Clinical, Biochemical, and Genetic Study of Patients with Hypertension Associated Kidney Disease

Abdelkader Ahmed Hashim[a],Tahia H. Saleem[b], Mohammed H. Hassan[c] , Doha Abd-Elraheem Salama[c]* , Marwa Abdelhady[d]

[a]Internal Medicine Department, Nephrology division, Faculty of Medicine, South Valley University, Qena, Egypt.
[b]Medical Biochemistry Department, Faculty of Medicine, Assiut University, Assiut, Egypt.
[c]Medical Biochemistry Department, Faculty of Medicine, South Valley University, Qena, Egypt.
[d]Internal Medicine Department, Faculty of Medicine, Luxor University, Luxor, Egypt

Abstract

Background: Hypertension (HTN) is the second leading cause of kidney failure, due to blood pressure (BP) rises and cause deterioration in kidney function. The biochemical and genetic mechanisms of HTN associated kidney diseases (HAKD) are still unclear.

Objectives: To asses circulating levels of fibroblast growth factor 23 (FGF23), αklotho and mircoRNA-126 (mir-126) in patients with HAKD, to evaluate the genetic profile of klotho G-395A (rs1207568), C1818T (rs564481), and wnt signaling pathway AXIN-1 C>T (rs9921222) single nucleotide polymorphisms (SNPs).

Patients and Methods: This cross-sectional case-control study included 50 patients have HAKD, and 50 healthy controls. Plasma concentrations of α klotho and FGF23 were assessed using ELISA assay kits, whereas mir-126 expression levels were quantified using reverse transcriptase quantitative polymerase chain reaction (RT-qPCR). Genetic analysis of rs1207568, rs564481 and rs9921222 SNPs were performed using conventional PCR techniques.

Results: The median FGF23 level in HAKD patients was significantly higher compared to controls, while the median αKlotho and mir-126 expression levels were much lower in HAKD patients compared to controls (P <0.01 for all). The genotyping of klotho rs1207568 showed significantly higher frequency of GG genotype in HAKD patients compared to the control group (P= 0.015), while regarding klotho rs564481, all participants have CT genotype. Wnt signaling pathway rs9921222 SNP showed a significant higher frequency of mutant homozygous TT genotype and mutant T allele in the HAKD group compared to controls, P =0.002, and 0.004 respectively). OR (95% CI) = 0.429 (0.24-0.766) for mutant T allele.

Conclusion: Up regulation of FGF23 and down regulation of αKlotho and mir-126 expressions may have role in HAKD. GG genotype of Klotho rs1207568 SNP, TT genotype of wnt signaling pathway rs9921222 SNP and its mutant T allele were found to be a genetic risk factors in development of HAKD.

Keywords: Fibroblast growth factor 23; αklotho; microRNA-126; Hypertension associated kidney diseases (HAKD), Polymerase chain reaction (PCR), Single nucleotide polymorphism (SNP).

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***Correspondence**: doha.abdelraheem91@gmail.com

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Introduction

Hypertension (HTN) is thought to be the second cause of kidney failure. Most hypertensive patients develop mild to moderate hypertensive nephrosclerosis. Nevertheless, the widespread occurrence of HTN in the overall population increased the incidence of renal failure caused by elevated blood pressure (BP). Hyalinosis, and glomerular damage have been associated with hypertensive kidney disease **(Sun et al., 2020).**

Bright was the first to propose that HTN causes increasing renal impairment. Chronic kidney disease (CKD) and high blood pressure almost always associated with each other **(Bright, 1836).** As previously stated elevated blood pressure accelerates the development and the deterioration in kidney function **(Ameer, 2022).**

Numerous experimental studies demonstrated the growing significance of the interacting Klotho fibroblast growth factor (FGF) 23 endocrine systems in regulating the formation of renal calcitriol and renal phosphate absorption. Deterioration of kidney function, calcifications of soft tissue, abnormal homeostasis of iron and vitamin D, vascular calcifications, and impaired angiogenesis were found in the clinical, pathological, and laboratory studies. These findings suggest that Klotho FGF 23 has a significant effect on the pathophysiology of kidney diseases **(Bernheim and Benchetrit, 2011).**

The wnt signal transduction pathway controls various biological phenomena during growth and adult life. It plays a major role in kidney development and nephron formation. Alterations in wnt signaling is involved in various kidney disease **(Al-Hakeim et al.,2021).**

MiRNAs are involved in many physiological and pathologic processes. Their expression has been observed to be altered in kidney diseases, which makes them useful as noninvasive, diagnostic biomarkers **(Zou and Zhang, 2018).** So, the current study aimed to investigate plasma levels of FGF23, αKlotho and mir-126 among patients with HTN- associated kidney diseases (HAKD). Also to assess genetic profile of Klotho gene SNP G-395A (rs1207568), C1818T (rs564481), and wnt signaling pathway SNP AXIN-1 C>T (rs9921222) SNP among such patients. Additionally, to correlate the measured biochemical, molecular and genetic assays with the degree of the control of HTN.

Patients and methods

Study design and participants

This cross-sectional case-control study involved 50 patients have HAKD and 50 age - sex and BMI-matched healthy controls. The research was conducted from March 2022 to August 2023 at the Nephrology Unit of the Internal Medicine Department, in collaboration with the Medical Biochemistry Department, Faculty of Medicine, South Valley University, Qena, Egypt.

Exclusion criteria: patients with a history of Diabetes Mellitus, acute infectious disease, systemic inflammatory diseases, hepatic dysfunction, vascular events, or malignancies.

Sample size calculation

Sample size calculated according to:

$$n\ (each\ group) = \frac{(p0q0 + p1q1)(z\ 1-\alpha/2 + z\ 1-\beta)2}{(p1 - p0)2}$$

p1= risk of exposure in cases

p0= risk if exposure in controls

q1= 1-p1

q0= 1-q0

$Z_{1-\alpha 2}$ = 1.96 for confidence interval 95%

$Z_{1-\beta}$ = 0.84 as desired power for our study 80%

so at least 48 patients should be present in the sample and we have included 50 patients. We have modified the sample size in order to get 80% statistical power and a 5% level of significance (type 1 error).

Data collection and clinical assessment

A detailed history was taken from the patient group including name, age, sex, history of anti-hypertensive medication and smoking, history of dialysis, history of co-morbidities as hepatic diseases and any autoimmune disease.

In the course of the general exams, the body mass index (BMI) was measured in kilograms per square meter **(Nuttall, 2015).** Additionally, blood pressure was measured in order to differentiate between controlled and uncontrolled HTN. An individual with HTN was considered to have controlled BP if their systolic blood pressure (SBP) was less than 140 mm Hg and their diastolic blood pressure (DBP) was less than 90 mm Hg **(Gillespie et al. 2013),** and were more likely to have uncontrolled BP if their SBP was more than or equal to 140 mmHg and/or their DBP was greater than or equal to 90 mm Hg **(Makukule et al., 2023).** An equation known as the MDRD (Medical Device Reprocessing Department) equation was utilized in order to determine the estimated glomerular filtration rate (eGFR) **(Levey et al., 2005):**

$$eGFR=175\times(Creatinine/88.4)^{-1.154}\times(Age)^{-0.203}\times(0.742 \text{ if female})\times(1.212 \text{ if Black})$$

Complete blood count, serum electrolytes (sodium, potassium, and calcium), liver function tests (ALT and AST), kidney function tests (urea, creatinine, and uric acid), lipid profile and urinary albumin-to-creatinine ratio (ACR) were among the routine laboratory data that were collected.

The renal echogenicity evaluated by the use of abdominal ultrasonography. This was accomplished by contrasting the echogenicity of the renal cortex, medulla, and pyelic sinus with that of the liver and spleen **(Fiorini and Barozzi, 2007).** Renal echogenicity categorized into four grades: Grade 0 denotes echogenicity lower than that of liver parenchyma, considered normal. Grade I reflects echogenicity comparable to that of liver parenchyma, also deemed normal. Grade II indicates increased echogenicity relative to liver parenchyma, categorized as pathological. Grade III shows echogenicity equivalent to that of the renal sinus **(Hricak et al., 1982).**

Blood samples and assays

Six milliliters of venous blood were collected from each participant, divided into two portions in EDTA tubes. The first part (4 milliliters) was centrifuged, at a speed of 3500 rpm for 15 min. The separated plasma was stored in cryotubes at -80°C, and used for biochemical and molecular analyses of α-klotho, FGF23, and mir-126. The second part (2 milliliters) was collected for genetic analysis of klotho and Wnt signaling pathway gene polymorphisms.

A. *Biochemical assays:*

An ELISA kit was supplied by Chongqing Biospes Co., China, with the catalogue number BZEK1920, was utilized to measure the α-klotho in plasma. Another ELISA kit was supplied by Elabscience Bionovation Inc. (catalogue number E-EL-H1116) was utilized to detect plasma FGF23 assay. An EMR-500 microplate reader from the United States was utilized in order to carry out the ELISA assays.

B. *Molecular assay of mir-126:*

Using the RNeasy Mini Kit (catalogue number 217204, Qiagen, Hilden, Germany), total RNA was extracted from plasma samples with addition of ploy (A) tail enzymes in accordance to the manufacturer instruction.

The High-Capacity cDNA Reverse Transcription (RT) Kit from Applied Biosystems for synthesizing cDNA using poly (T) adaptor, catalogue number 4368813 (Thermo-Fisher Scientific, Waltham, USA). Reverse transcription- quantitative polymerase chain reaction (RT-qPCR) was carried out with the Maxima SYBR Green/ROX PCR Master Mix available from Thermo Fisher Scientific number K0251

United States.

RT-qPCR was conducted using miRNA-specific forward and reverse primers. 1) Forward primer:5′-GGGCATTATTACTTTTGG-3′ and 2) miRNA reverse primer: 5′-TGCGTGTCGTGGAGTC-3′.

The gene expression study utilized the Applied Biosystems 7500 Fast RT-PCR System (Applied Biosystems, Germany), with U6 as the internal control. Following are the U6 primers: 5′-GCTTCGGCAGCACATATACTAAAAT-3′ is the sequence of the forward primer. the reverse primer, which is 5′-CGCTTCACGAATTTGCGTGTCAT-3′.

Following the completion of each cycle of the PCR, melting curves were prepared in order to verify the specificity of the products (**Fig.1**). The levels of gene expression were assessed by employing the comparative cycle threshold (CT) approach, also known as the ΔCT method. The calculation of ΔCT was as follows:

$\Delta CT = \Delta Rn_{\text{target gene}} - \Delta Rn_{U6}$, where ΔRn means delta normalized reporter dye fluorescence level

ΔΔCT = ΔCT (sample) – ΔCT (average control)

Relative fold-change in expression was computed using $2^{-\Delta\Delta CT}$

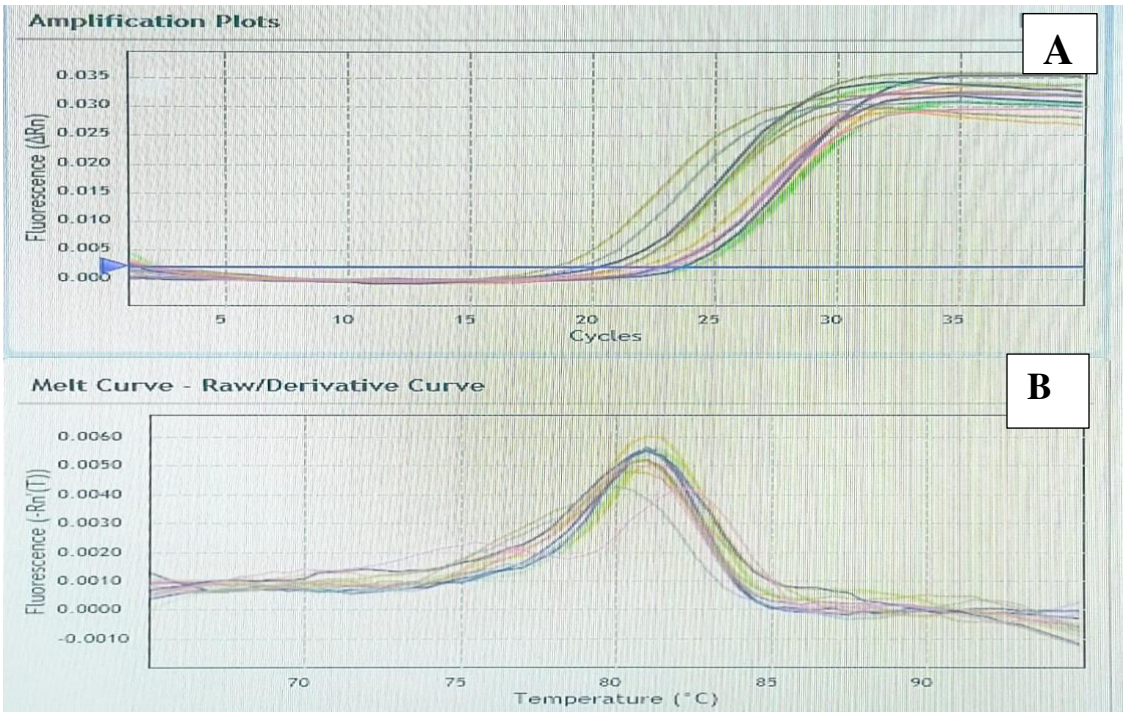

Fig.1. Quantitative assay of mir-126 expression using RTq-PCR; A) amplification plots ;B) Melt curve- Raw /Derivative Curve

C. Genetic assays of αKlotho wnt signaling genetic polymorphisms

The G-spinTM Total DNA Extraction Kit, produced by iNtRON Biotechnology, Inc. Korea, was used to extract DNA from whole EDTA blood samples using the manufacturer's stated methodology and preserved at a temperature of -80 ºC for later genetic analysis of the three SNPs G-395A (rs1207568), C1818T (rs564481), and AXIN-1 C>T (rs9921222).

1. G-395A (rs1207568) SNP.

The G-395A (rs1207568) SNP was analyzed using PCR with confronting two-pair primers (PCR-CTPP) according to the method reported by **Shimoyama et al.**

(2009). The primers utilized in the experiment were as follows: F1 primer: 5′GTTTCGTGGACGCTCAGGTTCATTC TC3′, F2 primer: 5′GAGAAAAGGCGCCGACCAACTTTC3 ′, R1 primer: 5′GATCCCGCCCCCAAGTCGGGA3′, and R2 primer: 5′GTCCCTCTAGGATTTCGGCCAG-3′. Using a Biometra thermal cycler (Germany, serial number 2603204) under the following conditions: The process began with an initial denaturation step at a temperature of 95ºC for duration of ten minutes. This was followed by 35 cycles of denaturation at the same temperature for one minute, annealing at 65 ºC for one minute, and elongation at 72 ºC for one minute. The final phase of the elongation process was carried out at a temperature of 72 ºC for a period of five minutes.

The PCR products were examined using 2% agarose gel to differentiate between the G and A alleles, with bands measuring 252, 175, and 121 base pairs, which corresponded to the G and A alleles. The occurrence of bands at 252, 175, and 121 base pairs indicates the existence of the heterozygous GA genotype. Conversely, bands at 252 and 175 base pairs indicate the presence of the homozygous GG genotype, while bands at 252 and 121 base pairs indicate the presence of the homozygous AA genotype (**Fig.2**).

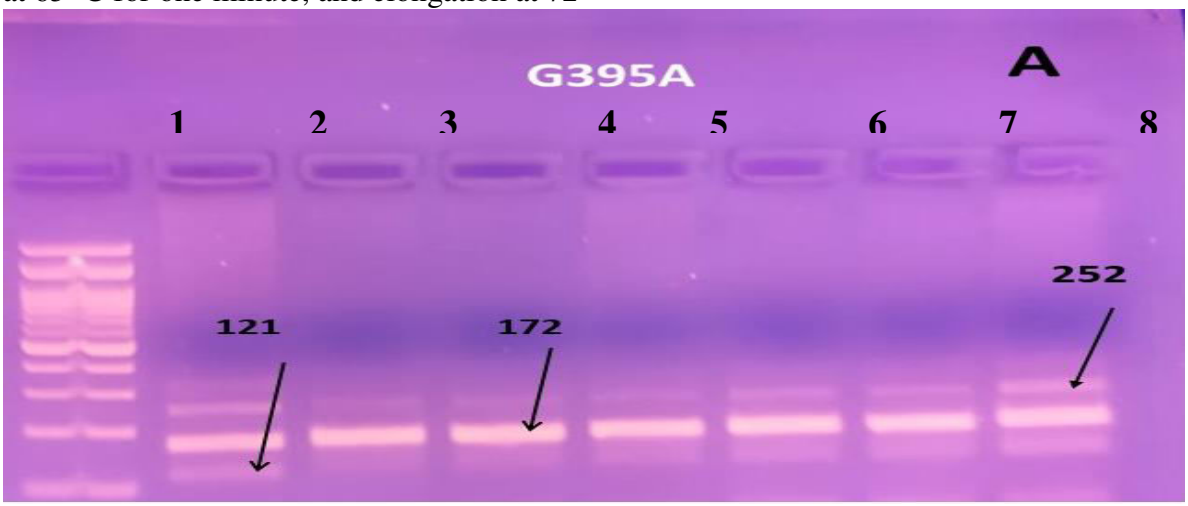

Fig.2. Gel electrophoresis for the G-395A (rs1207568) SNP was performed using PCR with confronting two-pair primers (PCR-CTPP). The gel lanes are labeled as follows: Lane 1 contains a 100 bp DNA ladder for size reference. Lanes 2, 6, 7, and 8 display the heterozygous GA genotype, characterized by bands at 252, 175, and 121 bp. Lanes 3, 4, and 5 show the homozygous GG genotype, with bands at 252 and 175 bp.

2. C1818T (rs564481) SNP

For the analysis of the C1818T (rs564481) SNP, PCR with confronting two-pair primers (PCR-CTPP) was utilized, as outlined by **Shimoyama et al. (2009).** The primer sequences used were: F1 primer: 5′CTCAGTTTACCGACCTGAATGTTTA CCTG3′, F2 primer: 5′CAGATCGCTTTACTCCAGGAAATGC AC3′, R1 primer: 5′GTCCAGGGAGAAGCGAAAATGTGT AACA-3′, and R2 primer: 5′GAGCTCTTGAAAGCACAGTCGGGC-3′.

The amplification process began with an initial denaturation at 95ºC for five minutes. This was followed by thirty-five cycles of denaturation at 94 ºC for thirty seconds, annealing at 61ºC for forty-five seconds, and elongation at 72 ºC for forty-five seconds. A last extension was carried out at a temperature of 72 ºC for a period of five

minutes.

PCR products were analyzed to identify fragment sizes of 416, 291, and 179 base pairs, which differentiate between C- and T-alleles. Bands at 416 and 291 bp indicate the homozygous CC genotype, bands at 416 and 179 bp represent the homozygous TT genotype, and bands at 416, 291, and 179 bp denote the heterozygous CT genotype. In our study, only the CT genotype was observed among participants **(Fig.3).**

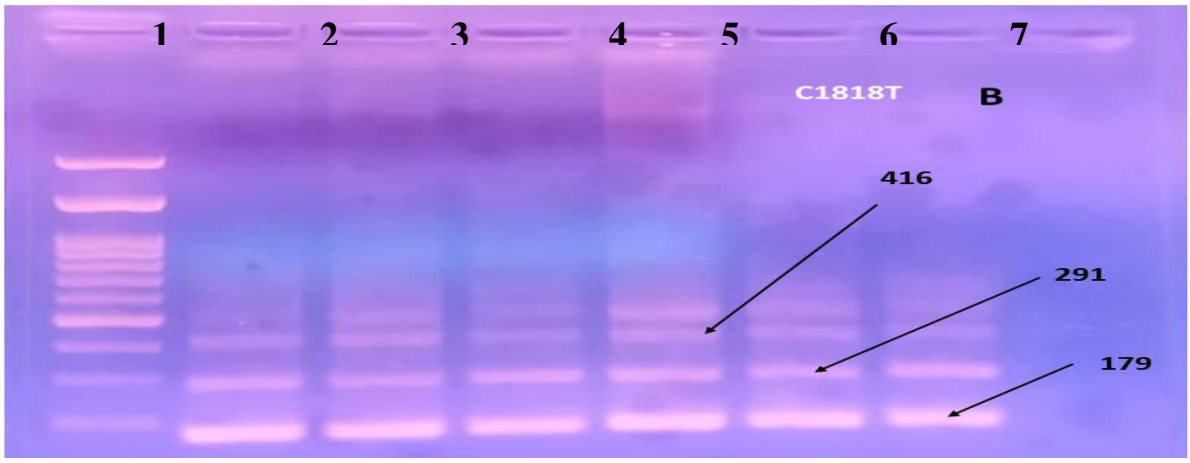

Fig. 3. The C1818T (rs564481) SNP is analyzed using the PCR with confronting 2-pair primers (PCR-CTPP) technique in gel electrophoresis. The lanes are labeled with numbers. Lane 1 displays a 100 base pair DNA ladder. Lanes 2-7 exhibit a heterozygous genotype (CT) with a band of 416,291,179 base pairs.

3. AXIN-1 C>T (rs9921222)

The method known as restriction fragment length polymorphisms-polymerase chain reaction (RFLP-PCR) was utilized in order to carry out the genetic study of the AXIN-1 C>T (rs9921222) SNP. According to **Said et al. (2020)**, the primers that were utilized were as follows: Forward primer 5′CCCAGTCCAGACACAAACCT3′ and Reverse primer 5′GGGCCAGTTGTCAGTATTGC3′.

Following an initial denaturation at 95 ºC for five minutes, the conditions for the amplification process consisted of thirty-five cycles of denaturation at 95 ºC for thirty seconds, annealing at 72 ºC for thirty seconds, and elongation at 72 ºC for thirty seconds. A last extension was carried out at a temperature of 72 ºC for ten minutes, the PCR products would be 475 base pairs.

Following the digestion of the PCR products using the restriction enzyme Catalogue No. R0517, (BspHI, New England Biolabs, USA), a reaction mixture consisting of 10 µL of PCR product, 1 µL of enzyme, 2 µL of enzyme buffer, and 15 µL of nuclease-free water was utilized. This reaction mixture was then incubated at 37°C for a duration of 24 hours. The CC genotype had bands at 229 and 246 base pairs; the CT genotype, had bands at 475, 229, and 246 base pairs; and the TT genotype had a single band at 475 base pairs (**Fig. 4**).

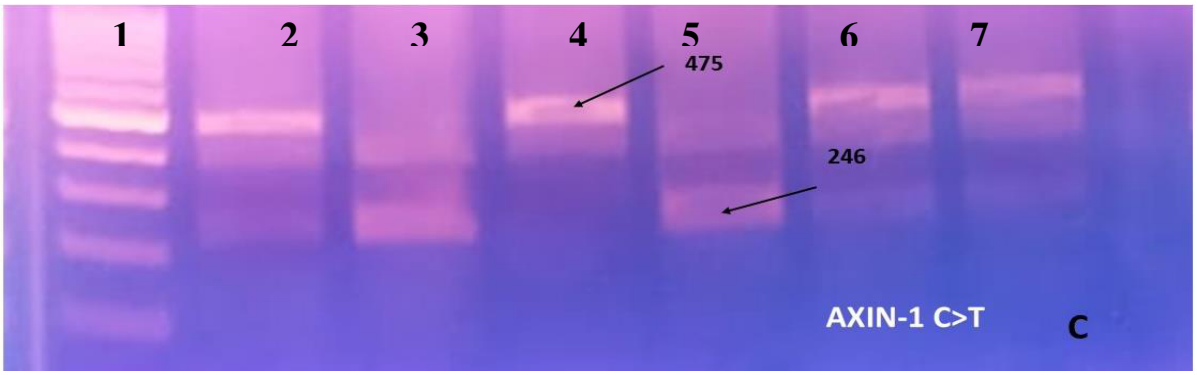

Fig.4. Agarose gel electrophoresis (2%) showing PCR amplification product of AXIN-1 C>T (rs9921222), Lanes are denoted with numbers. Lane 1 shows 100 bp DNA ladder; Lanes 3 are heterozygous genotype (CT) with 475,246,229 bp band. Lane 2-4 are homozygous genotype (TT), lane 5 are homozygous genotype (CC).

Ethical considerations

The study was conducted in accordance to the ethical guidelines laid down in the Declaration of Helsinki. The Institutional Review Board (IRB) of Faculty of Medicine, South Valley University has approved the current study (Ethical approval code: SVU-MED-MBC004-4-247-870). Before involvement in the study, an informed written consent was obtained from each participant.

Statistical analysis

In order to undertake the analysis of the data, SPSS version 27.0 (SPSS Inc., Chicago, Illinois, USA) was utilized. The Kolmogorov-Smirnov and Shapiro-Wilk tests were utilized in order to determine the normality of the data. Data were expressed as number, percentages, the mean ± SD for parametric data, or the median and IQR (interquartile range) for non-parametric parameters.

Unpaired T test was used to compare the means between two groups in parametric data. Regarding non-parametric data, the Mann-Whitney U test and Spearman's correlation were utilized. Chi-square test was used for nominal data. A two-tailed p-value of less than 0.05 is considered significant. The studied SNPs were followed Hardy-Weinberg equilibrium.

Results

Demographic and clinical data of the studied groups:

Demographic and clinical data were demonstrated in **(Table.1)**. The cases were matched to controls regarding age, gender, and BMI as indicated by insignificant differences between 2 groups (P >0.05 for all). Systolic and diastolic blood pressure measurements show significant elevations in the HAKD group compared to controls, (P < 0.001).

Table 1. Demographic and clinical data of the included participants

Variables	Cases (n=50)	Controls (n=50)	P value
Age (mean±SD, years) **(UT)**	49.66±8.18	51.52±6.28	0.145
Gender: • Male N(%) **(X^2)** • Female N(%)**(X^2)**	 25(50%) 25(50%)	 25(50%) 25(50%)	1.000
BMI (mean±SD, kg/m^2)	27.15±2.12	26.81±4.22	0.188
Residency			

• Rural N(%)(X^2) • Urban N(%)(X^2)	27(54%) 23(46%)	23(46%) 27(54%)	0.424
Family history of HTN • Yes N (%) • No N (%)	9(18%) 41(82%)	-----	----
Smoking • **No** N (%) • **Yes** N (%)	41(82%) 9(18%)	44(88%) 6(12%)	0.575
Dialysis • **No** N (%) • **Yes** N (%)	44(88%) 6(12%)	-------	-----
Blood Pressure measurements (UT) • **Systolic** (mean±SD, mmHg) • **Diastolic** (mean±SD, mmHg)	130±12.62 95±11.11	115.2±5.05 72.4±7.97	<0.001* <0.001*
Hypertension • **Controlled** N (%) • **Uncontrolled** N (%)	15(30%) 35(70%)	-----	-----

X^2: Chi-square test; **UT:** unpaired t test; * significant; HTN: hypertension; BMI: body mass index

Routine laboratory and imaging data of the included participants:

(**Table.2**) presents routine laboratory data showing 90% of HAKD patients have abnormal ultrasound echogenicity, with all abnormalities bilateral. As regards to the grades of kidney echogenicity, grade 0 and I have equal frequency (10% for each), and grade II and III have equal frequency (40% for each).

Table 2. Routine laboratory and sonographic data among the HAKD patient and control

Variables		Cases (n=50)	Controls (n=50)	P value
CBC parameters	**Hb** (mean ±SD, g/dL) **(UT)**	9.38±1.23	13.31±1.61	<0.001*
	MCV(mean±SD, fl) **(UT)**	83.95±6.21	81.18±5.92	0.004*
	WBC(mean±SD, 10^3 /µL) **(UT)**	6.44±1.3	6.17±2.52	0.012*
	Platelets(Median(IQR), 10^3 /µL) **(MW)**	318.5(233.75-456)	286(253-365)	0.199
Na (mean±SD, mmol/L) **(UT)**		140.16±5.73	138.12±5.79	0.076
K (mean±SD, mmol/L) **(UT)**		4.18±0.62	3.82±0.35	<0.001*
Ionized Ca (mean±SD, mg/dL**(UT)**		1.01±0.48	5.18±0.4	<0.001*
Liver function test	**ALT** (mean±SD, IU/L) **(UT)**	15.12±4.13	14.59±4.11	0.697
	AST (mean±SD, IU/L) **(UT)**	21.99±6.08	16.3±3.07	<0.001*
Lipid profile	**Cholesterol (mg/dL)** mean±SD **(UT)**	222.2±48.87	133.3±12.11	<0.001*
	TGD (mg/dL) mean±SD **(UT)**	187.26±32.06	122.6±20.43	<0.001*

	LDL (mg/dL) mean±SD **(UT)**	157.7±34.58	99.5±14.1	<0.001*
	HDL (mg/dL) (mean±SD, **(UT)**	38.46±13.09	60.2±6.65	<0.001*
Kidney function test	**Urea (mg/dL)** Median (IQR)**, (MW)**	76(75-91)	39(25-45)	<0.001*
	Creatinine (mg/dL) mean±SD **(UT)**	7.1(5.3-14)	0.6(0.4-0.8)	<0.001*
	eGFR (ml/min/1.73 m^2) (Median (IQR))**, (MW)**	7.6(6.8-9.8)	177.25(102.6-273.3)	<0.001*
	Uric acid (mg/dL) mean±SD, (UT)	7.26±2.44	4.3±1.29	<0.001*
	A/C Ratio (mg/g) Median (IQR) **(MW)**	3227(258-4752)	18.5(14.75-25.25)	<0.001*
Echogenicity by U/S **Normal** **Abnormal** • **Unilateral** N (%) • **Bilateral** N (%) **-Grades:** • **0** N (%) • **I** N (%) • **II** N (%) • **III** N (%)		 5(10%) 45(90%) 0(0%) 45(100%) 5(10%) 5(10%) 20(40%) 20(40%)	-----	-----

UT: Unpaired t test; MW: Mann-Whitney U test; * significant.
-Hb: hemoglobin, MCV: mean corpuscular volume, WBC: white blood cells, TGD: triglycerides, LDL: low density lipoprotein, HDL: high density lipoprotein, eGFR: estimated glomerular filtration rate

Circulating specific biochemical and molecular biomarkers among the studied groups

In the present study, the specific biochemical and molecular biomarker in the HAKD patient and control **(Table. 3)** showed that the median plasma FGF23 level for HAKD patients was significantly higher compared to controls, while αKlotho and mir-126 expression levels showed significantly reduced median levels in HAKD patients compared to controls, (P<0.001 for all).

Table 3.The plasma levels of FGF23, αklotho, and expression levels of mir-126 biomarkers among HAKD patients and controls

Variables	HAKD cases (n=50)	Control (50)	P value
FGF23 (pg/mL) Median(Q1-Q3) **(MW)**	91.68(47.29-270.73)	36.48(19.33-59.86)	<0.001*
αKlotho (pg/mL) Median(Q1-Q3) **(MW)**	133.97(82.14-212.76)	317.39(307.57-324.24)	<0.001*
mir-126 (pg/mL) Median (Q1-Q3) **(MW)**	0.35(0.21-0.65)	1.06(0.91-1.16)	<0.001*

MW: Mann-Whitney U test; * significant.

In our study the HAKD patients were categorized into controlled and uncontrolled subgroups in term of response to anti-hypertensive medication. Although the median plasma FGF23 levels were higher with lower expression levels of both αKlotho and Mir-126 among uncontrolled HAKD patients compared to the controlled HAKD subgroup, but didn't reached a significant level , p>0.05 for all.

Genetic profile of Klotho G-395A (rs1207568), C1818T (rs564481) SNPs and wnt signaling pathway AXIN-1 C>T (rs9921222) SNP among the studied groups

The frequency of the GG genotype of Klotho SNP G-395A (rs1207568) was significantly higher in HAKD patients compared to controls, p< 0.05. Allele frequencies revealed that the G allele was more frequent in HAKD patients in comparison to the controls; however, this difference did not reach a statistical significance, p> 0.05, (**Table.4**).

In term of response to anti-hypertensive medication, the frequency of the homozygous GG genotype was higher in controlled HAKD patients (40%) compared to the uncontrolled HAKD subgroup (17.1%), (p=0.168). The mutant AA genotype was detected in 5.7% of uncontrolled HAKD patients, but couldn't be detected in controlled HAKD group. Additionally, the wild G allele was present in 70% in controlled HAKD subgroup compared to 55.7% in uncontrolled HAKD patients. The mutant A allele frequency was higher in uncontrolled HAKD group (44.3%) compared to controlled HAKD group (30%), but still with insignificant difference (p = 0.181).

Table 4. Genotypes and allele frequencies of Klotho SNP G-395A (rs1207568) in HAKD patients and controls

Studied groups	**Genotype** N (%)							**Allele** N (%)	
	GG	**GA**	**AA**	**(GG+GA)**	**AA**	**GG**	**(GA+AA)**	**G**	**A**
Cases 50	12(24%)	36(72%)	2(4%)	48(96%)	2(4%)	12(24%)	38(76%)	60(60%)	40(40%)
Control 50	2(4%)	46(92%)	2(4%)	48(96%)	2(4%)	2(4%)	48(96%)	50(50%)	50(50%)
P value (X^2)	0.015*			1.000		0.004*		0.155(2.020)	
OR CI (95%)	-			1.000(0.135-7.392)		7.579(1.559-35.933)		1.500(0.857-2.626)	

X^2: Chi-square test, OR: odds ratio; CI: confidence interval; * significant.

Meanwhile, genetic disparities in the Klotho gene C1818T (rs564481) SNP between HAKD patients and controls revealed that all the patients and the control in the study had the CT genotypes.

Genotypes and allele frequencies of wnt signaling pathway SNP AXIN-1 C>T in HAKD patients and controls were showed in (**Table.5).** There was a significant lower prevalence of the CC genotype of the wnt signalling pathway SNP AXIN-1 C>T (rs9921222) SNP in HAKD patients compared to controls, p = 0.002. The frequency of C allele was shown to be significantly less frequent in HAKD patients when compared to controls, p<0.004.

However, for Genotypes and allele frequencies of wnt signaling pathway AXIN-1 SNP in the 2 subgroups of HAKD patients, the homozygous CC genotype

showed a non-significant decrease in controlled HAKD patients compared to the uncontrolled group, P = 0.688.

The C allele frequency found in 43.3% of controlled HAKD patients and 52.9% of uncontrolled HAKD patients. The T allele shows a significant increase in the HAKD group (50%) of alleles compared to 30% in uncontrolled group, P = 0.004.

Table 5. Genotypes and allele Frequencies of wnt signaling pathway SNP AXIN-1 C>T (rs9921222) in HAKD patients vs. controls:

Studied groups	Genotype N (%)							Allele N (%)	
	CC	CT	TT	(CC+CT)	TT	CC	(CT+TT)	C	T
Cases 50	13(26%)	24(48%)	13(26%)	37(74%)	13(26%)	13(26%)	37(74%)	50(50%)	50(50%)
Control 50	30(60%)	10(20%)	10(20%)	40(80%)	10(20%)	30(60%)	20(40%)	70(70%)	30(30%)
P value (X^2) *	0.002*			0.476		<0.001*		0.004*	
OR 95% CI	-			0.712(0.279-1.818)		0.234(0.100-0.547)		0.429(0.240-0.766)	

X^2: Chi-square test; OR: odds ratio; CI: confidence interval; * significant

Plasma levels of FGF23, αKlotho and expression levels of mir-126 in relation to Klotho G-395A (rs1207568) and wnt signaling pathway AXIN-1 C>T (rs9921222) genotypes among HAKD patient:

A significantly higher median Plasma FGF23 levels were observed in the GG genotype, than the AA genotype. For the klotho, median levels showed a decreased level in GG genotype compared to AA genotype, p = 0.045. GG genotype displaying a non-significant lower median Mir-126 expression levels to the AA genotype **(Table.6).**

Table 6. Plasma levels of FGF23, αKlotho, and expression levels of mir-126 in relation to αKlotho SNP G-395A (rs1207568) genotype among HAKD patient

Variable Median (Q1-Q3)	GG	AA	p-value
FGF23 (pg/mL) Median(Q1-Q3) **(MW)**	1571.66 (1570.36-0)	154(32.25-206.89)	0.028*
αKlotho (pg/mL) Median(Q1-Q3) **(MW)**	36.24(22.25-0)	221.17(68.58-226.49)	0.045*
mir-126 Median(Q1-Q3) **(MW)**	0.34(0.34-0)	0.4 (0.18-0.63)	1

MW: Mann-Whitney U test; * significant.

FGF23, αKlotho, mir-126 in relation to wnt signaling pathway SNP AXIN-1 C>T in HAKD patients' group, plasma FGF23 levels showed considerable variance across

genotypes. Individuals with TT genotype have higher levels at 224.34 pg/mL than those of the CC genotype (154.01 pg/mL), p < 0.001. αKlotho levels among the CC genotype showed the higher median level (221.3 pg/mL), in contrast to 130.3 pg/mL for TT without reaching a significant difference, P = 0.130. Mir-126 expression levels followed a similar trend of variation. The TT genotype showed a median of 0.13, significantly lower than in CC (0.19), P = 0.002, **(Table.7).**

Table 7. plasma levels of FGF23, αKlotho, expression levels of mir-126 in relation to wnt signaling pathway SNP AXIN-1 C>T (rs9921222) genotype among HAKD patients:

Variable Median (Q1-Q3)	CC genotype	TT genotype	p-value
FGF23 (pg/mL) Median(Q1-Q3) **(MW)**	154.01(32.5-174.25)	224.34(193.70-266.37)	<0.001*
αKlotho (pg/mL) Median(Q1-Q3) **(MW)**	221.36(70.50-228)	130.3(74.04-132.80)	0.130
mir-126 Median(Q1-Q3) **(MW)**	0.19(0.18-0.63)	0.13(0.09-0.17)	0.002*

(MW): Mann-Whitney U test * significant.

Correlation analysis of the studied biochemical and molecular markers:

There was a significant negative correlation of FGF23 with αKlotho levels in HAKD patients, **(Fig.5).** There were no any other significant correlations could be detected whether between the studied biomarkers with each other or in relation to the kidney function parameters (creatinine, urea, ACR and eGFR), p > 0.05 for all.

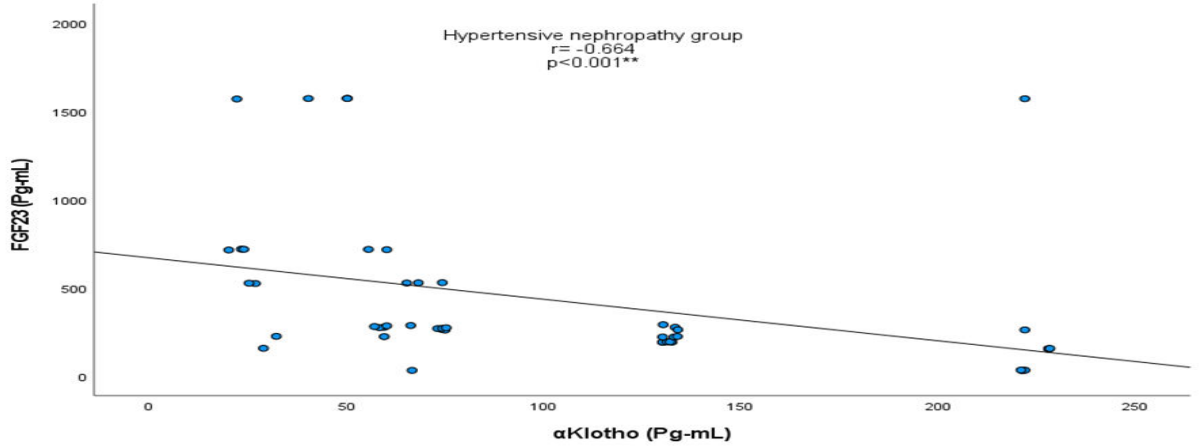

Fig.5. Spearman test for correlation between plasma levels of FGF232 and αKlotho

Discussion

Chronic HTN not treated well has been connected to a number of health problems, one of which is HTN associated kidney disease. Based on the findings of **Costantino et al. (2021),** it is the second most common cause of ESRD, following diabetes. According to **Kovesdy (2022),** advanced CKD usually includes HAKD. This is because HTN could worsen during the course of renal impairment. As the GFR falls, there is an increased incidence of HTN

that is resistant to treatment and difficultly managed. As a result of this persistent rise in blood pressure, the deterioration in kidney function gets accelerated, and permanent damage to the glomeruli results in persistent deterioration in the function of the kidney glomeruli **(Goldman, 2022).**

The primary purpose of the study was to assess the levels of FGF23, α-klotho, and mir-126 in the plasma of patients who have HAKD. Also, to explore the genetic characteristics of the klotho and Wnt signalling pathway SNPs that are present in these patients. When compared to the control group, the HAKD patients had significantly higher blood pressure than the control group. A research carried out by **Dogan et al. (2021)** was consistent with the findings of the study.

In our study, US imaging revealed that HAKD patients have more prevalent grades II and III kidney echogenicity compared to the control group, indicating significant renal damage. This finding aligns with **Majeed et al.'s (2022)**, who proposed that renal echogenicity can predict CKD severity and diagnose renal impairment.

In the current work, patients with HAKD had much lower calcium levels, higher levels of AST, highly elevated levels of urea and creatinine, urinary albumin-to-creatinine ratio (ACR) and uric acid levels with dramatically lowered eGFR compared to controls. In accordance with the findings of **Ibrahim et al. (2022),** who observed that hypertensive patients with a low eGFR had significantly higher levels of albuminuria, creatinine, the A/C ratio, and uric acid in comparison to the control group, our findings are in agreement with their findings. Similarly, **Dogan et al. (2021)** discovered that the levels of AST and calcium were significantly lower in the group of hypertensive nephropathy in comparison to the group that had only HTN. On the other hand, the levels of urea and creatinine were significantly higher in the group that had hypertensive nephropathy in comparison to the group that served as the control. This was accompanied by a significant decrease in the eGFR.

The findings of the current research revealed significantly higher levels of FGF23 in patients with HAKD compared to the control group. In agreement, **Akhabue et al. (2018)** discovered that higher levels of FGF23 were associated with an increase in both systolic and diastolic blood pressure over a period of time. Furthermore, they noted that individuals with elevated levels of FGF23 exhibited a greater incidence of HTN. **Li et al. (2018)** also reported that serum levels of FGF23 in CKD patients with increased mean arterial blood pressure were significantly higher than those of the control subjects. **Johnson et al. (2016)** reported that only persons having the highest decile of serum FGF23 had an increased incidence of HTN in CKD.

In our study, the levels of αKlotho were found to be significantly reduced in the HAKD patients. In line with our findings, **Drew et al. (2021)** also found that there was a negative connection between elevated baseline blood αKlotho levels and the HTN incidence.

Additionally, we found that the expression levels of miR-126 among he included HAKD patients were significantly lower when compared to the control group. Similar findings were reported by **Kontaraki et al. (2014).**

In the current study, there was a significant negative correlation between the plasma levels of FGF23 and αKlotho. **Rotondi et al. (2015),** reported similar correlation among patients with CKD. However, there were no studies could be traced in literature investigating the correlation between FGF23 and αKlotho in HAKD.

The prevalence of the GG genotype and G allele of αKlotho SNP G-395A (rs1207568) SNP were significantly frequent

in HAKD patients compared to controls, demonstrating that GG genotype of Klotho rs1207568 SNP could be considered as genetic risk factor in development of HAKD. In accordance, **Gao et al. (2015)** found that individuals with the recessive GA+AA genotype had a significantly decreased risk of HTN compared to those with the GG genotype. **Ezzat et al. (2024)** reported similar finding among children.

In terms of blood pressure control, the heterozygous GA genotype and A allele were more prevalent in the group of HAKD patients with uncontrolled BP as compared to the patients with controlled BP, however, the difference between the two groups did not reach a significant level. To the best of our knowledge, the frequency of A allele may be included in the resistance to anti-HTN drugs among HAKD patients; nevertheless, these findings require additional confirmatory researches.

Regarding the genetic differences in the Klotho SNP C1818T (rs564481) between HAKD patients and controls, the findings of the current study showed that all of the patients and the control had the CT genotype. The findings of **Akrabi et al., 2018** were consistent with our results.

In the current research, there was a significant lower frequency of the CC genotype and C allele of the wnt signalling pathway SNP AXIN-1 C>T (rs9921222) SNP among the included patients group compared to the controls, which suggests that this genotype may have a protective effect against HAKD while, TT genotype and its mutant T allele could be considered as genetic risk factors in development of HAKD. In agreement with our findings, **Said et al. (2020),** reported that the frequency of the CC and CT genotype for the AXIN-1 (C/T) (rs9921222) SNP was substantially greater in the subjects who served as controls as compared to the group of individuals with hypertensive nephropathy. Furthermore, the TT genotype was found to be significantly frequent in the group of individuals with hypertensive nephropathy.

Interestingly, in the present study, CC genotype and C allele of wnt signaling pathway rs9921222 SNP were more associated with the included HAKD patients who have uncontrolled blood pressure, highlighting that C allele may be included in the resistance to anti-HTN drugs among HAKD patients.

Regarding the possible effect of the genotypes of the studied SNPs on the expression levels of FGF23, αKlotho and mir-126, we for the first time reported that HAKD patients with the GG genotype of Klotho SNP G-395A have the highest levels of FGF23 and the lowest levels of αKlotho. Additionally, the TT genotype of AXIN-1 C>T (rs9921222) SNP is associated with the highest levels of FGF23 and lowest mir-126 expression levels.

Study limitations

Relatively small sized sample and lack of inclusion of HTN patients without nephropathy in the comparison were the main limitation of the study.

Conclusion

The study shows that individuals with hypertension associated kidney diseases (HAKD) have higher plasma levels of FGF23, while αKlotho and miR-126 levels decrease. The klotho SNP G395A genotype is more prevalent in HAKD patients, while CT and TT genotypes are more common in the Wnt signaling pathway. The A and C alleles of these SNPs may influence anti-hypertension medication responsiveness in HAKD patients.

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