Association Between Klotho Gene Polymorphism and Markers of Bone Metabolism in Patients Receiving Maintenance Hemodialysis in Iran

Aboalfazl Nazarian,[1] Meisam Hasankhani,[1]
Monireh Aghajany-Nasab,[2] Ali Monfared[3]

[1]Department of Clinical Biochemistry, Faculty of Medicine, Zanjan University of Medical Sciences, Zanjan, Iran
[2]Department of Biochemistry and Biophysics, Faculty of Medicine, Guilan University of Medical Sciences, Rasht, Iran
[3]Razi Hospital, Rasht, Guilan, Iran

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Introduction. Some genetic variations of Klotho have been reported as a risk factor for calcification and hyperphosphatemia in chronic kidney disease. Klotho polymorphism is also associated with outcome in patients receiving hemodialysis. This study aimed to evaluate the relationship between Klotho single nucleotide polymorphism (SNP) and bone metabolism as an early prognostic measure for chronic kidney disease.

Materials and Methods. Sixty patients receiving hemodialysis and 60 age-matched controls were enrolled in the study of the assessment of 2 types of Klotho polymorphism (G395A and C1818T). Serum biochemical parameters, including calcium, phosphate, urea, creatinine, parathyroid hormone, and 25-hydroxyvitamin D3 were measured.

Results. The frequency of being A carriers suggested marginal significances between the groups (GA and AA, 30% versus GG, 18.3%, $P = .06$), but such significant results were not found for the T allele carriers (CT and TT, 76.6% versus CC, 76.6%, $P > .99$). Homozygote and heterozygote individuals for the A allele at G395A SNP (A allele carriers) were more likely to be on hemodialysis (odds ratio, 1.43; 95% confidence interval, 0.60 to 3.30), but this association was not true for T allele carriers of C1818T SNP. Parathyroid hormone and serum calcium, phosphate, creatinine, and urea showed prominently higher levels in the patients receiving hemodialysis compared with control individuals.

Conclusions. The A allele of the G395A polymorphism of Klotho, which emerges the higher levels of phosphate, may be associated with the risk of mortality in Iranian patients receiving hemodialysis.

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INTRODUCTION

High morbidity and mortality rates have been reported in patients with end-stage renal disease (ESRD). Klotho, an aging process-related gene, has been proposed to have a role in atherosclerosis and cardiovascular diseases.[1] Klotho is expressed in distal convoluted tubules of the kidney and parathyroid cells, mediating the role of fibroblast growth factor (FGF)-23 in bone-kidney-parathyroid control of phosphate and calcium. Chronic kidney disease (CKD) can be a state of hyperphosphatemia-induced accelerated aging associated with Klotho deficiency. Patients with CKD experience decreased Klotho expression as early as stage 1 CKD. Klotho continues to decline as CKD progresses, causing FGF-23 resistance and provoking an increase in

FGF-23 and parathyroid hormone (PTH), as well as hypovitaminosis D3.

Secreted Klotho protein, formed by extracellular shedding, exerts FGF-23-independent phosphaturic and calcium-conserving effects through its paracrine action on the proximal and distal tubules, respectively. Klotho downregulation is the earliest biomarker of CKD and the initiator of CKD-mineral bone disorder. Maintaining normal phosphate levels with phosphate binders is expected in patients with CKD by declining Klotho expression to reduce mineral and vascular derangements.[2] Klotho deficiency is associated with progression and chronic complications in CKD, including vascular calcification, cardiac hypertrophy, and secondary hyperparathyroidism.[3] Available data indicate that FGF-23-FGF receptor/Klotho pathway can be a new drug target for disorders of phosphate and bone metabolism.[4]

The *KL* gene might be associated with survival in patients with ESRD.[5] Among factors influencing mortality rate of hemodialysis patients, genetic risk factor are of particular relevance.[6] Mutations in the *KL* gene in humans lead to a syndrome including severe vascular calcification with normal kidney function.[7] More recently, a gain-of-function mutation in the *KL* gene has been linked with severe hypophosphatemic rickets and hyperparathyroidism in humans.[8]

Circulating soluble-α-Klotho results from transmembrane-Klotho shedding and acts on phosphate and calcium tubular transport. Thus, the levels of soluble-α-Klotho could represent a marker for CKD-mineral bone disorder. Soluble-α-Klotho correlated positively with estimated glomerular filtration rate and serum calcium and negatively with serum phosphate, PTH, and FGF-23. These data indicate a negative effect of kidney disease on circulating soluble-α-Klotho starting very early in CKD. Assuming that soluble-α-Klotho mirrors transmembrane-Klotho synthesis, low circulating soluble-α-Klotho seems to reflect the ensuing of tubular resistance to FGF23, which accordingly is increased. Researchers endorse soluble-α-Klotho as an early marker of CKD-mineral bone disorder.[9]

In summary, α-Klotho, with its transmembrane and soluble forms, is deeply involved in the physiological regulation of mineral metabolism.[10] Both alleles of the G395A and CA1818T of the *KL* gene single nucleotide polymorphism (SNP) emerging deregulation on bone metabolism may be associated with the risk of mortality in patients receiving hemodialysis.[11,12] The Error in bone metabolism may initiate kidney failure. The aim of this study was to study the relationship between Klotho SNP and bone metabolism as early prognostic measure for CKD in Iranian patients.

MATERIALS AND METHODS

Study Population

The present case-control study included 2 groups: the hemodialysis group consisted of 60 patients receiving maintenance hemodialysis in September 2013, and control group consisted of 60 age-matched unrelated healthy volunteers who were selected randomly from individuals referring for routine laboratory tests. Individuals with liver and cardiovascular diseases and those with lipidemia were excluded from the study. All selected participants had never taken any medication related to bone turnover such as vitamin D and phosphate binders.

The study protocol was approved by the local Ethics Committee of Zanjan University of Medical Sciences. All investigations were performed in accordance with the Declaration of Helsinki. After obtaining informed consent, demographic and clinical data were collected.

Sample Collection and DNA Extraction

Whole blood samples for DNA extraction and serum samples for biochemical tests were kept at -20°C until use. Genomic DNA from whole blood was extracted using a Fermentase Genomic DNA Purification Kit (Cat No, 0512). The DNA quality was assessed by 260/280 OD ratios in all samples and were stored at -70°C.

Genotyping

Polymorphism selection. Among all known polymorphisms of Klotho, 2 SNPs (G395A and C1818T) were selected on the basis of the studies which showed that these SNPs were very common in Asian populations.[1,13] Both SNPs are listed in the National Center for Biotechnology Information SNP database, which happened to be very common (minor allele frequencies: A = 0.16 and T = 0.25). On the other hand, G395A and C1818T SNPs were included because they had previously been associated with CKD and CKD-related diseases

such as cardiovascular disease and osteoporosis.

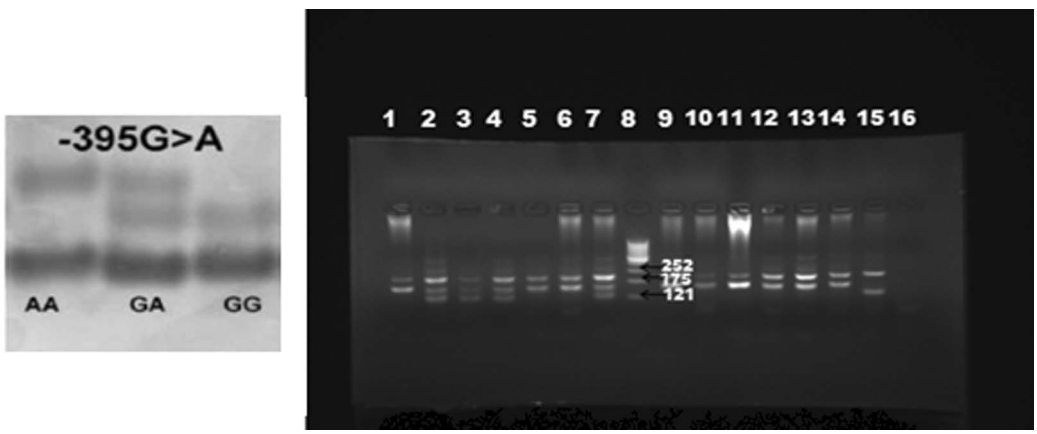
Polymerase chain reaction. In this study, polymerase chain reaction with confronting two-pair primers assay was used for genotyping of G395A (rs1207568) in the promoter region and C1818T (rs564481 or rs7596111 merged form) in exon 4. Four pair primers used for both SNPs were as described by Lorente and colleagues.[14] Polymerase chain reaction was carried out using the polymerase chain reaction Master Mix 2× (Fermentase; Cat No, K0171) according to the manufacturer's protocol for a reaction mixture of 25 µL containing 500 ng of total DNA template. Amplification was performed with a thermal cycler (Analytic Jena, Germany) under the following conditions: 95°C for 10 minutes, followed by 30 cycles of 94°C for 45 seconds, 61°C for 45 seconds, and 72°C for 45 seconds, followed by a 5-minute 72°C final extension. Polymerase chain reaction products were electrophoretically separated on a 2.5% agarose (Invitrogen, USA) gel and visualized with an ultraviolet trans-illuminator after staining by DNA safe stain (Cinna Gen, Iran). Polymerase chain reaction products sizes for G395A (Figure) included 252 bp and 175 bp for GG genotype, 252 bp and 121 bp for AA genotype, and 252 bp, 175 bp, and 121 bp for GA genotype. For C1818T, the particles were 416 bp and 291 bp for CC genotype, 416 bp and 179 bp for TT genotype, and 416 bp, 291 bp, and 179 bp for CT genotype.

Biochemical Parameters

25-hydroxyvitamin D3 was measured using a Euroimmune kit (Germany; Order No, EQ 6411-9601) that is based on competitive enzyme-linked immunosorbent assay and uses anti-25-hydroxyvitamin D3 antibody-coated plates for measuring the active form of vitamin D3 according to the calibrators concentration. Measurement of PTH was performed using a Diamentra kit (Germany) that is based on enzyme-linked immunosorbent assay, too. The enzyme-linked immunosorbent assay reader was STST 4200. Serum levels of calcium and phosphate were measured with Pars Azmoon kits (Tehran, Iran) using BT3000 auto-analyzer (Biotechnica Instruments, USA) on the basis of Arsenazo and ammonium molybdate methods, respectively, both of which measure free calcium and phosphate.

Statistical Analysis

The qualitative and quantitative results were expressed as the frequency and mean ± standard deviation, respectively. Statistical analysis was performed using the SPSS software (Statistical Package for the Social Sciences, version 22.0, IBM Corp, New York, NY, USA). The genotype frequencies of both SNPs were estimated by allele counting for all the participants, and the Hardy-Weinberg equilibrium was estimated using the chi-square test. Linkage disequilibrium and haplotyping were analyzed using the CubeX software (Online Encyclopedia for Genetic Epidemiology Studies).[15] Differences in the frequency of the genotypes between the groups were tested using the chi-square test. The association between groups and



Polymerase chain reaction products sizes for G395A. Columns 16 and 8 are negative control ladder, respectively; columns 1, 5, 6, 9, 12, 13, and 14 indicate GC genotype; columns 2, 3, 4, and 7 show GA genotype, and column 15 demonstrates AA genotype. The 252-bp bands were common to all columns as control, and 175-bp bands with 121 bp expressed AA genotype and 252-bp, 121-bp, and 175-bp bands expressed GA genotype.

biochemical factors was evaluated using regression logistic binary test. Differences in serum PTH, 25-hydroxyvitamin D3, calcium, and phosphate levels between the two groups were tested using independent the Student *t* test or the Mann-Whitney test, as appropriate. *P* values less than .05 were considered significant.

RESULTS

Participants' Characteristics

Characteristics of 120 participants (60 in each group) are shown in Tables 1 and 2. Although PTH and phosphate levels were significantly higher in the hemodialysis group than the control group, the baseline levels of serum calcium and 25-hydroxyvitamin D3 were not significantly different between the two groups.

Genotypes and Haplotype Structures

Using polymerase chain reaction with confronting two-pair primers assay, genotype frequencies of the two SNPs (G395A and C1818T) in the hemodialysis and control groups were calculated (Table 3). Given the low expression of Klotho in *A* and *T* allele carriers of both SNPs, participants in the G395A SNP were divided into A allele carriers versus noncarriers (GA and AA versus GG), and in the C1818T SNP, into T allele carriers versus noncarriers (CT and TT versus CC). However, the genotypes and alleles distribution of G395A and C1818T polymorphisms had no significant difference between the two groups ($P = .28$ for G395A and $P = .90$ for C1818T). The frequency of being A carriers suggested marginal significances between the groups (GA and AA, 30% versus GG, 18.3%, $P = .06$), but such significant results were not found for the T allele carriers (CT and TT, 76.6%

Table 3. Genotype and Allele Frequencies in Hemodialysis and Control Groups

Genotype	Hemodialysis Group	Control Group	*P*
G395A			
G/G	42 (70)	49 (81.7)	
G/A	15 (25)	10 (16.7)	
A/A	3 (5)	1 (1.6)	.28
G395A			
GG	42 (70)	49 (81.7)	
GA and AA	18 (30)	11 (18.3)	.06
C1818T			
C/C	14 (23.3)	…	
CT	43 (71.6)	…	
T/T	3 (5)	…	.90
C1818T			
CC	14 (23.3)	14 (23.3)	
CC and TT	46 (76.6)	46 (76.6)	> .99

Table 1. Characteristics of Hemodialysis Patients

Characteristic	All	Men	Women
Number of participants	60	27	33
Age, y	58.63 ± 11.51	56.88 ± 15.75	52.41 ± 9.80
Fasting blood glucose, mg/dL	112.16 ± 28.64	114.07 ± 24.10	113.97 ± 31.90
Blood urea, mg/dL	50.20 ± 10.52	52.44 ± 9.54	48.55 ± 10.65
Serum creatinine, mg/dL	5.27 ± 1.21	5.48 ± 1.29	5.09 ± 1.18
Serum calcium, mg/dL	9.46 ± 0.74	9.47 ± 0.83	9.43 ± 0.63
Serum phosphorus, mg/dL	4.61 ± 0.71	4.06 ± 0.59	4.66 ± 0.79
Vitamin D, U/L	21.97 ± 7.52	24.42 ± 7.86	19.43 ± 6.62
Parathyroid hormone, pg/mL	80.23 ± 23.00	89.78 ± 32.00	72.50 ± 33.60

Table 2. Characteristics of Age-matched Healthy Individuals

Characteristic	All	Men	Women
Number of participants	60	27	33
Age, y	55.00 ± 11.13	55.83 ± 11.33	55.22 ± 11.12
Fasting blood glucose, mg/dL	104.73 ± 14.73	104.37 ± 14.63	104.11 ± 14.74
Blood urea, mg/dL	17.60 ± 5.53	17.60 ± 5.93	17.80 ± 5.49
Serum creatinine, mg/dL	1.20 ± 2.11	1.26 ± 2.51	1.35 ± 2.36
Serum calcium, mg/dL	9.46 ± 0.56	9.47 ± 0.53	9.46 ± 1.31
Serum phosphorus, mg/dL	4.03 ± 0.49	4.025 ± 0.05	4.025 ± 0.49
Vitamin D, U/L	25.30 ± 14.09	25.36 ± 13.71	25.36 ± 14.35
Parathyroid hormone, pg/mL	47.60 ± 12.24	47.68 ± 14.90	47.80 ± 15.24

versus CC, 76.6%, $P > .99$). Thus, compared with the GG genotype, the A carriers were associated with an increased likelihood of being on hemodialysis. The allele frequencies were 0.9 and 0.1 for G and A, respectively, and 0.6 and 0.4 for allele C and T as well. The Hardy-Weinberg equilibrium was tested for G395A SNP, and no deviation was found from this assumption ($P = .57$), but C1818T was significantly out of Hardy-Weinberg equilibrium ($P < .05$).

Linkage disequilibrium analysis was performed on G395A and C1818T sites of the *KL* gene. Linkage disequilibrium was calculated for the whole population ($D' = -0.1$; $r^2 = 0.0034$) indicating that linkage disequilibrium was markedly lower in the participants across the gene region. There was no significant association between the four possible haplotypes and risk of hemodialysis ($P = .58$).

Klotho Polymorphisms and Risk of Hemodialysis

Homozygote and heterozygote individuals for the A allele at G395A SNP (A allele carriers) were more likely to be on hemodialysis (odds ratio, 1.43; 95% confidence interval, 0.60 to 3.30), but this association was not true for T allele carriers of C1818T SNP. As can be seen in Table 4, clear trends were observed for both groups in all biochemical parameters except for vitamin D3 and fasting blood sugar ($P < .05$). Hyperphosphatemia increased the risk of hemodialysis by about 70%. The mean phosphate level was 0.585 ± 0.22 mg/dL higher in the hemodialysis group than the control group (Table 3).

DISCUSSION

In this study, we evaluated 2 SNPs (G395A in promoter region and C1818T in exon 4) of *KL* gene to find an association with biochemical parameters of Iranian patients receiving hemodialysis. Although there were no differences between genotypes in the hemodialysis and control groups, the frequency of being an A carrier had marginal statistical significance between the two groups, indicating the importance of these genetic variations in bone mineral metabolism in patients receiving hemodialysis. On the other hand, the need for hemodialysis was associated with high levels of PTH, creatinine, urea, phosphate, and being an A carrier. The *KL* gene was clearly correlated with PTH and 25-hydroxyvitamn D3 for regulation of calcium, and phosphorus, and its expression was dramatically decreased in patients with CKD.

Table 4. Logistic Regression Model of Factors Associated With Hemodialysis

Parameter	Odds Ratio (95% Confidence Interval)	*P*
Vitamin D3	1.025 (0.993 to 1.057)	.12
Parathyroid hormone	0.959 (0.940 to 0.978)	< .001
Serum calcium	0.997 (0.619 to 1.606)	.99
Serum phosphorus	0.322 (0.583 to 0181)	< .001
Serum creatinine	0.259 (0.171 to 0.391)	< .001
Blood urea	0.706 (0.605 to 0.824)	< .001
Fasting blood glucose	1.003 (0.996 to 1.011)	.39

Rhee and colleagues showed that Korean women with A allele at G395A SNP had higher blood pressure compared with noncarriers. However, Bostrom and coworkers did not find any association of A allele carriers for G395A SNP with nondiabetic ESRD in African Americans,[16] although A allele carriers were associated with the severity of ESRD in this population. Our results showed that the A allele carriers were at risk of receiving hemodialysis. This genetic background of being A carrier may lead to decrease Klotho expression resulting in high blood pressure and consequently kidney damage.

It has been shown that aging process originating from Klotho or FGF-23 deficiency are consequences of phosphate toxicity. In addition, it has been reported that high levels of phosphate in CKD patients were associated with a stepwise increase in mortality compared with those having lower serum phosphate levels.[17-19] On the other hand, serum phosphate independently predicts decline in renal function in early CKD.[20,21] Higher mean level of phosphate in hemodialysis patients compared with healthy ones in our study and increased susceptibility to hemodialysis by a chance of 70% resulting from serum phosphate level could explain the importance of phosphate toxicity and its prediction power. Some in vitro and human studies have shown that hyperphosphatemia, which is common in CKD patients, may lead to increased cardiovascular mortality by inducing vascular calcification.[22]

Yilmaz and colleagues found a diagnostic value for PTH levels in CKD patients receiving dialysis that is consistent with our results indicating higher PTH level in patients receiving hemodialysis.[23] Secondary hyperparathyroidism is a common

complication of CKD and it is clear that changes in the bone-kidney-parathyroid axis showing as phosphate elevation could be seen during PTH elevation.[24,25] To address such a change, Oliveira and colleagues used phosphate binders to adjust PTH in CKD patients, because PTH is progressively increased as kidney function fails as a result of phosphate retention. Therefore, not only PTH level may be a good prognostic factor, but also it could be a good marker for management of the disease.[26]

In contrast to our study, Yamada and coworkers showed, in a cohort study, an association between G395A SNP and mineral bone disorder in postmenopausal Japanese women and reported that the mineral bone disorder tended to be lower in subjects with GG genotype than in those with AA genotype or those with combined GA and AA genotypes, but they did not find such association in premenopausal women.[27] As reported repeatedly, Klotho protein is involved in aging processes such as osteoporosis in postmenopausal state, but it has been shown that A carriers of G395A SNP have lower Klotho protein involving in aging phenotype. On the other hand, bone and mineral complications of CKD reflected in mineral bone disorder and expected to be lower in A carriers. Although, both calcium and 25-hydroxyvitamin D3, the most common coordinators of mineral bone disorder, were lower compared with the control group in our study, we did not find any significant differences for these biochemical parameters in the studied groups.

The C1818T is a silent mutation located in exon 4. It has been shown that the T allele carriers of C1818T SNP were associated with a risk of coronary artery disease and bone density in postmenopausal period.[22,28] We did not find any associations for the T allele carriers in CKD patients compared with healthy subjects and with biochemical parameters. Despite the lack of evidence for a direct association of this polymorphism, it is possible that these differences might be related to different genetic backgrounds in various populations and different confounders such as the use of active vitamin D3 and phosphate binder or dietary states.

The A allele carrier status of G395A polymorphism was associated susceptibility with disease progression and mortality in Asian population.[29] The expression of the A allele of the Klotho G395A polymorphism was significantly downregulated in the hypertension group compared to the control group.[30] This gene encodes a type I membrane protein. Reduced production of this protein has been observed in patients with chronic kidney failure, and this may be one of the factors underlying the degenerative processes, arteriosclerosis, osteoporosis, and skin atrophy seen in CKD.

CONCLUSIONS

This is the first study in Iranian patients with CKD which shows that PTH and phosphate along with creatinine and urea could serve for management of the disease. Genetic background of being an A carrier could increase the susceptibility to hemodialysis. These findings prove the hypothesis that patients with higher levels of Klotho expression prior to the progression to ESRD may be protected by an enhanced ability to regulate phosphate homeostasis, because it has been demonstrated that A carriers of G395A SNP had lower Klotho protein.

CONFLICT OF INTEREST

None declared.

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Correspondence to:
Aboalfazl Nazarian
Department of Clinical Biochemistry, Faculty of Medicine, Zanjan University of Medical Sciences, PO Box 4513956111, Zanjan, Iran
Tel: +98 24 334 40301
Fax: +98 24 334 49553
E-mail: nazarian@zums.ac.ir

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