KL rs9536314 variant influencing mineral homeostasis and oxidative stress in diabetic CKD

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Abstract
Chronic kidney disease (CKD), often accompanied by mineral and bone disorders, is worsened by diabetes. The Klotho-Fibroblast growth factor 23 (KL-FGF23) axis regulates calcium and phosphorus balance and is linked to CKD progression. This study investigated the rs9536314 KL gene polymorphism, serum KL and FGF23 levels, oxidative stress and bone markers in pre-dialysis diabetic CKD patients. A total of 100 subjects, comprising of 50 pre-dialysis diabetic patients and 50 healthy people, were enrolled in the present cross-sectional study. Patients showed significantly reduced serum KL (2.55±0.77 ng/mL vs. 4.51±0.65 ng/mL, $P < 0.001$) and elevated FGF23 (84.96±9.62 ng/mL vs. 22.83±6.74 ng/mL, $P < 0.001$) compared to the control group. In addition, patients exhibited higher parathyroid hormone and phosphorus levels, indicating disturbed mineral homeostasis. Oxidative stress markers, including malondialdehyde and pro-oxidant/antioxidant balance, were significantly elevated, whereas glutathione peroxidase activity was reduced in pre-dialysis patients, depicting a pronounced oxidative stress state.

Genetic analysis of the rs9536314 KL polymorphism revealed no significant differences in genotype or allele frequencies between groups (TT: OR = 0.77, 95% CI: 0.33–1.75; TG: OR = 1.40, 95% CI: 0.63–3.11; GG: OR = 0.65, 95% CI: 0.10–4.08; allele G: OR = 0.99, 95% CI: 0.55–1.78; all $P > 0.4$), indicating that this polymorphism may not directly contribute to diabetic pathogenesis in pre-dialysis patients. Overall, while the KL rs9536314 polymorphism did not show a significant association with disease risk, the pronounced alterations in Klotho, FGF23 and oxidative stress markers reveal their potential role as biomarkers and therapeutic targets in CKD progression.

Keywords: Diabetes mellitus, Klotho, Fibroblast growth factor 23, Pre-dialysis patients, Polymorphism.

Introduction

Diabetes mellitus (DM) is a chronic metabolic condition with continuous hyperglycemia and unnecessarily increased blood glucose levels. It could be brought on by decreased insulin secretion, resistance to insulin's peripheral effects, or a combination of the two[49]. Higher blood glucose levels result from defects in insulin synthesis or activity[38]. The two main subtypes of DM are type 1 and 2 diabetes mellitus (T1DM and T2DM) and both are primarily caused by deficiencies in insulin secretion and/or action[1]. DM is proving to be a burden on public health globally, with estimates predicting that by 2040, there will be an additional 200 million cases[9]. Diabetes complications raise the risk of death and produce a lower quality of life. These complications can affect multiple organs and the entire body including the heart, kidneys, nerves and blood vessels.

Some of these problems clearly contribute to the rise in diabetes-related mortality[25]. One of the primary causes of chronic kidney disease (CKD), which is defined by a persistent decline in kidney function, is DM. In recent years, CKD has become a major health concern that affects people on many continents[37]. New molecular mechanisms that could affect the development and severity of diabetes have been proposed by recent research. Fibroblast growth factor-23 (FGF23) and α-klotho (KL) are two molecules of interest that are involved in these processes[35,45].

KL is a single-pass transmembrane protein (130 kDa), which was initially introduced as an anti-aging molecule. In 1997, Kuro-o et al[20] discovered α-KL, a new gene that slows down aging. The KL protein, encoded by the *KL* gene, is a regulatory protein that plays a main role in maintaining the body's metabolic balance, especially in regulating phosphate homeostasis[13]. KL directly interacts with FGF receptor 1 (FGFR1) and regulates the biological activities of FGF23[19,41]. Any alteration in the expression of the KL gene results in skin atrophy, arteriosclerosis, infertility, or a shortened lifespan. This suggests that KL is essential to the impact of FGF23[21].

The kidneys and the brain's choroid plexus are where KL is primarily expressed. It was also expressed in the pituitary, parathyroid glands, heart and reproductive organs, although in small amounts in other tissues. Podocytes and cells that make up the proximal tubule of the nephron all contain KL in the kidneys. Additionally, KL is lost into the lumen of the proximal tubule[23,43]. According to studies, KL plays a part in the central regulation of energy balance, controls the metabolism of fat and glucose and inhibits insulin signaling[4,10].

KL is predominantly expressed in the kidney. Therefore, its deficiency has a critical role in the pathogenesis and

development of various kidney diseases such as chronic kidney disease (CKD)[46]. According to previous reports, KL level decreases in patients in the early and intermediate CKD stages, preceding the elevation of serum creatinine level; the KL level decreased further in advanced stages[26]. Lower KL levels in pre-dialysis patients are linked to greater negative kidney outcomes, such as CKD progression, renal replacement therapy, mortality, serum creatinine doubling and CKD-mineral bone diseases (CKD-MBD)[26,46].

Therefore, KL is thought to be a promising biomarker for the early diagnosis and prognosis of CKD. KL is a FGF23 co-receptor and the FGF23–KL system is crucial in reducing hypophosphatemia by increasing renal phosphate excretion through the activity of renal phosphate transporters. Abnormal calcium and phosphorus metabolism or significant vascular calcification are associated with KL insufficiency[48].

By considering the main actions and roles of KL and FGF23, their gene polymorphisms are associated with several diseases. The main objective of the present work was to investigate the association between KL gene polymorphisms and calcium-phosphate homeostasis in pre-dialysis diabetic patients.

Material and Methods

Materials: 3,3′,5,5′-tetramethylbenzidine (TMB), dimethyl sulfoxide (DMSO), sodium hydroxide (NaOH), hydrogen peroxide (H_2O_2; 30%), hydrochloric acid (HCl), sodium acetate (CH_3COONa) and acetic acid (CH_3COOH) were purchased from Sigma Aldrich (USA). Chloramine T (ChT) and uric acid were purchased from Merck Co. (Germany). Peroxidase enzyme was purchased from Applichem (230 U/mg, A3791, 0005, Darmstadt, Germany).

Patients and samples: A total of 50 pre-dialysis patients aged 25–80 years who were not undergoing dialysis treatment, were referred to the Nephrology Department, affiliated to Tabriz University of Medical Sciences and 50 healthy people were included in the present cross-sectional study after taking written informed consent from the patients prior to the study. The ethics committee of Tabriz University approved this study (ethical code: IR.TABRIZU.REC.1403.067). A power analysis based on previous studies found that 80% statistical power at a 5% significance level required at least 45 volunteers per group. For enhancing statistical robustness and accounting for potential participant dropout, the sample size was adjusted to 50 subjects per group, resulting in a total of 100 individuals.

Participants must be at least 18 years old, have a diagnosis of chronic irreversible renal failure and have made at least one visit to a pre-dialysis clinic. The exclusion criteria were pregnancy, serious viral infections (e.g. HIV, CMV and HBV) and age under 18 or over 80 years. The control group, sourced from Hakim Laboratory in Tabriz, consisted of adults over 30 years old without a CKD history. To enhance the validity of comparative analysis, a matching strategy for control subjects and pre-dialysis patients was utilized based on five key characteristics: age, sex, body composition indices, smoking habits and metabolic health markers. These additional factors were integrated into the analytical model to bolster the robustness of our findings and reduce potential confounding effects.

The statistical study also included other parameters that were gathered through questionnaires including medication history, food habits and physical activity. We certify that all procedures followed the Declaration of Helsinki and all other applicable rules and laws.

Biochemical analysis and clinical assessments: 5 mL of fasting venous blood samples was collected from both patients and control subjects. All the samples were drawn into EDTA and gel separator with clot activator tubes. Then, the collected blood samples were centrifuged at 3000 rpm for 15 min and the separated serum and plasma were stored at –80 °C in a deep freezer. The prepared fasting serum samples were used to determine serum levels of urea, creatinine, calcium (Ca), phosphorus (P), parathyroid hormone (PTH), alkaline phosphatase (ALP), blood sugar and lipid profile such as triglycerides (TG), cholesterol, low-density lipoprotein (LDL) and high-density lipoprotein (HDL) were determined using advanced analytical equipment (Roche Cobas C311 system).

Furthermore, the serum concentrations of KL and FGF23 were estimated using commercially available human enzyme-linked immunosorbent assay kits (ELISA) (Human FGF23 and KL ELISA Kits, ZellBio, GmbH, Germany). With a detection threshold of 0.25 ng/ml and reliability metrics indicating variation below 5% within tests and 8% between tests, this testing technique showed great precision.

Pro-oxidant anti-oxidant balance (PAB) analysis: PAB assays were performed according to the previously reported method[2,3]. For this purpose, three different solutions including TMB, cationic TMB (TMB^+) and working solutions, were prepared. The stock solution of TMB was prepared by dissolving 0.06 g of the TMB powder in 10 mL of DMSO. The TMB solution was developed by adding 200 µL of TMB stock solution in 10 mL of acetate buffer (50 mM; pH 5.8). Furthermore, to prepare TMB^+, 400 µL of the prepared TMB stock solution was added to 20 mL acetate buffer (50 mM, pH 4.5) and then 70 µL fresh ChT (100 mM) was added to the reaction solution and shaken well. After incubating the mixture for 2 hours in a dark place at 25 °C, 25 U of peroxidase enzyme was mixed with the prepared solution. This mixture was aliquoted into 1 mL and stored at –20 °C until used for analysis.

The working solution was prepared by mixing 1 mL of TMB^+ and 10 mL of TMB solution and incubating in a dark place for 2 min at 25 °C. In each well of a 96-well plate, 10

µL of each separated serum sample, standard solution (prepared by inserting different proportions (0-100%) of H_2O_2 (250 µM) to 3 mM uric acid in 10 mM NaOH) or blank (distilled water) and 200 µL working solution were mixed well and incubated in a dark place for 12 min at 37 °C. Subsequently, 100 µL of HCl (2 N) was mixed with the prepared mixture and the optical density of the samples was recorded using an Elisa reader (Bio Tek instruments, Inc., USA) at 450 nm, with a reference wavelength of 620 or 570 nm.

The values of the PAB were expressed in arbitrary Hamidi-Koliakos (HK) units, which represent the percentage of H_2O_2 in the standard solution. The values derived from the standard curve above, plotted using the standard samples, were then used to compute the values of the unknown samples.

DNA extraction and genotyping of the KL gene: Venous blood samples were obtained for genomic DNA isolation which was performed using the SimEXTM Blood DNA Extraction Kit (Simbiolab®, Iran). The extracted DNA was analyzed for the concentration and purity by NanoDrop 2000 spectrophotometer (DeNovix, USA) at 260/280 nm and gel electrophoresis (on a 2% agarose gel). The rs9536314 polymorphism was identified by Tetra-primer ARMS-PCR by using two common forward outer and reverse outer primers and two T and G allele-specific primers for the simultaneous detection of two alleles in a single reaction and two forward and reverse inner allele-specific primers (Table 1). The primers for this study were designed using the NCBI database, Primer Blast and Gene Runner software.

PCR was done in a thermal cycler (Sensoquest, GmbH, Germany) in a 0.2 mL PCR tube. The reaction mixture was of 1.0 µL of DNA template (100 ng/µL), 0.7 µL of each primer, 6.0 µL Green PCR master mix (2x) and 4.3 µL DNase-free water (a total volume of 12 µL). PCR was run in the thermal cycler according to the following conditions: DNA denaturation at 95 °C (5 min) accompanied by 30 cycles of 95 °C for 30 secs, annealing at 58 °C for 30 secs, extension at 72 °C for 30 secs and an ultimate extension at 72 °C for 10 min. The PCR products were visualized by electrophoresis on a 2.0% agarose gel. Genotypes were determined based on the presence of allele-specific bands.

Table 1
Designed T-ARMS-PCR primers for rs9536314 genes

SNP ID	Primer set and sequence ($5' \rightarrow 3'$)	Tm (°C)	Annealing T (°C)
rs9536314	FO: CAGGTGTCCATTGCCCTAAG	60	58
	RO: GAGAACTTTACCCACAATCATACCT	63	
	FI: AACCTTTCATCTATTCTGCCTGATT	61	
	RI: GATGACCTTTTTCTCAGATTCAGTAAC	64	

Table 2
Clinical characteristics of the population studied (diabetic patients and control subjects).

Parameters		Patients Mean±SD (n=50)	Control Mean±SD (n=50)	P value
Age (years)		61.00±16.58	59.39±15.87	0.616
Sex	Female; n (%)	21 (42)	24 (48)	0.549
	Male; n (%)	29 (58)	26 (52)	
FBS (mg/dL)		139.56±12.44	87.36±10.64	0.0001***
Urea (mg/dL)		104.50±14.94	38.10±13.95	0.0001***
HDL (mmol/L)		38.24±3.73	40.14±3.97	0.021*
Creatinine (mg/dL)		3.50±1.65	1.04±0.20	0.0001***
Cholesterol (mg/dL)		179.33±22.60	168.41±17.75	0.007**
TG (mg/dL)		199.45±31.39	102.13±23.04	0.0001***
PTH (pg/mL)		298.28±14.89	53.69±17.80	0.0001***
LDL (mmol/L)		121.50±17.83	108.18±15.40	0.030*
Ca (mg/dL)		9.07±0.68	8.85±1.08	0.501
P (mg/dL)		7.98±1.73	4.42±1.04	0.0001***
ALP (U/L)		334.48±33.43	220.14±35.02	0.0001***
MDA (nmol/L)		5.49±1.16	1.88±0.67	0.0001***
GPx (U/g Hg)		12.69±4.97	14.91±4.26	0.025*

Statistical analyses: IBM SPSS Statistics 25 was used for statistical analysis on the collected data and the findings were displayed as mean ± standard deviation (SD). All continuous variables were tested for normal distribution with the Kolmogorov-Smirnov test. Comparisons between two studied groups (patients and control groups) were assessed with the student t-test or Mann-Whitney U test and the χ^2-test for categorical variables. To investigate the correlations between different variables, Pearson's rank or Spearman correlation analyses were applied. The association between genotypes for the rs9536314 SNP in patients and control individuals was evaluated by odds ratios (OR) and 95% confidence intervals (CI) with homozygotes major alleles as references using additive models. A two-tailed $P < 0.05$ was considered statistically significant. The receiver operating characteristic (ROC) curve was conducted to investigate the potential of the variables as biomarkers.

Results

Biochemical analysis: We studied 50 pre-dialysis patients with chronic renal failure and 50 healthy people in the present study. The demographic, clinical and biochemical characteristics of the patients and control subjects are presented in table 1. The demographic analysis demonstrated balanced group characteristics. As can be seen, the age profiles of the patients and reference group were 61.00±16.58 (ranging from 25-85) and 59.39±15.87 years (ranging from 27-83 years) (p=0.616). The male-to-female ratio was 58% vs. 42% in the pre-dialysis patients group (p=0.549).

The control group matched with the patient group in terms of age and gender. The results indicated that no significant differences were observed in the serum concentrations of LDL, Ca and cholesterol between the two studied groups. However, FBS, urea, P, PTH, FGF23 and ALP concentrations were significantly elevated in pre-dialysis patients in comparison with reference group (P<0.01). In addition, a significant reduction was detected in the serum levels of HDL, creatinine, TG and KL in patients when compared to the healthy group (P<0.01).

Pro-oxidant anti-oxidant balance (PAB) assay results: The mean PAB value in pre-dialysis patients was 96.84±15.52 and in healthy volunteers, it was 63.74±19.33 HK, which was significantly higher in pre-dialysis patients with CKD when compared to control subjects (P < 0.001). In addition, pre-dialysis patients exhibited higher concentrations of FGF23 (84.96 ± 9.62 vs. 22.83 ± 6.74 ng/ml, p < 0.001) and decreased serum levels of KL (2.55 ± 0.77 vs. 4.51 ± 0.65 ng/ml, p < 0.001) (Figure 1).

The results indicated that serum FBS values were positively correlated with FGF23 (r = 0.392, P=0.018) in pre-dialysis group. Furthermore, there was also negative correlation between creatinine and age (r = –0.378, P= 0.009), creatinine and cholesterol (r = –0.331, P=0.046) and cholesterol and KL (r = –0.332, P=0.037). Also, the results showed statistically significant and positive correlation between GPx and cholesterol (r = 0.384, P=0.017) (Figure 2).

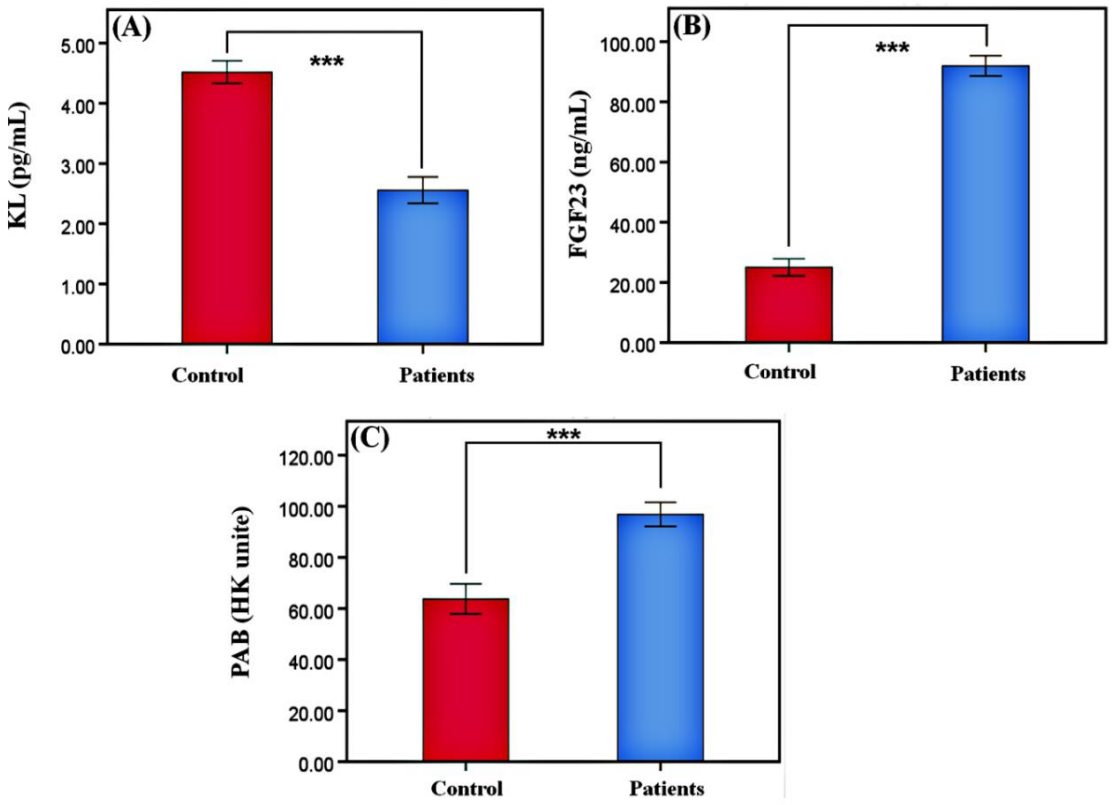

Figure 1: The serum levels of KL (A), FGF23 (B) and PAB values (C) in pre-dialysis diabetic patients and control group. HK unit: Hamidi-Koliakos arbitrary unit based on the percentage of H_2O_2 evaluated in standard solution.

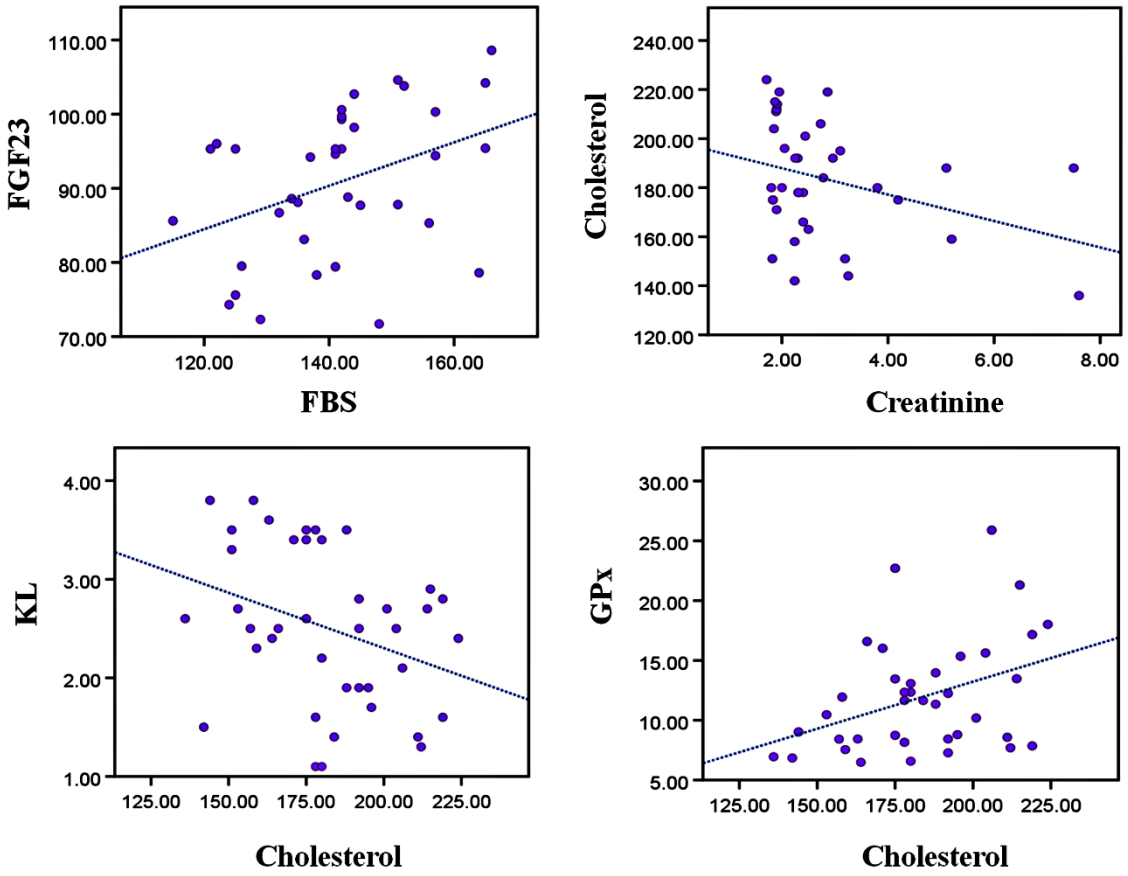

Figure 2: The correlation analysis between different studied biochemical factors.

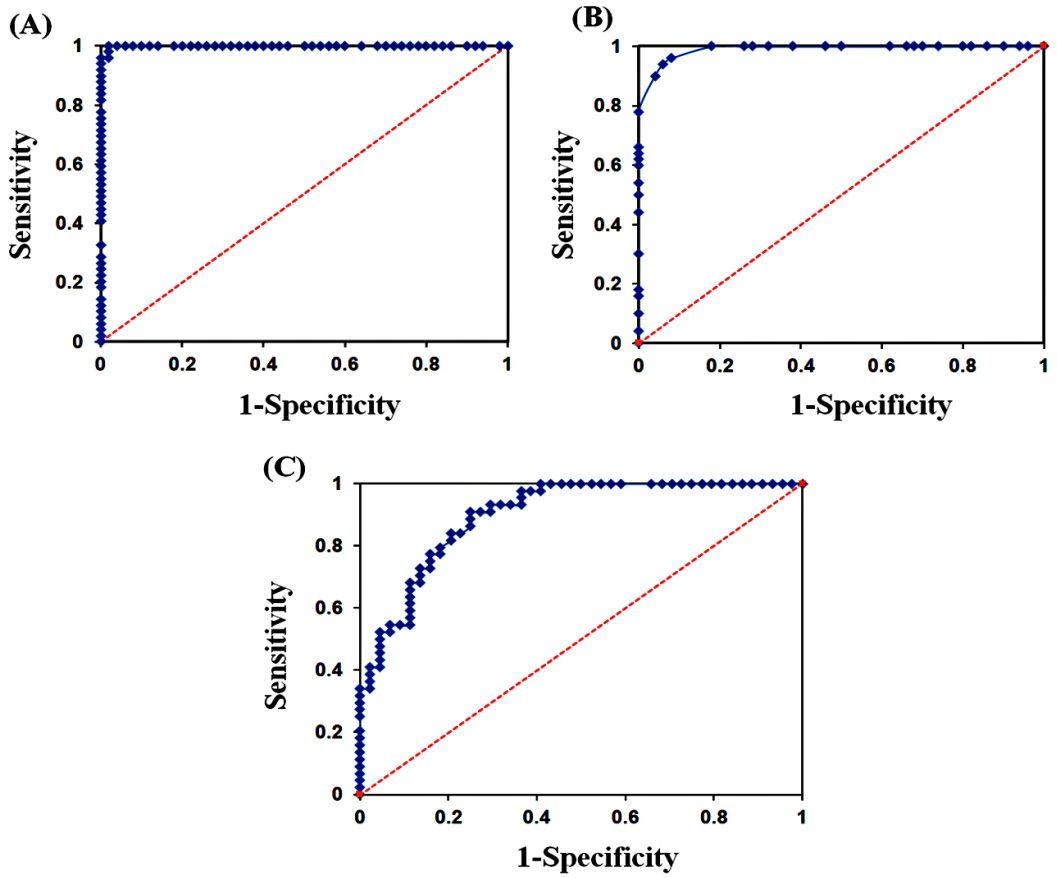

Figure 3: The ROC curves for evaluating the performance of (A) FGF23, (B) KL and (C) PAB in predicting CKD.

In addition, the ROC curves were conducted for FGF23, KL and PAB to evaluate the potential of these factors as biomarkers to predict the incidence of CKD in pre-dialysis patients. The calculated area under the curves (AUCs) were 0.99, 0.98 and 0.90 for FGF23, KL and PAB respectively. These results confirmed the predictive power of FGF23 and KL with a sensitivity of 99% and 96% and specificity of 98% and 92% respectively. While PAB (sensitivity=84%, specificity=79%) showed potential as a biomarker, its performance was weaker compared to FGF23 and KL (Figure 3). This difference suggests that FGF23 and KL may be more reliable indicators for CKD, offering better diagnostic or prognostic utility.

Genotype and allele frequency distribution: The genotype and allele frequency distribution of the rs9536314 variant between each group was studied to evaluate the potential association between the studied gene polymorphism and the risk of CKD. Table 4 shows the results of the statistical analysis of the data. According to this table, genotypic and allelic frequency analysis of rs9536314 polymorphism showed that none of the genotypes (TT, TG and GG) or alleles (alone or in combination) have a significant difference between the CKD patients and the control group (P>0.05). Thus, this polymorphism cannot be specifically associated with CKD in the pre-dialysis patients.

Effects of KL gene polymorphism on clinical, hormonal and bone metabolism biomarkers: The possible effects of KL genetic polymorphism on the serum levels of clinical, hormonal and bone metabolism biomarkers were investigated and the outputs are presented in table 4. The KL TT genotype resulted in a statistically significant rise in the FBS, urea, TG, PTH, ALP and FGF23 levels and a significant decrease in the serum concentrations of HDL, P and KL (P < 0.05). In addition, in individuals with the KL TG genotype, the serum levels of FBS, urea, LDL, TG, PTH, creatinine, ALP and FGF23 significantly increased (P < 0.05). However, a statistically significant decrease was detected in the serum levels of HDL, P and KL (P <0.05).

Unlike TT and TG genotypes, in the GG genotype, no significant differences were observed in the biochemical or hormonal parameters between the control group and the patients (p>0.05 for all parameters). This means that the GG genotype may have a protective effect or at least have no specific effect on metabolism and disease progression. Patients with this genotype are biochemically similar to the control group. The results indicated that the TT and TG genotypes showed significant changes in most parameters between patients and controls. This indicates the potential role of these genotypes in disease severity or progression.

Discussion

In this work, we studied the association of the rs9536314 KL gene polymorphism with bone metabolic markers and PAB in pre-dialysis diabetic patients in comparison with healthy control group. Our data (Tables 2 and 3) indicated that diabetes causes significant changes in markers of bone metabolism, renal function and oxidative stress. The findings of this study revealed that the pre-dialysis diabetic patients have lower serum concentrations of soluble KL and elevated levels of FGF23 compared to healthy subjects. Kumar et al[18] studied the association between KL and cardiac abnormalities in pre-dialysis CKD patients. They indicated that the serum concentrations of KL and FGF23 were decreased and increased in pre-dialysis CKD patients respectively (P < 0.001). According to reports, FGF23-KL signaling pathway prevents renal phosphate reabsorption in the kidney by internalizing sodium-dependent phosphate cotransporters and inhibits the synthesis of 1,25-dihydroxyvitamin D [1,25(OH)2D] by changing the enzymes that metabolize vitamin D. Loss of KL causes severe hyperphosphatemia and hypervitaminosis D by impairing the binding of FGF23 to FGFR1.

Table 3
Genotype and allele frequencies of rs9536314 polymorphisms in CKD patients and control group

SNP	Frequencies n (%)		OR (95% CI)	*P* value
	Control (50)	Patient (50)		
rs9536314				
TT	16 (32)	19 (88)	0.77 (0.33-1.75)	0.529
TG	32 (64)	28 (56)	1.40 (0.63-3.11)	0.414
GG	2 (4)	3 (6)	0.65 (0.10-4.08)	0.646
Alleles				
T	36 (36)	34 (35.8)	-	-
G	64 (64)	61 (64.2)	0.99 (0.55-1.78)	0.976
TT	16 (32)	19 (88)	-	-
TG+GG	34 (68)	31 (62)	0.77 (0.33-1.75)	0.529
TG	32 (64)	28 (56)	-	-
TT+GG	18 (36)	22 (44)	1.40 (0.63-3.11)	0.414
GG	2 (4)	3 (6)	-	-
TG+TT	48 (96)	47 (94)	0.65 (0.10-4.08)	0.646

Table 4
Clinical characteristics and bone metabolism biomarkers levels in pre-dialysis patients with CKD and controls

Parameters	TT (mean±SD)			TG (mean±SD)			GG (mean±SD)		
	Control	Patients	*P*	Control	Patients	*P*	Control	Patients	*P*
Age	59.06± 16.82	63.94± 12.67	0.289	59.12± 14.93	57.78± 16.02	0.740	50.00± 12.72	60.00± 17.43	0.800
FBS	87.75± 12.63	135.55± 12.90	0.0001***	86.75± 12.40	145.90± 13.13	0.0001***	83.50± 6.36	131.50± 7.37	0.333
Urea	38.81± 12.51	107.20± 16.20	0.0001***	39.90± 14.80	101.92± 12.78	0.0001***	26.50± 4.94	96.01± 8.88	0.200
HDL	42.18± 3.50	37.94± 4.50	0.004**	39.00± 3.91	38.32± 3.24	0.466	42.00± 0.05	39.33± 3.78	0.400
LDL	109.30± 16.30	114.75± 11.45	0.312	111.56± 13.84	123.17± 15.38	0.010**	106.00± 11.31	102.80± 11.30	0.800
Cholesterol	168.58± 16.69	184.87± 20.48	0.043	167.03± 19.41	180.76± 25.75	0.510	169.00± 12.72	170.66± 25.79	0.800
TG	100.40± 22.84	191.61± 25.51	0.0001***	99.53± 23.65	200.90± 27.98	0.0001***	124.02± 12.72	231.66± 4.72	0.200
PTH	52.20± 15.91	289.72± 18.92	0.0001***	57.86± 18.23	293.59± 17.72	0.0001***	47.50± 26.16	321.20± 8.20	0.333
Creatinine	0.98± 0.13	2.92± 1.54	0.0001***	1.07± 0.23	3.14± 1.82	0.0001***	0.99± 0.07	2.28± 0.03	0.200
Ca	8.86± 0.52	9.16± 0.69	0.119	8.81± 1.31	8.77± 1.25	0.645	9.25± 0.07	9.40± 0.26	0.800
P	4.28± 0.86	7.37± 2.40	0.001***	4.45± 1.15	6.66± 2.65	0.0001***	5.00± 0.01	6.63± 1.60	0.400
ALP	209.68± 31.70	326.36± 37.17	0.0001***	227.37± 33.66	337.92± 31.16	0.0001***	188.00± 66.46	353.66± 22.36	0.200
KL	4.57± 0.69	2.48± 0.90	0.0001***	4.47± 0.62	2.61± 0.67	0.0001***	4.80± 1.27	2.50± 1.00	0.200
FGF23	25.15± 11.85	90.15± 9.28	0.0001***	24.88± 8.41	93.00± 11.75	0.0001***	26.80± 0.03	92.01± 11.15	0.500
GPx	15.02± 4.28	13.89± 5.43	0.309	15.21± 4.23	12.24± 4.68	0.021*	9.90± 2.47	9.20± 2.83	0.800
MDA	2.01± 0.95	5.36± 1.12	0.0001***	1.83± 0.52	5.61± 1.19	0.0001***	1.62± 0.39	5.31± 1.96	0.333
PAB	68.62± 19.61	95.00± 16.59	0.0001***	60.63± 18.81	96.08± 13.08	0.0001***	69.43± 28.15	121.91± 22.83	0.200

Additionally, KL is expressed in the parathyroid gland where the production and release of PTH are inhibited by FGF23-KL axis. Therefore, reducing KL production in pre-dialysis patients can increase PTH levels by blocking the FGF23-KL axis[17,28]. In accordance with these reports, in the present study we discovered that the serum level of KL reduced and the concentrations of FGF23 and PTH got elevated in pre-dialysis CKD patients. Studies have consistently shown that low serum KL levels are associated with the progression of CKD and enhanced tissue damage, including vascular calcification and cardiovascular dysfunction[26,31,39].

KL is intimately linked to a development of T2DM and plays a role in systemic glucose metabolism and adipocyte maturation[6]. In both T1DM[15,42,50] and T2DM[8,32,47], KL levels exhibit a declining tendency and in diabetic individuals, the pancreas is even depleted. It should be mentioned that in patients with type 2 diabetes, decreased levels of circulating KL are strongly linked to declining renal function; this trend is more noticeable in those undergoing hemodialysis. Additionally, research has shown that in the early stages of diabetic nephropathy, there is a reduce in KL expression in both plasma and urine; a further decline could be a sign that diabetic nephropathy is developing[16,43,44]. According to reports, KL overexpression protects against a variety of pathological phenotypes, particularly kidney illness while downregulation considerably shortens longevity.

In order to maintain phosphate homeostasis, FGF23 and KL are essential. The kidneys and parathyroid glands are the organs most affected by FGF23. When there is no illness, this hormone increases the excretion of phosphorus in the urine by preventing the kidney from producing sodium-dependent phosphate transport protein 2A (NaPi2A), which reduces proximal tubular reabsorption of phosphate[7,11]. In

addition, the secretion of FGF23 by osteocytes is regulated by P, Ca, PTH and 1,25$(OH)_2D$[14]. In the present study, we discovered that the serum levels of P increased significantly in pre-dialysis patients. However, the serum calcium level was normal, but PTH level was increased which can affect the production of FGF23.

KL has generally been demonstrated to have anti-inflammatory and anti-fibrotic effects, to protect against oxidative stress, to downregulate apoptosis and to regulate the metabolism of calcium and phosphate[26,43]. KL-deficient mice showed mineral metabolism disorders including phosphate retention, hypercalcemia and elevated FGF23 levels, which are ubiquitous in CKD. Due to the kidney dysfunction, the homeostasis of phosphorus, calcium and associated hormones is significantly disrupted in CKD, particularly in the pre-dialysis stages. CKD-MBD (chronic renal disease-mineral and bone problem) is the term for this condition. These alterations have been extensively studied and are pivotal in understanding CKD pathophysiology[33,36].

Olauson et al[34] evaluated a mouse model with partial deletion of KL in distal tubular segments. They discovered that the mice had high levels of FGF23, were hyperphosphatemic and had a lot of sodium-phosphate cotransporter Npt2a expressed at the brush boundary membrane. They also discovered no changes in the levels of calcium and 1,25-dihydroxyvitamin D3 in the blood. They come to the conclusion that homeostatic regulation of mineral metabolism depends on renal FGF23-KL signaling which is impaired in CKD.

Mineral homeostasis in CKD is significantly impacted by abnormalities in KL and FGF23 expression. It has been demonstrated that rats with experimentally induced kidney damage exhibit downregulated renal KL expression[12,40]. Additionally, compared to healthy persons, nephrectomy tissues and arteries from CKD patients had decreased levels of KL expression. Gutiérrez[12] reported that over-expression of KL increased urinary phosphate excretion and lowered serum phosphate concentrations in animals with moderate CKD[12]. In conditions like inflammation or metabolic stress, which are common in many chronic diseases and in advanced CKD, both FGF23 and PTH can increase together. This could be due to changes in calcium and phosphate metabolism triggered by inflammatory pathways.

On the other hand, we observed that the serum level of MDA (as a byproduct of lipid peroxidation) increased and the catalytic activity of GPx decreased in pre-dialysis patients with CKD, indicating increased oxidative stress in the patients. Oxidative stress can directly or indirectly affect the regulation of hormones related to phosphate and calcium metabolism, such as FGF23, KL and PTH. It has been reported that KL is also downregulated by oxidative stress[5]. In individuals with diabetic nephropathy, it has been noted that KL levels decreased as oxidative stress and inflammation levels increased[43]. Inflammatory factors, including TNF-α and the uremic toxin indolyl sulfate, can increase ROS production and downregulate klotho expression through a NF-κB-dependent mechanism[41]. It has been reported that the increased levels of KL reduce oxidative damage in animal models[45]. The observed increase in the pro-oxidant/antioxidant balance (elevated MDA and reduced GPx activity) in our study indicates a state of oxidative stress, which has several important implications for the pathophysiology and biochemical alterations.

Furthermore, no significant correlation was observed between KL and FGF23. The lack of correlation between these factors in our study could have several possible reasons. FGF23 increases in response to hyperphosphatemia or other metabolic disturbances, but in conditions like CKD, reduced KL levels may occur independently of elevated FGF23. Furthermore, we studied the rs9536314 KL gene polymorphism in pre-dialysis patients. Our findings showed that there were no appreciable differences in the frequency distributions of either genotype or allele between patients and healthy people. This lack of association suggests that KL gene polymorphisms might not be directly involved in the etiology or development of CKD in our population.

In addition, no significant OR was found between studied groups. We discovered that pre-dialysis diabetic individuals with a high frequency of homozygous GG and TT genotypes were 1.66 times more likely to acquire CKD than the controls, but this difference was not statistically significant (95%CI: 0.80, 3.38). In line with our findings, some studies have also reported no significant association between the KL gene polymorphism and CKD, whereas others have suggested a possible role for this variant in disease susceptibility. Marchelek-Myśliwiec et al[29] discovered no significant association between KL polymorphism rs9536314 with Ca, P, PTH, or FGF23 in patients on long-term hemodialysis. Mendoza-Carrera et al[30] found that the rs9536314 G allele was associated with a protective effect against hypo-HDL-C, albuminuria and CKD. Also, they observed no differences in genotype or allele frequencies between patients and the reference population.

Like any scientific study, ours might have some limitations that could impact the results. The sample size may limit the statistical power to detect subtle or complex relationships such as correlations between KL, FGF23 and biochemical factors. Larger studies with more participants could provide more robust results. While important indicators including KL, FGF23, MDA and GPx were examined, other relevant variables like inflammatory markers and FGFR1 were not included. Considering these markers could provide a more complete understanding of the pathways involved. KL gene polymorphism assessment did not show significant differences between pre-dialysis patients and healthy groups. Broader genetic investigations, such as assessing several polymorphisms or genome-wide relationships, may uncover more subtle effects that were missed in our investigation.

Conclusion

This study explored the relationship between KL gene polymorphism, bone metabolic markers and oxidative stress in pre-dialysis diabetic patients in comparison with healthy individuals. The findings revealed that the KL polymorphism rs9536314 in pre-dialysis patients was not associated with the risk CKD. We discovered that serum KL levels were decreased while FGF23, PTH and phosphorus levels were increased, indicating the disturbed mineral metabolism that is common in CKD. The rise in MDA and decrease in GPx activity indicated increased oxidative stress, which causes mineral imbalances and probably leads to kidney injury.

Although FGF23 and KL were altered, no significant correlation was observed between them. Furthermore, our findings indicated that FGF23 and KL can be used as promising biomarkers for assessing kidney dysfunction. These findings could be helpful in the development of novel diagnostic and treatment approaches for CKD patients, as well as in lowering the risks of bone problems in these individuals.

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