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Association of rs11746443 (RGS14) Polymorphism with Susceptibility to Calcium Kidney Stones Disease

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ABSTRACT. Although the exact cause of kidney stones is unclear and complicated, it is believed to be related to the Receptor of G Protein Signaling 14 gene (*RGS14*). To determine the polymorphism's eventual role in calcium stone formation, attempted to evaluate the association between calcium stone disease and the *RGS14* (G/A rs11746443) polymorphism in the Babylon Province population. Method: From January to December 2020, blood samples were obtained from 60 patients admitted to the Ibn Al-Nafees laboratory and Hillah Hospitals in Babylon Province, Iraq. Furthermore, normal people were used as a control group (40 samples). A polymerase chain reaction technique was used to genotype RGS14 single-nucleotide polymorphisms (SNPs), followed by single-strand conformation polymorphism. As a result, DNA sequencing was used to verify these DNA polymorphisms. Results: Due to the existence of SNPs within the studied area, the conformational haplotypes of RGS14 were obtained using SSCP-PCR in four patterns: 2-bands, 3-bands, 4-bands, and 5-bands. The sequencing results of this region revealed that were five SNPs. one of these SNP is rs11746443 , which is located at the chromosomal site 177371305 G/A (Intron 12). This SNP may be associated with renal calcium-containing stone diseases because it is positioned upstream of the *SLC34A1* and *AQP1* genes, which play a role in kidney task and the concentration of urine. Whereas, other SNPs in the exon 13 leads to change four amino acid residues of RGS14. These changes in amino acids were thought to not affect the calcium stones formation. Conclusion: These results indicate that the *RGS14* SNP (rs11746443) maybe play a role in kidney stone disease susceptibility.

Keywords: Regulator of G Protein Signaling 14, Renal Calcium-containing stones ,Single nucleotide polymorphism.

INTRODUCTION

Kidney stones are a form of crystal concretion that develops in the kidneys [1]. Urolithiasis affects approximately 12 percent of the global population at certain period in their lives [2]. It affects people of all ages, genders, and races, but men between the ages of 20 and 49 are more affected than women [3]. Around 75% of kidney stone disease is

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caused by calcium-based calculi, and their prevalence is increasing, implying that environmental and dietary influences are acting on a preexisting genetic history. Modern genetic studies have effectively recognized genes that may be involved in the development of kidney stones [4]. The disorder has a known familial nature and significant heritability, and genetic studies have clearly detected genes that may be involved in the formation of kidney stones [4].

In humans, the RGS14 gene codes for a member of the G-protein signaling regulator family. The G protein signaling family of regulators includes RGS14 (Regulator of G Protein Signaling 14). It is found on chromosome 5q35.3 and has 15 exons. The neuronal system was mainly involved in the function [5]. Urabe *et al*. used a three-stage genome-wide association study to find the RGS14 gene polymorphism in nephrolithiasis in the Japanese population [6]. The significance of the correlation was then reaffirmed [7]. The RGS14 polymorphism is embroiled in the causes of nephrolithiasis, according to these and other observations, and thus can be used as a genetic marker.

There is no information about the association between the *RGS14* gene polymorphism and the development of renal calcium-containing stones disease in the Babylon Province population, Iraq. As a result, we conducted a study to look into the risk factor for renal calcium-containing stone disease in *RGS14* rs11746443 patients.

METHODS

Sampling

A case-control study was conducted from January to December 2020. Blood samples were taken from patients at the Ibn Al-Nafees laboratory and the Hillah Hospitals in Babylon, Iraq. A total of 100 blood samples were used in this study. A total of 60 blood samples were taken from patients with Calcium Kidney Stone Disease, with a control group of 40 samples taken from apparently healthy people. The patients ranged in age from 17 to 75 years old. The control group, on the other hand, ranged in age from 18 to 60. A specialist physician examined all of the participants.

SSCP-PCR technique to identify genotypes

After extracting DNA from blood samples, the study groups were genotyped using the SSCP-PCR technique. A Geneaid extraction and purification kit was used to extract and purify DNA from blood. *RGS14* (rs11746443), was identified by amplification of the targeted DNA sites using design-specific primers obtained from Macrogen company in South Korea. Forward primer: 5’-CCACAGCCAGGCGAGAAA-3’, and reverse primer: 5’-ATCCCCAGCTCATCCACCAT -3’.

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To get the reaction volume up to 20 µl, 1 µl of both forward and reverse primer, 12.5 µl of Green Master Mix, 3 µl of Genomic DNA, and 2.5 µl of nuclease-free water were added to the reaction volume of PCR. Amplification was conducted in a thermocycler (Biometra, Germany) with the following settings: 3 min pre-denaturation at 94°C; 30 cycles with denaturation for 30 seconds at 94°C, annealing for 30 seconds at 60°C, extending for 30 seconds at 72°C; and a final extension of 3 min.

PCR products were electrophoresed in 1 percent agarose at 75 V using gel electrophoresis (cleaver science – UK) and visualized with ethidium bromide (0.4µg/mL). A gel documentation system (Cleaver Scientific –UK) was used to take photos. After electrophoresis of exon 4 and part of intron 3 of the RGS14 PCR fragment, the clear and bright bands were found. These amplified fragments are then suitable for SSCP experiments.

Single Strand Conformation Polymorphism (SSCP)

As an ideal cheap method of detecting unknown single nucleotide polymorphisms (SNPs), the PCR-SSCP technique was used to doing experiments with post-PCR screening for all 100 PCR amplicon. The critical PCR-SSCP protocol was followed as defined by Al-Shuhaib *et al*., [8] , [9]. Followed by 30 cycles of PCR amplification, an equivalent volume of SSCP denaturing-loading buffer was applied to 10 of each PCR amplicon (95 percent formamide, 0.05 percent bromophenol blue, and 0.05 percent xylene cyanol, and 20 mM EDTA, pH 8). After being denatured for 8 minutes, PCR amplicons were directly put on ice and kept freezing at the very least 10 minutes. The samples were then loading onto polyacrylamide gels that were neutral. Afterward, gels were stained with a silver staining procedure that was extremely sensitive [10] .

The single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) bands were observed in the higher part of the gel and the lower part of the gel, respectively. The genetic pattern of each amplicon is determined by the variation of ssDNA in SSCP gels, and the SSCP-PCR conditions were 8 percent polyacrylamide gel electrophoresis power applied: 200 V, 90 mA for 160 minutes. After that, silver staining was used to visualize the gels.

Sequencing

According to Macrogen sequencing laboratory instructions, From both the forward and reverse termini, each PCR amplicon reflecting a particular PCR-SSCP banding pattern was sent for Sanger sequencing reactions. (South Korea). All observed PCR-SSCP banding pattern sequences have been aligned with the program DNA Bio Edit 7.2.5 and edited.

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Statistical analysis

All statistical analyses were performed using SPSS statistical software (version 23), and $p \leq 0.05$ are considered statistically important.

RESULTS

As a first step, DNA was extracted from a blood sample to separate the genomic DNA of patients and healthy controls. Figure 1 depicts the electrophoresis of genomic DNA on a gel.

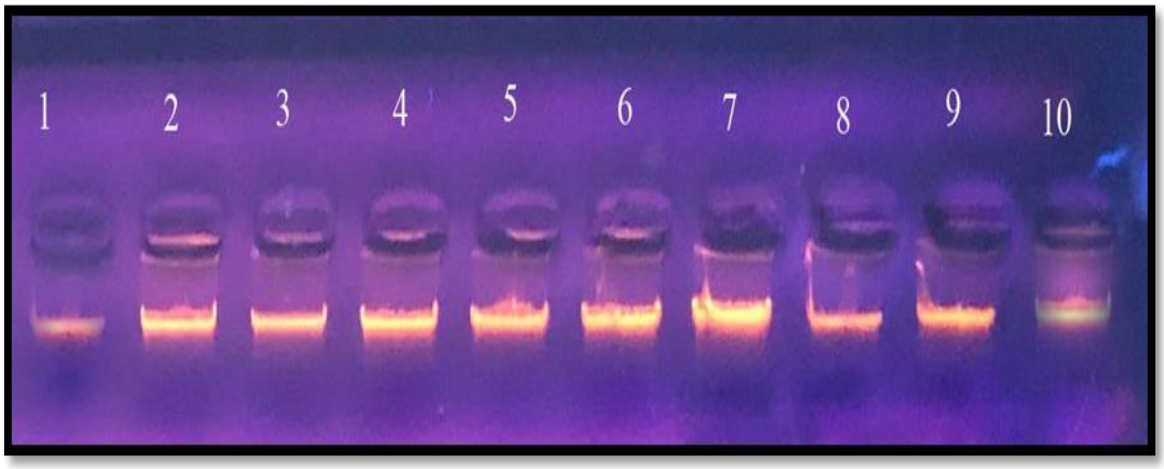

Figure 1: Genomic DNA extracted from blood samples of renal calcium-containing stone disease patients and healthy control groups on an agarose gel electrophoresis pattern. 1 percent agarose, 75 V, 20 mA for 1 hour, stained with ethidium bromide electrophoresis conditions. Sample 1-5 refer to patients while 6-10 represent healthy control.

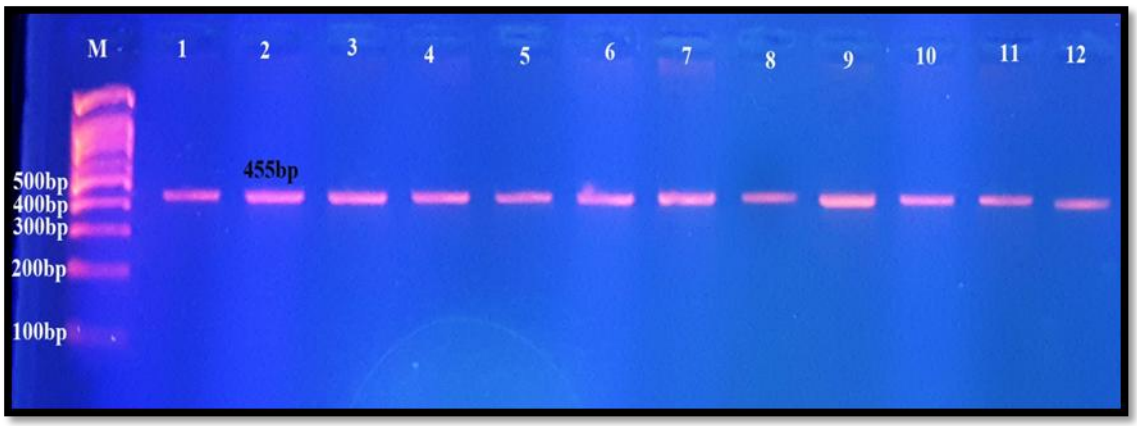

Figure 2. The amplified product patterns of the regulator of G protein signaling 14 (*RGS14*) gene in patients and controls on an agarose gel. Lanes 1 to 12 refer to RGS14-PCR product patterns; M: refers to DNA scale marker (221bp). Conditions for electrophoresis: 75 V, 20 mA for 120 minutes; 1% agarose concentration Precast

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ethidium bromide was used as a staining tool. Samples 1-5 refer to patients while 6-12 refer to healthy control.

For *RGS14* genotyping by the RCR-SSCP technique, genomic DNA from patients with renal calcium-containing stone disease and control groups was amplified using precise primers designed according to the target region (Exon-12, 13, and intron 12) of RGS14 of *Homo sapiens* reference ID: AC140179.3. Under optimal conditions, the thermocycler apparatus were used to conduct DNA amplification. In an agarose gel containing a *danger* region of the *RGS14* target sequence, the findings revealed the presence of a single band (455bp) (Fig. 2) (rs11746443).

Based on the number of bands on the SSCP gel, the presence of different conformational DNA polymorphisms constitutes 2-bands, 3-bands, 4-bands, and 5-bands, as shown in Fig.3 of PCR-SSCP gel electrophoresis.

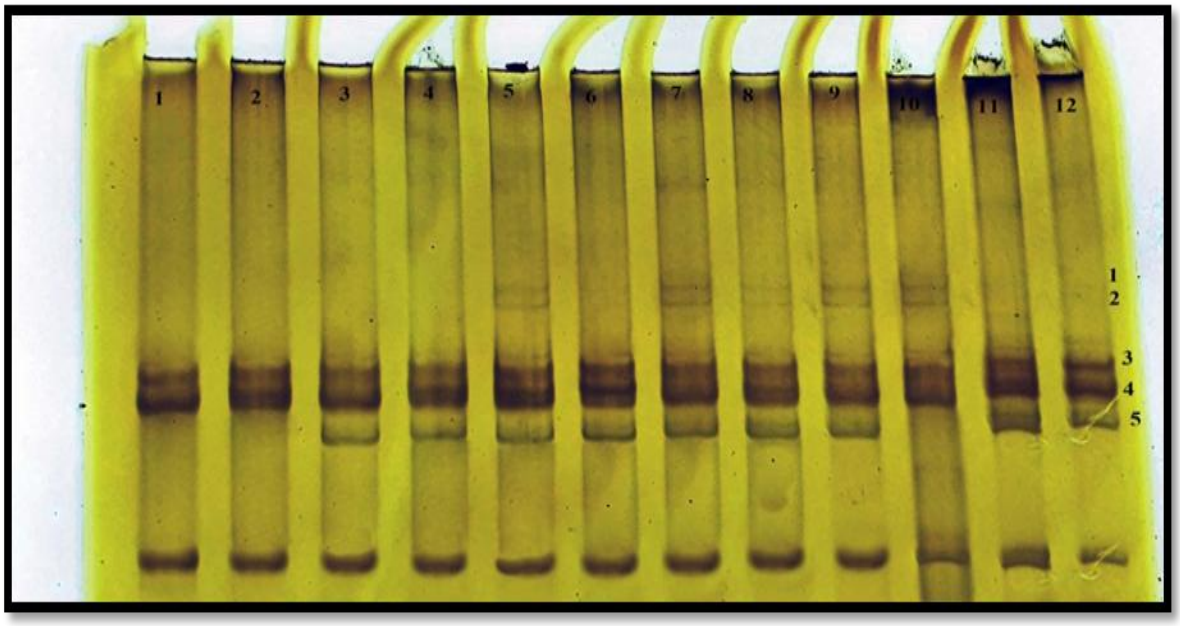

Figure 3: DNA polymorphisms in the RGS14 gene's Exon-12 and 13 regions, sorted by band number using the PCR-SSCP technique. The numbers 1 through 5 refer to the band's location. Lanes 1–12 reflect the target site within Exon-12 and 13 of the RGS14 gene PCR-SSCP haplotype patterns. Lanes 1, 2 and 11 represent 2-band haplotypes, while Lane 11 represents a 3-band haplotype in a control. 3-bands haplotype of patient groups are represented by Lanes 3,4,6 & 12; 4-bands haplotype of patient groups are represented by Lanes 10; 5-bands haplotype of patient groups are represented by Lanes 5, 7,8 & 9. Electrophoresis conditions: 8 percent polyacrylamide gel concentration; 200V (7.5V/cm) – 100mA; run time: 125 minutes; silver nitrate staining technique

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Conformational polymorphism ratios across haplotypes in the patient community were 2-bands 33.33 %, 3-bands 30%, 4-bands 25%, and 5-bands 11.66 %, respectively, compared to 2-bands 47.5 %, 3-bands 7.5 %, 4-band 30 %, and 5-band 15 % in the control group. There is a correlation between DNA polymorphisms and the numbers of band in patients as compared to control groups, as depicted in Table 1.

Table 1: The number of bands in the PCR-SSCP haplotype distribution of the RGS14 gene, as well as their relationship with patient and control group

Conformational haplotype polymorphism	**patient group No. (%)**	**Control group No. (%)**	**P-value**	**OR**	**95% CI**
2 bands [a]	20(33.33%)	19(47.5%)	0.013*	5.70	1.442-22.524
3 bands	18 (30%)	3 (7.5%)			
4 bands	15(25%)	12 (30%)	0.732	1.18	0.443-3.18
5 bands	7(11.66)	6 (15%)	0.872	1.10	0.314-3.9
3 bands [a]	18 (30%)	3 (7.5%)	0.032*	0.20	0.049-0.878
4 bands	15(25%)	12 (30%)			
5 bands	7(11.66)	6 (15%)	0.050*	0.19	0.037-1
4 bands [a]	15(25%)	12 (30%)	0.918	0.93	0.247-3.524
5 bands	7(11.66)	6 (15%)			
Total number	60	40			

*P ≤ 0.05; OR: Odds Ratio ; 95%CI: 95% Confidence Interval.

Nevertheless, using only the gel visualization, it may be hard to establish the pattern of wholly resolved SSCP bands. As a result, sequencing must be used to validate these DNA polymorphisms. Many SNPs (Fig.4 and Table 2) were found between the one resolved haplotype and the RGS14 (exon 12,13, and intron 12) for reference sequences ID: XM 024454328.1, according to the sequencing results.

The sequencing results of the *RGS14* gene shown there were five SNPs . one of these SNP is previously recorded in NCBI as rs11746443 , which is located at the chromosomal site 177371305 G/A (Intron 12). this SNP may be associated with renal calcium-containing stones diseases because it is situated upstream of the *SLC34A1* and *AQP1* genes, which are involved in kidney function and urine concentration. Whereas, other SNPs is novel and not recorded previously like that located at the chromosomal site 177371436 (T/C), 177371441 (T/C), 177371448(T/A), 177371463 (A/G), and 177371554 (G/T) according to the human reference sequence alignment *RGS14* gene ID: XM_024454328.1. all of these SNPs are found in exon 13 as shown on fig. 4 and table 2.

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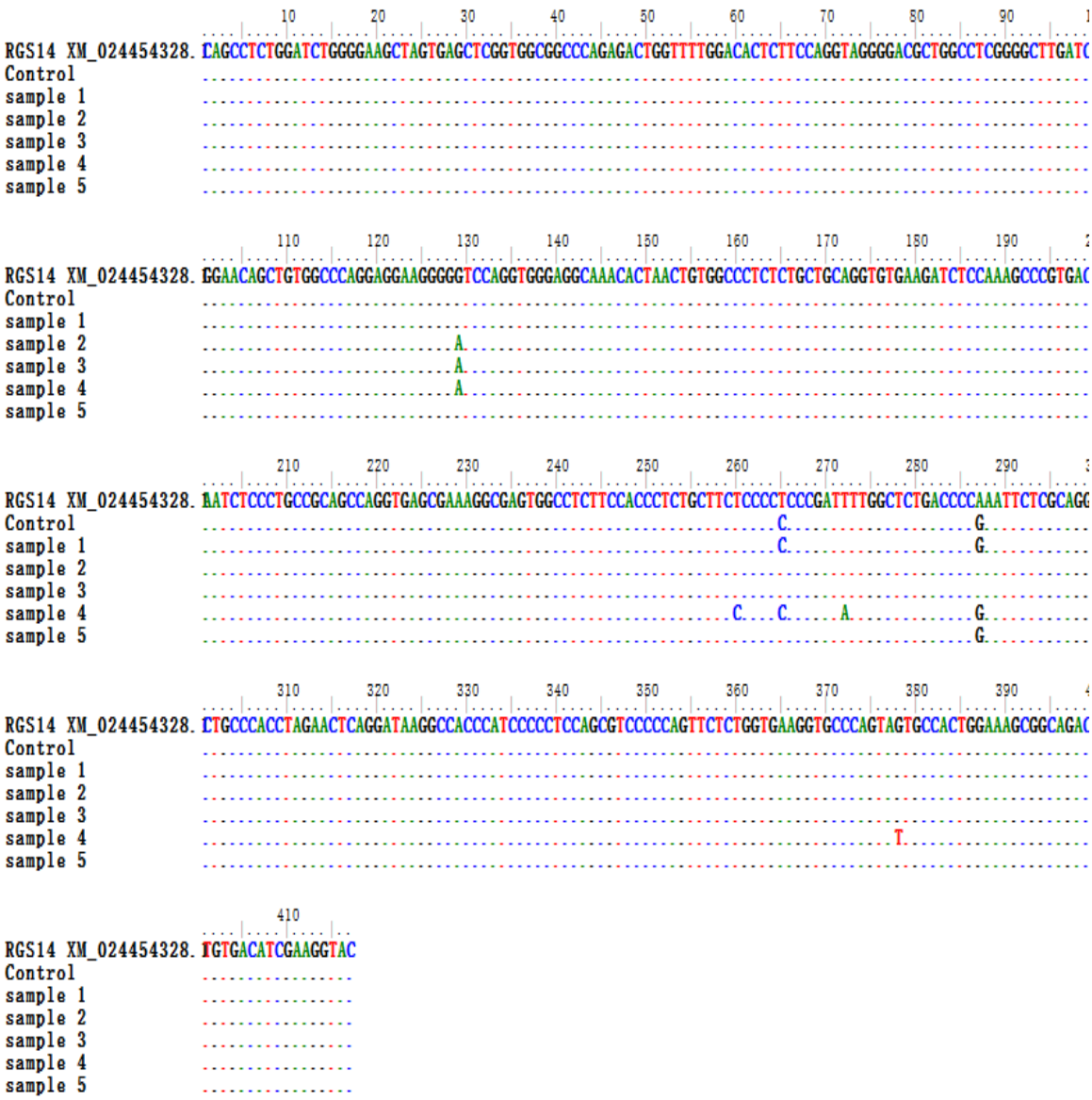

Figure 5: Bio Edit software version 7.2.5 sequence alignment results for *Homo sapiens* RGS14 gene fragment. Sample 1-5 represent a patient with renal calcium-containing stone diseases .

Table 2: All SNPs in the target sequence of *the RGS14* gene.

Location	SNP	CODE	Amino acid	Code	Amino acid	Seq. BASE/AA	Previously Recorded SNPs
177371305 (Intron 12)	G/A	-	-	-	-	129/-	**rs11746443**
177371436 (exon 13)	T/C	TCC	S	CCC	P	260 /30	-

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177371441 (exon 13)	T/C	CCT	P	CCC	P	265 /31	-
177371448 (exon 13)	T/A	TTT	F	ATT	I	272/34	-
177371463 (exon 13)	A/G	AAA	K	GAA	E	287/39	-
177371554 (exon 13)	G/T	AGT	S	ATT	I	378/69	-

The results appeared to modify four amino acid residues of regulator of G protein signaling 14 (*RGS14*) when interpreting the DNA sequence using Bio Edit software version 7.2.5 according to the reference sequence alignment of the human RGS14 gene ID: XM 024454328.1 as shown in fig. 5. These amino acid replacement were Ser (S) 30→ Pro (P) 30 in sample 4, Phe (F) 31→Ile (I) 31 in sample 4, Lys (K) 39→ Glu (E) 39 in sample 1,4,5, and control . Finally, Ser (S) 69→Ile (I) 69 in sample 4.

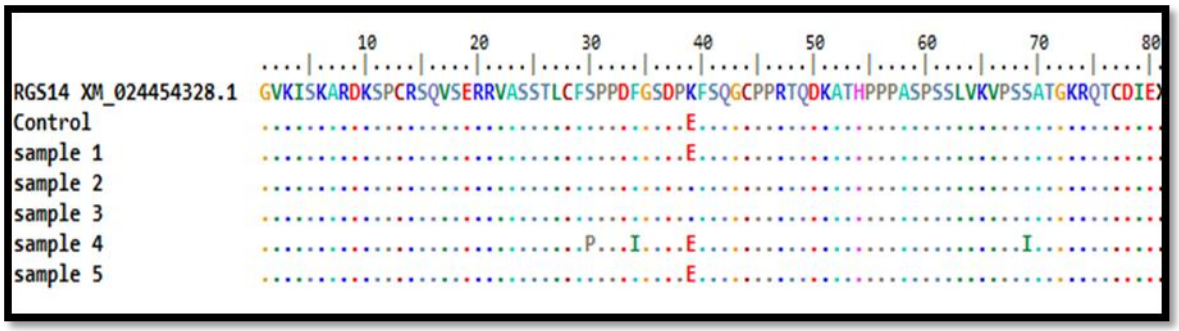

Figure 5: RGS14 amino acid pair sequence alignment based on Bio Edit software version 7.2.5 alignment. The patient's sample ranges from 1 to 5.

DISCUSSION

A total of 5,892 nephrolithiasis cases and 17,809 Japanese controls were used in a three-stage genome-wide association study (GWAS). *RGS14-SLC34A1-PFN3-F12* on 5q35.3 (rs11746443; P = $8.51*10^{-12}$, OR = 1.19), *INMT-FAM188B-AQP1* on 7p14.3 (rs1000597; P = 2.16*0-14, OR = 1.22), and *DGKH* on 13q14.1 (rs4142110; P = $4.62*10^{-9}$, OR = 1.14) were discovered as novel loci for nephrolithiasis . Following that, studies of 21,842 Japanese subjects showed a connection between SNP rs11746443 and a decrease in estimated glomerular filtration rate (eGFR) (P = 6.5461028), implying that this variation in renal function plays an important role. They did not, however, detect a connection between rs11746443 and serum phosphorus [7]. The results of our current study are consistent with what was mentioned above .

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The SLC34A1 gene, which is situated in the RGS14-SLC34A1-PFN3-F12 region, encodes NPT2a, a type IIa sodium-phosphate cotransporter that is greatly expressed in the kidney. Phosphate homeostasis is dependent on the NPT2a protein family, which is located on the apical membrane of renal proximal tubular epithelial cells [11] .

Previous research has linked *SLC34A1* gene mutations to renal activity [12] , serum phosphorus levels [7] , and PTH [13] , suggesting that they may induce hypophosphatemia, nephrolithiasis, and osteoporosis.

In NPT2-deficient mice, increased phosphate (Pi) urinary excretion, a 70-80 percent decrease in the Na/ Pi co-transport, and hypophosphatemia have all been observed [14]. Hypophosphatemia can raise the level of 1, 25-(DH) 2D in the blood, and intestinal Ca channel overexpression can cause intestinal Ca hyperabsorption, resulting in hypercalcemia and hypercalciuria. Furthermore, parathyroid hormone(PTH), a well-known danger factor for nephrolithiasis, due to the negative feedback of hypophosphatemia, is at a low stage [15].

Wang *et al*. used 624 patients with nephrolithiasis and 1008 control subjects in a case-control sample in the Chinese Han population. They were examined for SNPs linked to nephrolithiasis, such as rs12654812 and rs11746443 on 5q32.3; rs12669187 and rs1000597 on 7q14.3; rs7981733, rs4142110, and rs17646069 on 13q14.1; and rs4293393 on 16p12. They reported that no one of the eight previously revealed SNPs were significantly associated with the threat of urolithiasis in the Chinese Han population, implying that the causes of calcium urolithiasis may vary among Chinese and Japanese ethnic groups [16] .

CONCLUSION

These findings indicate that the rs11746443 in the intron 12 of the *RGS14* gene can play a key role in renal calcium-containing stone disease susceptibility.

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