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Identification of Genetic Variants Associated with Chronic Kidney Disease Using Restriction Fragment Length Polymorphism

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ABSTRACT

Chronic kidney disease (CKD) is one of the most common kidney diseases that poses serious health risks. **Objective:** To identify the genetic variants associated with CKD. **Methods:** A cross-sectional study was conducted among 183 participants at Ibn-e-Sina Hospital, Multan, from Nov 2023 to May 2024. *Klotho (KL), Catalase (CAT), Cyclophilin (Cyp),* tumor protein p53 (p53), and Superoxide dismutase 1 (SOD1) genes were selected for polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP). Primers were designed via primer3plus and amplified via Snap Gene. In-silico RFLP involved NEB cutter and Snap Gene for the construction of the sequence map and the mutated band. Wet lab RFLP involved DNA cleavage by restriction enzymes. After PCR incubation, samples were visualized via gel electrophoresis. The T-Coffee tool aligns sequences, enabling the identification of variations in amino acids. **Results:** The target genes were successfully amplified in the CKD patients before proceeding to RFLP analysis. TaqI, EcoRI, AlwI, and AccI restriction enzymes were selected from in-silico Sequence Maps to cut the band of *SOD1, Cyp, KL,* and p53 genes, respectively. Cleave bands of mutated sequences were obtained. The alignment of wild-type to mutated with T-Coffee tool revealed six amino acid substitutions in CAT (Asp/Asn, Asp/Gly, Tyr/Asp, Lys/Arg, Gln/Ser and Ala/Val) and single amino acid substitutions in KL (*Phe/Val*), Cyp (*Ser/T*), SOD1 (*Gly/Arg*) and p53 (*Leu/unknown*). **Conclusions:** The current study identified genetic variants in *KL, CAT, Cyp, p53,* and *SOD1* genes that may influence CKD progression and therapy.

INTRODUCTION

Chronic kidney disease (CKD) is a common and serious medical condition affecting millions of people worldwide [1]. It is characterized by a progressive loss of kidney function over time, leading to complications such as hypertension, anemia, bone disease, and cardiovascular disease [2]. The best available indicator of overall kidney function is glomerular filtration rate (GFR), measuring the fluid filtered through functioning nephrons [3]. CKD is a non-communicable disease strongly associated with chromosome 22 [4]. CKD patients experience heightened genomic instability, leading to increased genetic and chromosomal damage from radiation and impaired DNA repair. This genetic damage may be both cause and consequence of CKD [5]. Several genes associated with

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CKD have been identified, affecting the risk and progression of the condition [6]. Genetic variations can have various effects on the body, ranging from no noticeable effect to serious health conditions [7]. It may impact protein function and lead to organ damage that influences disease development and progression [7]. For example, variations in the klotho gene can lead to a reduction in the expression of the klotho protein, which has been associated with a higher risk of age-related diseases, including CKD and Alzheimer's disease [8]. Genomic studies are critical in finding a correlation between a normal genome and its variations, as the genomic variant of a diseased person is present in non-coding regions [7]. Understanding the effects of specific variations in genes can help researchers to develop targeted treatments and therapies that address the underlying genetic causes of disease [9]. The use of molecular biology techniques in genetic research has contributed significantly to the identification of genetic variations associated with CKD [10]. Restriction Fragment Length polymorphism (RFLP) analysis is a key technique for identifying genetic variations. It can detect DNA sequence variations that alter DNA fragment lengths produced by restriction enzymes [11]. In addition to identifying genetic variations, RFLP analysis has several advantages over other genetic analysis methods. RFLP analysis can detect variations in genes even when the specific mutation is unknown. It can also be used to detect multiple variations (haplotype) in a single gene simultaneously [11]. Previously, RFLP has been successfully identified as an association of several polymorphisms in IL-6, *GPX1*, *SOD2*, and NRF2 genes in patients with CKD and end-stage renal disease (ESRD) [12, 13].

Pakistan does not have any extensive genetic association studies that focus on the contribution of *KL*, *CAT*, *Cyp*, *p53*, and *SOD1* gene variants to chronic kidney disease, and the bulk of local research is conducted on clinical and biochemical parameters but not regarding the molecular genetic phenotype. This study aimed to identify genetic variants associated with *CKD* by analysis of PCR-RFLP, which may help in creating targeted therapies and personalized treatment plans through the functional implications of detected polymorphisms.

METHODS

This cross-sectional study was conducted among 183 participants at Ibn-e-Siena Hospital and Research Institute, Multan, Pakistan, from Nov 2023 to May 2024. CKD was defined by the Kidney Disease: Improving Global Outcomes (KDIGO) guidelines. Ethical approval was obtained from Ibn-e-Siena Hospital and Research Institute, Multan, with the IRB/IEC number of C-70-1021-03. Informed consent was obtained from all participants according to the Declaration of Helsinki. Participants were eligible for inclusion based on the following criteria. Above the age of 18 years and willing to provide written informed consent were selected for this study. Along with this, for cases, confirmed CKD patients were selected with one of the following comorbid conditions associated with increased risk of CKD, such as diabetes, hypertension, cardiovascular disease, arthritis, and glomerulonephritis. While for the control age of 18 years, without CKD and associated risk factors, was included in this study. Participants were excluded from the study if they required dialysis therapy, were undergoing specific immune or steroid therapies, or had been diagnosed with uterine fibroids or cancer. Additional exclusion criteria included clinical histories of rheumatoid arthritis, systemic lupus erythematosus, acute infection, septic shock, hypotension, or a positive diagnosis of COVID-19. The present study included 183 participants. The sample size was determined to estimate the population parameter with a 95% confidence level and 5% absolute precision. The sample size was determined using Cochran's formula with finite population correction as described by Shemsu *et al.* [14]. The formula of Cochran's formula with Finite Population Correction is given below. n = NZ2P(1-P)/ d2(N-1) + Z2 P(1-P), where n = sample size with finite population correction (183), N = population size (349), Z = Z-value (1.96 for a 95% confidence level), P = expected proportion (0.5) and d = margin of error or precision (0.05). Whole blood sample (5mL) was collected from all participants in EDTA tubes. DNA extraction from blood samples was performed using a mammalian genomic DNA extraction kit (Axygen Biosciences, California, USA), following the manufacturer's recommendations, and stored at −80°C till the assay was performed. *Klotho* (*KL*), *Catalase* (*CAT*), *Cyclophilin* (*Cyp*), tumor protein *p53* (*p53*), and *Superoxide dismutase 1* (*SOD1*) genes were selected for polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP). The genetic sequences of selected genes were retrieved from NCBI (http://www.ncbi.nlm.nih.gov/nucleotide). The primer3plus web interface (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) was used for designing the primers. Designed primers were confirmed via the computer software Snap Gene (https://www.snapgene.com/). For in-silico RFLP, firstly, a sequence map of the coding DNA sequence (CDS) was extracted by NEB cutter (http://nc2.neb.com/NEBcutter2/), and then Snap Gene software was used to cleave the bands of the mutated sequences. In wet lab RFLP, restriction enzymes cleave DNA into fragments. Tubes were then undergone in PCR incubation at 37°C for 1-2 hours, followed by loading on 1% agarose gel electrophoresis for visualization. DNA sequencing by the Sanger Sequencer was employed to determine the precise nucleotide sequence (A, T, C, G) of

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the targeted genes within participants. For alignment of DNA sequences, the T-Coffee tool (https://www.ebi.ac.uk/Tools/msa/tcoffee/) was used. Identified nucleotide variations among samples and reference sequences, with variations highlighted in the obtained sequences.

SPSS version 25.0 and Microsoft Excel were used for data analysis. Quantitative variables (e.g., Age and Weight) were represented as mean ± standard deviation (SD) and statistically analyzed with the independent samples t-test, while qualitative variables were represented with frequency (n) and percentage (%) and statistically analyzed by Chi-square test and Fisher's Exact Test. To evaluate the association between genetic variants and CKD risk, genotype and allele frequencies were compared between groups. To determine the strength of the association, Odds Ratios (OR) and 95% Confidence Intervals (CI) were calculated. To account for the presence of zero values in the control group, the Haldane-Anscombe correction was applied by adding 0.5 to all cells in the 2x2 contingency tables. Statistical significance for all genotype and allele distributions was assessed using Fisher's Exact Test. p-values were reported to determine the statistical significance, with values less than 0.05 ($p<0.05$) indicating a significant association.

RESULTS

Out of 183 participants, 113 were CKD patients, and 70 were healthy controls. The average age was 46 ± 15 years within the CKD participants, while 42 ± 17 years within the control. The male-to-female ratio in the CKD group was 30:70, while in the control group, it was 40:60. CKD participants had a mean weight of 57 ± 15 kg, while controls had 58.5 ± 13 kg. The p-values for age, gender, and weight were greater than 0.05; these parameters were not statistically significant. The clinical and demographic characteristics of participants are given (Table 1).

Table 1: The Clinical and Demographic Characteristics of Participants

Parameters	CKD Patients (n =113), Mean ± SD/n (%)	Controls (n=70), Mean ± SD/n (%)	p-value
Age (Years)	46 ± 15	42 ± 17	0.05
Male	34 (30.1%)	28 (40.0%)	0.05
Female	79 (69.9%)	42 (60.0%)	0.05
Weight (kg)	57 ± 15	58.5 ± 13	0.05
Smoking	79 (69.9%)	10 (14.3%)	0.05
Ischemic Heart Disease	76 (67.3%)	22 (31.4%)	0.05
Family History	47 (41.6%)	34 (48.6%)	0.05
Hypertension	73 (64.6%)	0 (0%)	0.05
Type 2 Diabetes Mellitus	41 (36.3%)	0 (0%)	0.05
Hepatitis-B	7 (6.2%)	0 (0%)	0.05
Hepatitis-C	9 (8.0%)	0 (0%)	0.05

The genetic sequences of the *KL*, *CAT*, *Cyp*, *p53*, and *SOD1* genes were retrieved from NCBI, and their FASTA sequences were obtained for primer designing through primer3plus. The specificity and high efficiency of primers were achieved through the optimization of GC content, melting temperatures (Tm), and primer lengths. Because GC content and length are critical determinants of specificity and hybridization stability, these parameters were strictly controlled to ensure robust amplification during both PCR and DNA sequencing (Table 2).

Table 2: The Primers Used for Genotyping

Genes	Primer sequence	GC%	T_m	Restriction Enzyme
SOD1	F: CCAGTGCAGGGCATCATCAA	55	60.7	TaqI
	R: CAAGCCAAACGACTTCCAGC	55	60	
Tp53	F: TGAAGCTCCCAGAATGCCAG	55	60	AccI
	R: CAGTCAGAGCCAACCTCAGG	60	60	
CAT	F: AGTGATCGGGGGATTCCAGA	55	60	HinfI
	R: CCACCCTGATTGTCCTGCAT	55	60	
KL	F: TACAACAACGTCTTCCGCGA	50	60	AlwI
	R: GCTTAGGGCAATGGACACCT	55	60	
Cyp	F: CCGTGTTCTTCGACATTGCC	55	59.8	EcoRI
	R: TCGAGTTGTCCACAGTCAGC	55	60	

F: Forward; R: Reverse

Designed primers were confirmed via the computer software SnapGene. Successful amplification of genes was confirmed via gel electrophoresis. For *in-silico* RFLP, the sequence map of selected genes was obtained from the NEB Cutter tool. The sequence maps have different mutation sites and different enzymes (A-D). For mutation identification, TaqI, EcoRI, AlwI, and AccI restriction enzymes were selected from these Sequence Maps to cut the band of *SOD1, Cyp, KL,* and *p53* genes, respectively. Instead of performing the actual laboratory experiments, in silico RFLP software or online tools were utilized to simulate the digestion patterns based on the known recognition sites of the chosen restriction enzymes. To cleave the bands of the mutated genomic sequences, the current study created 1% gel, a 1 kb ladder, and used several enzymes via Snap Gene software. Specific bands were obtained, indicating that DNA fragments were cleaved by the selected restriction enzyme. TaqI enzyme cut the bands at 1 side, and two bands of 352bp and 80bp were obtained in the *SOD1* gene (E). The study cut the bands at 1 side, and two bands of 230 bp and 190 bp were obtained in the *p53* gene (F). The AlwI enzyme cut the bands at 1 side, and two bands of 691bp and 263bp were obtained in the *KL* gene (G). EcoRI enzyme cut the bands at 1 side, and two bands of 5872bp and 565bp were obtained in Cyp gene (H). The presence of cut bands suggests that the recognition site for the restriction enzyme is present in the DNA region of interest in the samples of CKD patients. A-D illustrate mutated sequence maps for the *SOD1*(A), *p53*(B), *KL*(C), and

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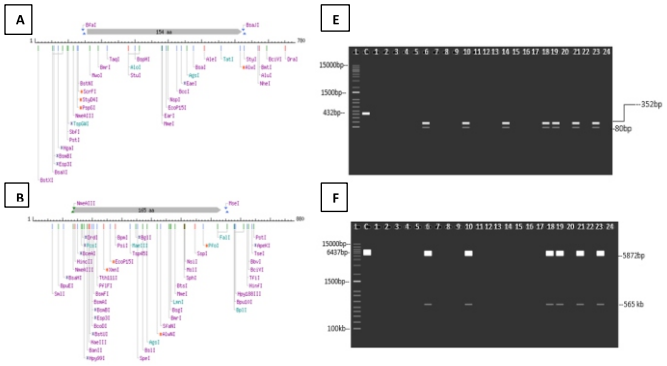
Cyp (D) genes, while E to H display the bands of mutated sequence *SOD1* (E), *p53* (F), *KL* (G), and *Cyp* (H) genes, respectively. L: Ladder, C: control, and Well 1-24: samples in E to H (Figure 1).



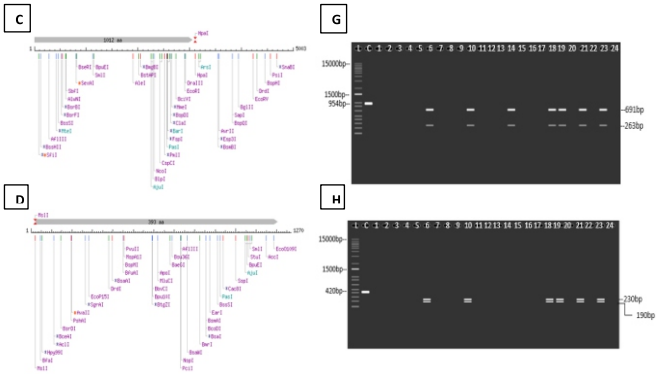


Figure 1: Mutated Sequence Map and Bands of *SOD1*, *p53*, *Klotho*, and *Cyclophilin* Genes

In the wet lab, selected restriction enzymes from in silico RFLP were used to cleave DNA into fragments, but the *CAT* gene failed to provide restriction enzymes from in silico RFLP. Therefore, we utilized the HinfI restriction enzyme for the *CAT* gene analysis. DNA sequencing was used to determine the precise nucleotide sequence (A, T, C, G) of the targeted genes within the CKD cohort. This approach allows for the identification of specific genetic variants that may serve as markers for disease susceptibility, phenotypic severity, and individualized treatment response. DNA sequencing revealed several distinct genetic variations within the CKD cohort. Among them, significant polymorphisms were observed in the *KL* (T/G; n=60), *SOD1* (G/C; n=22), and *p53* (unknown; n=17) genes; none of them were present in the control group. Conversely, polymorphisms in the Cyp (T/A; n=10 vs. n=2 in controls) and CAT (haplotype; n=4) genes were observed but did not reach statistical significance (Table 3).

Table 3: Frequency Distribution among CKD Patients and Control Groups

Genes	Genotype Frequency			Allele Frequency			Odd Ratio	95% CI	p-value
	Genotype	CKD (n=113)	Control (n=70)	Allele	CKD (n=113)	Control (n=70)			
SOD1	G/C	22 (19%)	0 (0%)	G	204 (90.3%)	140 (100%)	34.67	2.08-584.6	<0.05
				C	22 (9.7%)	0 (0%)			
p53	CTG/ NNN	17 (15%)	0 (0%)	CTG	209 (92.5%)	140 (100%)	25.57	1.52 - 428.37	<0.05
				NNN	17 (7.5%)	0 (0%)			
CAT	Multiple (Haplotype) (G/A, A/G, T/G, A/G, C/T, C/T)	4 (3.5%)	0 (0%)	Non-Haplotype	222 (98.2%)	140 (100%)	5.80	0.31-109.95	>0.05
				Haplotype	4 (1.8%)	0 (0%)			
KL	T/G	60 (53%)	0 (0%)	T	166 (73.5%)	140 (100%)	159.45	9.68-2617.49	>0.05
				G	60 (26.5%)	0 (0%)			
Cyp	T/A	10 (8.8%)	2 (2.8%)	T	216 (95.6%)	138 (98.6%)	2.68	0.66-10.84	>0.05
				A	10 (4.4%)	2 (1.4%)			

NNN indicates a non-specific variant fragment length detected by AccI

Based on the CLUSTAL W alignment, the mutated proteins exhibited specific amino acid substitutions. Among them, alignment of wild-type to mutated catalase protein sequences revealed six amino acid substitutions (A). The amino acid substitutions in CAT are: aspartic acid to asparagine, aspartic acid to glycine, tyrosine to aspartic acid, lysine to arginine, glutamine to serine, and alanine to valine (A). The CLUSTAL W alignment of wild-type to mutated *KL*, *Cyp*, *SOD1*, and *p35* protein sequences revealed single amino acid substitutions. *KL* shows an amino acid substitution of phenylalanine to valine (B). p35 shows an amino acid substitution of leucine to unknown (C). *Cyp*, show the amino acid substitution of serine to threonine (D). *SOD1* shows an amino acid substitution of glycine to arginine (Figure 2E). The observed variations differed in their structural impact; single substitutions (*SOD1*, *p53*, *KL*, *Cyp*) involve a point-specific residue change within a conserved sequence, whereas the multiple substitutions in CAT represent a complex haplotypic variation. Even a single amino acid substitution can significantly disrupt the protein function by altering structural stability or impairing enzymatic activity. T-Coffee alignment clearly shows substitutions in CKD patients, allowing researchers to pinpoint variations that may lead to functional loss or drug resistance (Figure 2).

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Figure 2: Variations in the Sequence by Protein Alignment via the T-Coffee tool. A-D Shows Mutation in the Sequence of *CAT* (A), *KL* (B), *p53* (C), *Cyp* (D), and *SOD1* (E) Proteins, Respectively

DISCUSSION

The current study investigation identified several genetic variations within the *KL*, *CAT*, *Cyp*, *p53*, and *SOD1* genes that demonstrate a strong association with CKD pathogenesis. Overall, our findings emphasize the importance of considering genetic factors in the development of CKD, as evidenced by genetic mutation analysis using RFLP analysis and Snap Gene software. Researchers utilized in silico RFLP analysis to investigate genetic variations in CKD, employing bioinformatics tools to simulate enzymatic digestion patterns of DNA sequences from CKD patient samples with specific restriction enzymes [15]. This *in silico* method predicts fragment patterns and identifies genetic variations linked to CKD susceptibility or progression. By integrating *in silico* RFLP analysis with experimental validation and clinical data, researchers seek to understand genetic factors influencing CKD diagnosis and treatment [16]. The current study used the BLAST tool on the NCBI database to identify mutated sequences in the genes of interest and then used Snap Gene software to analyze the sequences and cut them with different enzymes to identify mutations. Researchers identified specific polymorphisms within the CAT and *SOD1* genes in CKD patients. CAT and *SOD1* gene polymorphisms in CKD patients were also observed by Crawford *et al.* who reported that the SOD2 Val/Val genotype increased end-stage renal disease (ESRD) risk, which was even higher in combination with the *GpX1* polymorphism [17]. These findings are parallel with the existing research of Kidir *et al.* on acute kidney injury (AKI), where polymorphisms in SOD, *GpX1*, and CAT have also been observed [18]. AKI is marked by a rapid decline in glomerular filtration rate, leading to significant morbidity and possible chronic kidney disease onset. The overlap of antioxidant genes across both CKD and AKI underscores the critical role of oxidative stress as a common driver in the progression of various forms of renal disease [19]. Researchers' analysis found a strong association between *KL* gene variations and CKD, aligning with previous studies [20, 21]. The polymorphism of the *KL* gene was most prevalent among detected CKD patients compared to other genes in our study. Hassan *et al.* also found a polymorphism of the *KL* gene within CKD patients that shows the association of the *KL* gene with CKD [21]. The current study analysis revealed that genetic variation in the *Cyp* gene was also strongly associated with CKD, but the results of *Cyp* gene polymorphism were non-significant in our study and were also observed in 2 control patients, which suggests that this gene may play a role in the development and progression of CKD. Based on previous findings, *Cyp* genes play a critical role in protein folding and are significantly upregulated during kidney injury, acting as a stress response mechanism [22, 23]. The association of these genetic variants with decreased renal function highlights the potential of *Cyp* genes not only as markers of injury but as key players in the progression of renal dysfunction, supporting the hypothesis that dysregulated protein folding contributes directly to the pathogenesis of chronic renal failure. Recent studies have suggested that variations in the *p53* gene may be associated with CKD development [24]. This is particularly underscored by the work of Wu *et al.* who demonstrated that targeting the Tp53RK pathway can effectively mitigate renal fibrosis. Collectively, these results suggest that *p53*-mediated pathways, specifically those governing apoptosis and fibroblast activation, are central to the maladaptive repair processes that drive CKD progression [25]. The consistency between current results and earlier reports strengthens the understanding of the pathogenesis of CKD and highlights the significance of these genetic factors in the disease. The current study enhances the understanding of the genetic basis of CKD and reveals genetic variants that have potential for personalized interventions based on genetic profiles to improve patient outcomes. The study successfully identified genetic variants in target genes using cost-effective RFLP analysis, known for its specificity and sensitivity.

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However, limitations include a relatively small sample size that may hinder the detection of all variations and a cross-sectional design that prevents establishing causal relationships between the variations identified and CKD. Despite these limitations, our findings have important clinical implications. Future research should explore the significance of these genetic variants and validate findings in diverse populations. Additionally, methods like RFLP analysis should investigate further genetic variants related to CKD, potentially impacting prevention and treatment strategies.

CONCLUSIONS

The current study identified genetic variants related to CKD using RFLP analysis, including variations in the *KL*, *p53*, *CAT*, *SOD1*, and *Cyp* genes. The current findings suggest that these genetic variants may influence CKD development and progression, suggesting that targeting them could offer new therapeutic strategies.

Authors' Contribution

Conceptualization: ZHQ
Methodology: ZHQ, AJ
Formal analysis: ZHQ, MI, AI, BF
Writing and Drafting: ZHQ, MI, AI, AJ, SA, MJK
Review and Editing: ZHQ, MI, AI, BF, AJ, SA, MJK

All authors approved the final manuscript and take responsibility for the integrity of the work.

Conflicts of Interest

All the authors declare no conflict of interest.

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