanger sequencing, and analyzed via gel electrophoresis. Bioinformatic tools such as BLAST, PolyPhen, and Mutation Taster identified potentially pathogenic variants. Mutations in *CLDN 19* may disrupt magnesium transport, while *SCNN1A* variants may affect sodium balance, both contributing to stone formation. These findings support the importance of genetic screening in high-risk populations and highlight the potential for improved diagnostics and personalized treatment strategies for nephrolithiasis.

Keywords: Mutational Analysis, CLDN19, Nephrolithiasis, PCR, Gene.

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Introduction

Solid mineral and salt aggregates called renal calculi, or kidney stones, develop in the urinary tract and renal system. A multitude of biochemical, environmental,

epidemiological, and genetic factors contribute to the multifactorial process of these stones' development. Because kidney stones directly affect patients' quality of life, healthcare expenses, and the strain on the healthcare system, their occurrence has been rising continuously worldwide and has grown to be a serious healthcare concern [1]. Kidney stones frequently cause significant clinical symptoms, such as hematuria, renal colic, and, in more severe cases, the development of chronic renal failure [2]. Kidney stones remain a major problem for both patients and healthcare professionals, despite improvements in medical research and treatment. According to the epidemiology of kidney stones, 7% of women and 13% of men will experience renal calculi at some point in their lives. Calcium-based substances, such as calcium phosphate and calcium oxalate, are frequently the cause of kidney stones and can make up as much as 80% of cases. A lesser percentage of kidney stone production is caused by other types of stones, such as struvite, cystine, and uric acid. Notably, kidney stones based on calcium are disproportionately common in some ethnicities. About half of all urological problems in nations like Pakistan are renal calculi [3]. Further investigation into the genetic aspects and causes of kidney stone development is now necessary, with a focus on the role that genetic abnormalities play in the aberrant handling of vital ions like calcium and magnesium. Magnesium (Mg^{2+}) is essential for preserving homeostasis and cellular function, which includes controlling the body's calcium levels. Through both paracellular and transcellular pathways, magnesium is mostly absorbed in the thick ascending limb (TAL) of the renal tubules in the kidneys. Controlling the flow of ions across the renal tubular epithelium depends on tight junctions (TJs), which are made up of different membrane proteins. Claudins are one of these proteins that are very crucial for controlling the paracellular route and guaranteeing that magnesium is reabsorbed properly. The renal tubules express claudins, such as *CLDN16* and *CLDN19*, especially in the TAL, where they create ion-selective pores that control the absorption of magnesium [4]. Mutations in these two claudins have been connected to a number of kidney diseases, and their interaction is essential for preserving magnesium homeostasis [5]. Rare inherited disorders, including Familial Hypomagnesemia with Hypercalciuria and Nephrocalcinosis (FHHNC), have been linked to mutations in the claudin genes, including *CLDN16* and *CLDN19*. Kidney stones, low magnesium levels, and high calcium excretion in the urine are among the indicators of this illness. If treatment is not received, FHHNC, which is usually inherited in an autosomal recessive fashion, can cause gradual kidney impairment. The genetic alterations linked to FHHNC impede paracellular magnesium and calcium transport by interfering with the regular function of claudin proteins. Nephrocalcinosis and kidney disease are further exacerbated by mutations in additional ion transport-related genes, such as *SCNN1A*, which codes for the alpha subunit of the epithelial sodium channel (ENaC), which have been connected to problems affecting sodium, potassium, and water balance. The genetic foundation of renal stone formation has been extensively studied worldwide; however, there is a noticeable lack of research that focuses on the molecular processes of magnesium homeostasis impairment in places like Pakistan. For the creation of targeted therapeutics and individualized medical treatments, it is crucial to comprehend the genetic variations that lead to diseases such as FHHNC and pseudo hypoaldosteronism type 1(PHA1). Finding the genetic variants linked to nephrolithiasis may also aid in early identification and improved treatment of the condition, thereby averting long-term kidney damage and enhancing patient outcomes [6]. With emphasis on the *CLDN 19* and *SCNN1A* genes, this work attempts to close the information gap about the

genetic foundations of magnesium homeostasis and nephrolithiasis [7]. We intend to gain a better understanding of these genes' functions in nephrolithiasis and their effects on diseases like FHHNC and PHA1 by examining the genetic variants in these genes. The results of this study may potentially have important ramifications for genetic counseling and the creation of better treatment alternatives for individuals with these crippling illnesses. In areas where nephrolithiasis is a major public health concern, our goal is to better understand kidney stone formation and its genetic factors in order to enhance patient care and outcomes [8].

Materials and Methods

Enrolment of families

Twenty families from various parts of Punjab, Pakistan, who had a known history of kidney stones were enrolled in this study. The selection of the families was based on the fact that each family had at least one impacted member. To make sure ethical guidelines were followed, each family member provided written informed consent. In-depth clinical and family histories were obtained, including details on the age at which kidney stones first appeared, their recurrence, and the medical care that was received. Standard diagnostic techniques such as CT scans, X-rays, ultrasonography, and urine crystal analysis were used to confirm the clinical diagnosis. To enable genetic analysis, the study's inclusion criteria for participants required that both normal and afflicted members of the enrolled families be included.

Collection of blood samples

Using an intrusive sampling procedure, blood samples were taken from the enrolled families affected and unaffected members. A skilled phlebotomist used a 5cc syringe to draw around 3 ml of blood from each person. To stop clotting, the blood was put into vacutainers with 200 µl of 0.5 M EDTA (ethylene-diamine-tetra-acetic acid) as an anticoagulant. To maintain confidentiality, each container was meticulously tagged with the participant's name or a special code. To maintain the integrity of the DNA until further processing, the obtained blood samples were kept in a freezer at -20°C.

Sterilization of apparatus

Throughout the study, all glassware and plasticware were autoclave sterilized to prevent contamination and guarantee the correctness of the findings. The following was the procedure for sterilization: First, every item, including the tips of micropipettes, flasks, and glass beakers, was thoroughly cleaned with detergent. The things that were to be autoclaved were placed in suitable containers, like glass bottles and jars, and the containers were sealed to prevent contaminants [9]. Distilled water was added to the machine to prepare the autoclave, making sure the water level was higher than the autoclaving platform [10]. The autoclave was filled with materials to be sterilized and operated for 15 minutes at 121°C and 15 psi. After the sterilizing procedure was finished, the autoclave was cautiously opened, allowing the materials to cool to ambient temperature before being stored in a sterile setting [11].

DNA extraction

The organic DNA extraction method was used to extract the genomic DNA from the blood samples [12]. The extraction of high-quality DNA appropriate for further genetic investigation is guaranteed by this technique. The following actions were taken to extract DNA.

Materials used for DNA extraction

The reagents and instruments used for DNA extraction are Proteinase K, Lysis Buffer, SDS (sodium dodecyl sulfate), AI Buffer,

Sodium chloride (NaCl) solution, Phenol-Chloroform-Isoamyl (PCI), 70% Ethanol, Isopropanol, Sterilized deionized water, Micro-pipettes and tips, Eppendorf tubes, Water bath incubator, and Paraffin film for sealing [13].

Preparation of solutions for DNA extraction

Several solutions were prepared to aid in the DNA extraction process: Lysis Buffer: 1 g of Potassium bicarbonate (KHCO3) was mixed with 8.29 g of Ammonium chloride and 200 µl of EDTA (ethylene-diamine-tetra-acetic acid), and the final volume was adjusted to 1 liter with distilled water. EDTA Solution: 18.62 g of EDTA was dissolved in sterilized distilled water to make a final volume of 500 ml.10N Sodium Hydroxide: 400 g of Sodium hydroxide was dissolved in sterilized distilled water to make 1 liter of solution.6M Sodium Acetate: 492 g of Sodium acetate was dissolved in sterilized distilled water to make a final volume of 1 liter, with the pH adjusted to 5.2 using acetic acid [14]. Buffer AI: 2 ml of 0.5 M EDTA, 5 ml of 1 M Tris-HCl, and 40 ml of 1M sodium chloride were added and made to a final volume of 500 ml with sterilized distilled water [15]. Proteinase K Solution: 100 mg of Proteinase K was dissolved in 10 ml of TE buffer to make a 10 mg/ml solution. This solution was stored at -20°C.TNE Buffer (Tris-NaCl-EDTA): 5 ml of Tris base, 40 ml of 6M sodium chloride, and 2 ml of EDTA were dissolved in sterilized distilled water to make a final volume of 500 ml [16].

DNA extraction protocol

The steps for DNA extraction were as follows: The frozen blood samples were either thawed at ambient temperature or for a few minutes in a water bath set at 37°C. A 1.5 ml Eppendorf tube was filled with 200 µl of blood. After adding 1 milliliter of lysis buffer to the tube, it was centrifuged for 20 minutes at 4°C at 6500 rpm [12]. After discarding the supernatant, the pellet was carefully fractured by tapping the tube. Until a clean white pellet of white blood cells was obtained, the washing procedures (i.e., adding the lysis buffer and centrifuging) were carried out three to four times.25 µl of Proteinase K, 150 µl of AI Buffer, and 30 µl of 10% SDS solution were used to resuspend the pellet. The Eppendorf tube was wrapped in paraffin film and left in a water bath at 55°C for the night to digest [17]. Following digestion, 100 µl of 6M sodium chloride was added, and the mixture was then incubated on ice for 20 minutes [18]. The mixture was centrifuged for 20 minutes at 6500 rpm at 4°C, and the supernatant was moved to a new tube. Double the volume of PCI (Phenol: Chloroform: Isoamyl alcohol) was added, and the mixture was centrifuged once more at 6500 rpm for 15 minutes at 4°C. The DNA-containing upper aqueous layer was moved into a new tube. DNA was precipitated by adding double the volume of isopropanol or absolute ethanol, and the tube was gently inverted until DNA threads appeared [19]. The supernatant was disposed of after the sample was centrifuged for 20 minutes at 6500 rpm and 4°C.150 µl of 70% ethanol was added to the pellet, followed by another centrifugation for 15 minutes at 6500 rpm at 4°C. The supernatant was discarded, and the pellet was air-dried for several hours to remove any residual ethanol [20]. The DNA pellet was dissolved in 50 µl of nuclease-free water and incubated in a water bath at 70°C-72°C for 15-20 minutes. The DNA samples were stored at -20°C until further use.

Quantification of the extracted genomic DNA

Two methods were employed to quantify the genomic DNA obtained after extraction.

Spectrophotometer: The absorbance of DNA samples was measured using a

Thermo Fischer Scientific NanoDrop spectrophotometer. The DNA samples were measured at an OD 260/280 ratio, and the device was calibrated using 1 µl of nuclease-free water as a blank. For pure DNA, a standard ratio of 1.8 was thought to be optimal; values greater or lower, suggested possible RNA or protein contamination [21].

Gel Electrophoresis: Agarose gel electrophoresis was also used to evaluate the quantity and quality of DNA. After preparing a 0.8% agarose gel, 3 µl of the DNA sample was loaded onto it using 3 µl of 6X loading dye. To verify the existence and caliber of the extracted DNA, the gel was run at 100 V for 30 minutes and examined under a UV lamp [22]. To ensure that the extracted DNA was of the right quality and concentration for use in subsequent processes, both techniques were applied.

Primer designing and amplification of selected exons of *CLDN 19* and *SCNN1A* gene

Primers were created to amplify all the *SCNN1A* gene's exons and two of the *CLDN19* gene's exons. The exon data were taken from Ensembl, while the primer sequences were taken from the NCBI database. To guarantee specificity, the primers were constructed using Primer3 software and examined for self-complementary and cross-complementary sequences [23]. The Macrogen Company synthesized the primers [24]. Exon 2 (*CLDN 19* gene): Forward primer: GTCCTCAGCGTAGTTGGCAT; Reverse primer: CTGCCAGGATGAAGAGGGC Exon 4 (*CLDN 19* gene): Forward primer: AAGCACCCACCCTGCAATCT; Reverse primer: TGGACAAGTCCCTGTTGTGC. *SCNN1A* gene: 14 exons were selected, and primers were designed for them. Each of the primer pairs amplified a ~500bp fragment.

Polymerase chain reaction (PCR)

Using the specified primers, the DNA samples were amplified via Polymerase Chain Reaction (PCR). To maximize the target region's amplification, PCR settings were adjusted. Denaturation at 95°C for 3 minutes, 35–40 cycles of 95°C for 30 seconds, annealing at 55°C - 60°C (optimized for each primer), and extension at 72°C for 40 seconds were the usual PCR conditions [25]. A final extension was performed for ten minutes at 72°C.

Analysis of PCR amplicons

After amplification, PCR products were analyzed through gel electrophoresis on a 2% agarose gel in TAE buffer. A 1 Kb ladder was included for size comparison. The gel was run at 110 V for 30 minutes and visualized under a UV transilluminator [26].

Purification and sequencing of PCR products

After gel electrophoresis, absolute ethanol was added, the pellets were centrifuged at 13,500 rpm, and the PCR amplicons were precipitated and purified. Following purification, the amplicons were transferred to the Center of Applied Molecular Biology, University of Punjab, Lahore, for sequencing using the Sanger Chain Termination Method and an ABI 3130 Genetic Analyzer [27].

Bioinformatics analysis

Bioinformatics tools were used to evaluate the obtained DNA sequences. BLAST was used to align the sequences with NCBI and Ensembl reference sequences. PolyPhen and MutationTaster were used to find SNPs and mutations and assess their possible functional impact. Using the programs CLUSTAL W and PyMOL, additional homology and structural analysis were performed [28].

Results

Collection of samples and patient details

Twenty kidney stone patients in all were recruited from different clinics and hospitals. Family history, biochemical analysis, radiographic results (X-ray, ultrasound, CT scan), and clinical symptoms were used to corroborate their diagnosis. Table 1 contains demographic information, such as patient. Of the 20 people, 5 were women and 15 were men. The average age of those impacted was determined. In every instance, kidney stones were confirmed to be present by radiological imaging methods.

Estimation and quantification of genomic DNA

Each patient had 200µl of blood drawn in order to extract DNA. Two techniques were used to analyze DNA:

Agarose Gel Electrophoresis: Qualitative analysis showed that certain DNA samples included contaminants. Nanodrop spectrophotometry: Acceptable purity was indicated by optical density (OD) values, which varied between 1.70 and 1.88. The range of concentrations of stock DNA was 67.7 ng/µl to 411.5 ng/µl.

Table 1: Details of 20 individuals confirmed for the presence of a kidney stone.

S/No	ID	Sex	Age	Family History	Diagnostic test	Caste
1	KS-1	M	27	-VE	X-ray, USG	Rajput
2	KS-2	M	30	-VE	X-ray, USG	Sandhu
3	KS-3	M	34	+VE	X-ray, USG	Mughal
4	KS-4	F	15	-VE	X-ray, USG	Jatt
5	KS-5	M	32	-VE	X-ray, USG	Rajput
6	KS-6	M	30	-VE	X-ray, USG, CT-Scan	Arain
7	KS-7	M	37	-VE	X-ray, USG	Jatt
8	KS-8	F	25	-VE	X-ray, USG	Khokhar
9	KS-9	M	45	+VE	X-ray, USG	Arain
10	KS-10	M	27	-VE	X-ray, USG	Rajput
11	KS-11	M	48	+VE	X-ray, USG	Arain
12	KS-12	M	24	-VE	X-ray, USG, CT-scan	Arain
13	KS-13	M	18	+VE	X-ray, USG	Rajput
14	KS-14	F	29	+VE	X-ray, USG	Kumhar
15	KS-15	M	34	+VE	X-ray, USG	Rajput
16	KS-16	M	25	+VE	X-ray, USG, CT-scan	Rajput
17	KS-17	M	40	-VE	X-ray, USG	Rajput
18	KS-18	M	22	+VE	X-ray, USG	Jatt
19	KS-19	F	31	-VE	X-ray, USG, CT-scan	Arain
20	KS-20	M	19	+VE	X-ray, USG, CT-scan	Bhatti

ID, sex, age, family history, diagnostic tests, and caste.

Primer designing for *CLDN 19* gene

The Primer 3 online tool was used to generate primers for the *CLDN 19* gene's exons 2 and 4. A range of 40% to 60% was maintained for the GC content. Gradient PCR was used to optimize the designed primers at temperatures between 55 and 60°C.

Standard PCR

Following optimization, a Bio-Rad Thermocycler was used to conduct standard PCR. PCR reactions were carried out at a total volume of 25 µl. Each reaction contained approximately 100 ng of template DNA, 1× PCR buffer, 2.5 mM $MgCl_2$, 200 µM of each dNTP, 300 nM of each primer, and 2 units of Taq DNA polymerase. The reactions were performed under standard thermal cycling conditions, a preheat temperature of 95C for three minutes and 35-40 cycles of denaturing temperature 95C for 30 sec, an annealing temperature of 57°C for 30 sec and extension temperature 72C for 40 sec. On a 2% agarose gel, amplicons were found and seen using a UV transilluminator.

Sequencing results

PCR amplicons of *CLDN 19* and *SCNN1A* genes were sequenced. BLAST analysis of exon 2 of the *CLDN 19* gene showed complete sequence similarity with the reference sequence, confirming the absence of mutations. Similarly, exon 4 of the *CLDN 19* gene also exhibited no detectable sequence variations.

- c.2018del (deletion mutation).
- c.1697T>C (non-synonymous mutation).

These mutations were identified in two separate families. Pedigree analysis of affected families (KS-2 and KS-8) was depicted in Figures 1 and 2. *SCNN1A* Variants Identified in 2 families.

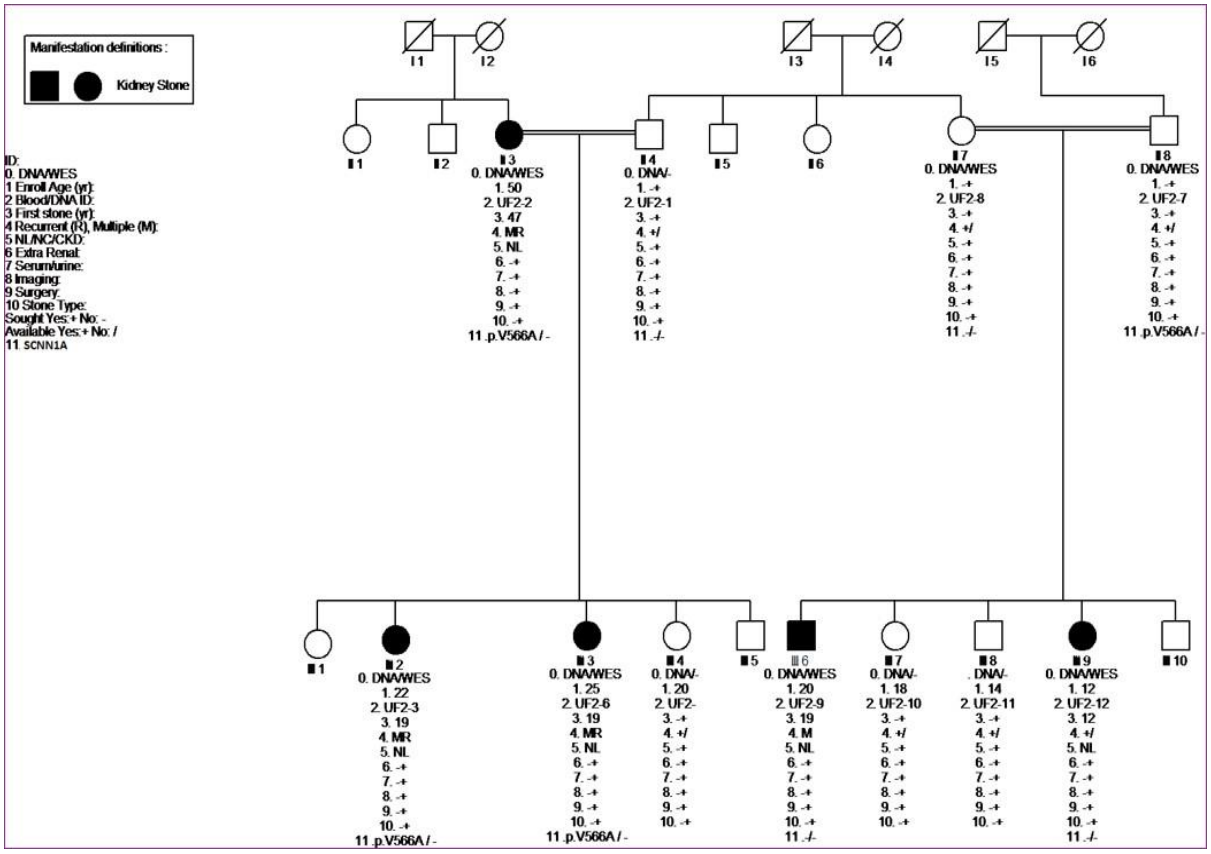

Figure 1: Pedigree of the KS 2 family.

Multiple sequence alignment

Multiple sequence alignment of *SCNN1A* variants showed that mutations causing Pseudo hypoaldosteronism (PHA) were distributed across the protein, while Bartter syndrome and Liddle syndrome mutations clustered in specific regions. Notably, kidney stone-associated mutations were localized at the end of the alignment shown in Figure 3.

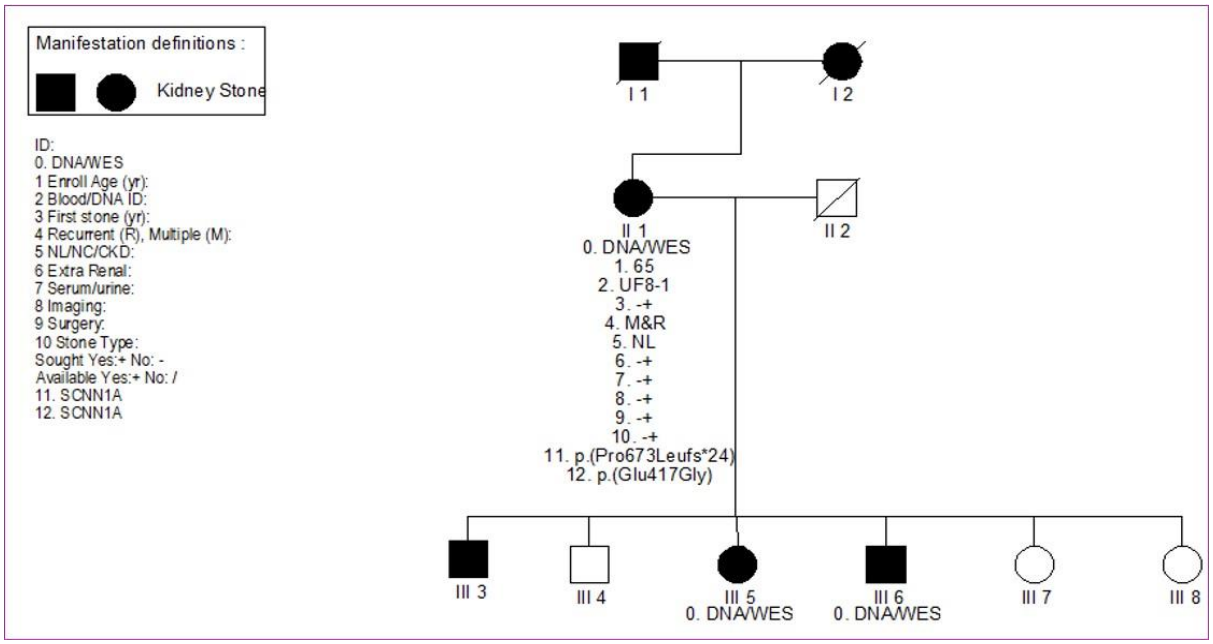

Figure 2: Pedigree of the KS 8 family.

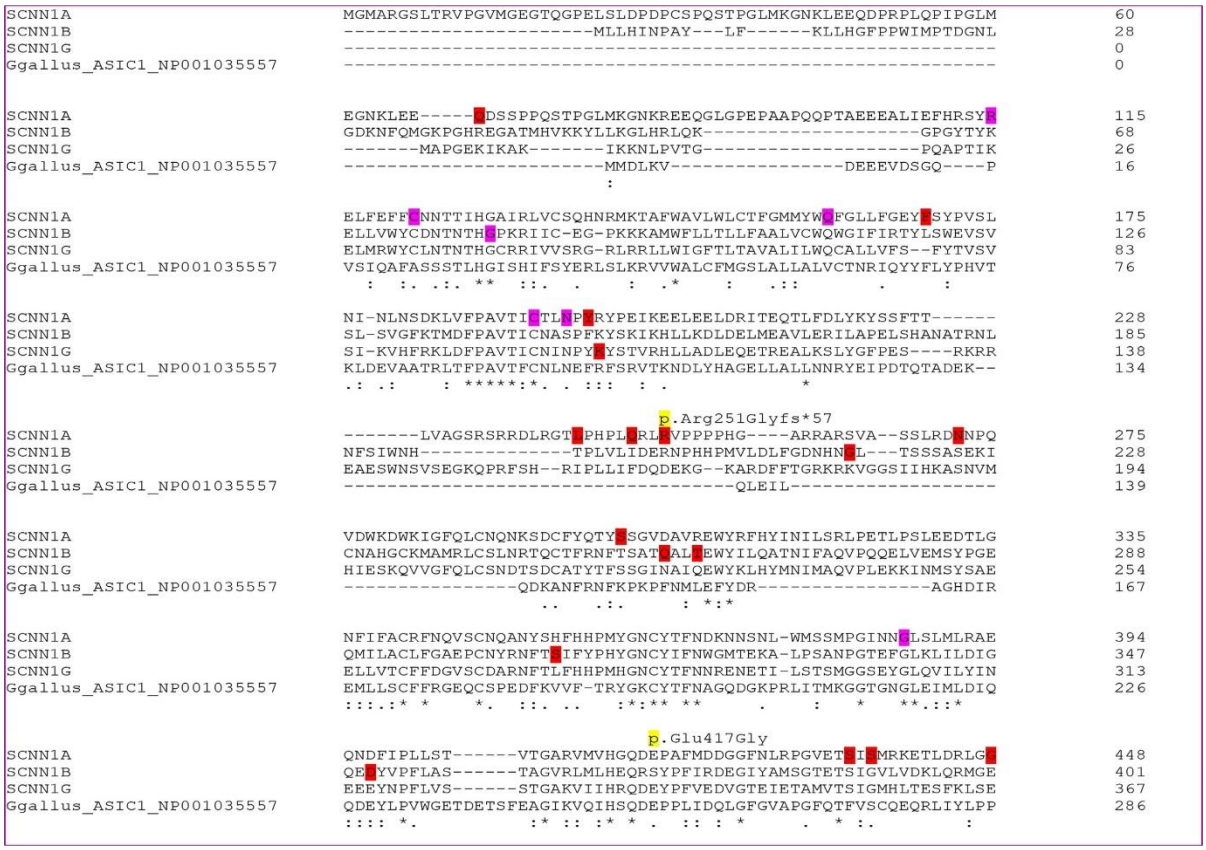

Figure. 3: Multiple sequence alignment result of the *SCNN1A* gene**.**

Discussion

A complex condition, kidney stone disease is impacted by both environmental and genetic factors. This study's main goal was to find out how genetic variants in the *CLDN 19* and *SCNN1A* genes affect the development of kidney stones. Our research advances our knowledge of the pathogenesis of renal stones and offers important new information about the genetic susceptibility to nephrolithiasis, especially in the Pakistani population [29]. In renal tubules, the *CLDN 19* gene is recognized to be essential for preserving calcium and magnesium balance. However, our study's *CLDN 19* gene sequencing data showed no alterations in the kidney stone patients we looked at. This implies that either the mutations present are uncommon and require a larger sample size for discovery, or *CLDN 19* mutations may not be a common cause of kidney stone production in the population under study. Additionally, our in silico comparative investigation showed that *CLDN 19* is highly conserved across species, such as dogs, cats, and humans [30].On the other hand, the *SCNN1A* gene's sequencing results showed two different mutations: a non-synonymous mutation, c.1697T>C in exon 6, found in the KS2 family, and a deletion, c.2018del in exon 12, found in the KS8 family. These alterations raise the possibility that *SCNN1A* plays a part in nephrolithiasis [31]. A subunit of the epithelial sodium channel (ENaC), which is necessary for sodium reabsorption and fluid homeostasis in the kidneys, is encoded by the *SCNN1A* gene [32]. Hypercalciuria is a known risk factor for kidney stone formation and can result from dysfunctional sodium management. Our findings are consistent with other research showing that changes in potassium and sodium balance can make people more susceptible to nephrolithiasis. Stone formation may be encouraged by the discovered mutations in *SCNN1A*, which could lead to an imbalance in potassium and sodium levels. Prior research has demonstrated that hypercalciuria, which causes calcium crystal aggregation in the urinary system, is correlated with increased sodium excretion. Supplementing potassium has also been proposed as a possible treatment approach to reverse this impact. This emphasizes the necessity of more investigation into the therapeutic modification of potassium and sodium channels in order to stop the production of stones in vulnerable people. Additionally, our work highlights the importance of genetic screening for kidney stone patients. Early diagnosis and individualized treatment plans may benefit from the identification of genetic variants in *SCNN1A* and other associated genes. Understanding the genetic basis of nephrolithiasis can assist with predicting disease susceptibility and guide focused therapies, reducing recurrence rates and improving patient outcomes [33].

Furthermore, the results of this research add to the increasing amount of data indicating that ENaC plays a part in renal stone disease. Given that *SCNN1A* has conserved mutations across species, animal models may be used to further explore the protein's functional implications. To verify the pathogenicity of these mutations and investigate possible treatment targets, larger cohorts and functional assays are required in future research. Our research offers new information about the genetic variables linked to kidney stone illness. The discovery of *SCNN1A* mutations emphasizes the possible involvement of sodium channel dysregulation in nephrolithiasis, even if no changes were found in *CLDN 19* [34]. These results open the door for further research into the genetic and molecular pathways behind kidney stones and highlight the significance of genetic studies in comprehending kidney stone pathogenesis.

Conclusion

In the *SCNN1A* gene, this study discovered a non-synonymous mutation (c.1697T>C) and a unique deletion mutation (c.2018del) that may contribute to kidney stone development. There were no mutations found in the *CLDN 19* gene. The functional significance of *SCNN1A* mutations warrants additional exploration, and these findings advance our understanding of the genetic risk in kidney stone disease.

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