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RESEARCH ARTICLE

KLOTHO IN CHRONIC KIDNEY DISEASE IN INDIAN POPULATION

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ABSTRACT

Introduction: Klotho, an anti-aging protein primarily expressed in the kidney is the suppressor of the expression of multiple aging phenotypes.

Aims and Objectives: Present study aimed at analyzing the soluble alpha-Klotho levels in serum, prevalence of its KL-VS (F352V) and promoter (G-395A) polymorphisms in chronic kidney disease (CKD) patients & controls and their influence on soluble alpha-Klotho levels.

Materials and Methods: CKD (N=100) and healthy Controls (N=97) were enrolled for the study. Soluble alpha-Klotho levels were analysed by ELISA at recruitment of subjects and further in CKD stage 1 & 2 (N=10), stage 3 & 4 (N= 14) and stage 5 (N=48) at 6 month. Genotyping of T42568G SNP (rs 9536314, F352V, of the KL-VS variant) and G-395A SNP (rs1207568) of the promoter polymorphism of Klotho gene was carried out by Taqman genotyping assay using Real Time PCR. The influence of the genotypes on alpha-Klotho levels was also examined.

Results: As compared to the controls, there was a significant decrease in alpha -Klotho levels in CKD stage 1 & 2 (42.1%, p=0.01), stage 3 & 4 (74.2%, p=0.001) and in stage 5 (78.2%, p=0.001) in serum. However no significant change was seen in alpha -Klotho levels in patients at six months as compared to the levels at recruitment. The prevalence of heterozygous and homozygous genotypes for both these SNPs was low as compared to wild type genotype. The distribution of genotypes did not show any significant difference between CKD and Control groups and alpha-Klotho levels were not influenced by genotypes of both the polymorphisms.

Conclusion: Soluble alpha-Klotho levels were found to decrease significantly at initial CKD stages. Thus, analysis of soluble alpha-Klotho levels may help in identifying CKD patients much before the severity sets in.

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INTRODUCTION

Klotho gene was first introduced as an age suppressor gene in 1997 by Kuro-o and colleagues (Kuro-o *et al*., 1997). In their experiment, defect in its expression in mice was shown to be associated with various features of premature aging (Kuro-o *et al*., 1997). Apart from these, Klotho has also been demonstrated to be associated with phosphate imbalance and play important role in mineral bone disease associated with chronic kidney disease (CKD) and in other diseased conditions (Kurosu *et al*., 2006). Klotho, or more precisely alpha-Klotho (α-Klotho) is a single-pass transmembrane protein that is expressed predominantly in kidney tubular epithelium (Aizawa *et al*., 1998 and Kato *et al*., 2000), and to a lesser extent in the parathyroid gland (Hofman-Bang *et al*., 2010 and Krajisnik *et al*., 2010) and epithelial cells of the choroid plexus (Li *et al*., 2004). The protein has a large extracellular amino-terminal domain and a small intracellular carboxy-terminal domain. Circulating Klotho results either from direct secretion by the cell or from cleavage of the intracellular domain of the full length protein by secretases (Matsumura, *et al*., 1998). The secreted Klotho protein with glucuronidase activity controls multiple ion channels and growth factor signaling pathways, including insulin, IGF-1, and Wnt signaling (Tatar *et al*., 2003; Unger *et al*., 2006; Yamamoto *et al*., 2005; Liu *et al*., 2007). This signaling activity in tissues shows close association with

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the extended life span and is known to regulate various metabolic processes. The transmembrane form of Klotho protein functions as an obligatory co-receptor for a bone derived phosphaturic hormone, Fibroblast growth factor-23, (FGF-23). Klotho is required by FGF-23 for its functioning. The Klotho protein binds to multiple FGF receptors and increases their affinity for FGF-23 and helps in excreting phosphorus from kidney (Kurosu *et al*., 2006). Thus FGF-23 and Klotho are crucial components for the regulation of blood phosphate levels. Klotho protein is encoded by a 50-kb gene on chromosome 13q12, which consists of 5 exons (Matsumura, *et al*., 1998). A common variant of the human Klotho gene (*KL*) is reproducibly associated with longevity and healthy aging (Di Bona, *et al*., 2014 and Arking *et al*., 2002). A haplotype, "KL-VS" composed of six single nucleotide polymorphisms (SNPs), spanning exon 2 and its flanking sequence was reported by Arking *et al*. (2002). Two of these SNPs result in amino acid substitutions: F352V (rs9536314) and C370S (rs9527025). "KL-VS" refers to the V and S alleles of these SNPs respectively, and since all six SNPs occur in perfect linkage disequilibrium, a single variant, F352V, can be used to tag the haplotype. Arking's *et al*., (2002) who identified this variant also carried out a transient transfection assay, and have reported that secreted levels of Klothoharboring V352 are reduced 6-fold, whereas extra cellular levels of the S370 form are increased 2.9-fold. The V352/S370 double mutant exhibits an intermediate phenotype (1.6-fold increase), providing a rare example of intragenic complementation in cis by human SNPs. The remarkable conservation of F352 among homologous proteins suggests that it is functionally important (Bonafè *et al*., 2009). This finding and its potential role in phosphate imbalance further demands study of this polymorphism in CKD. Another polymorphism, G-395A substitution in the promoter region has been reported to affect DNA–protein interaction which might affect the level of expression of Klotho (Kawano et al., 2002). Kawano *et al*. (2002) have reported through the dual-luciferase reporter assay that the -395A carrier of a 498-bp DNA fragment (containing the G-395A site) upstream of the Klotho gene had higher relative luciferase activity than the -395G carrier. This suggest increased expression of the Klotho gene with -395A allele. The present study thus aimed at analyzing the soluble levels of alpha-Klotho, prevalence of KL-VS and G-395A polymorphisms of alpha-Klotho gene and their influence on soluble alpha-Klotho levels in CKD patients & controls in Indian population.

MATERIALS AND METHODS

Study Population

Hundred patients with CKD between 18 to 70 years of age with or without any co-morbidity and 97 healthy individuals were enrolled for this study. They were selected as per the inclusion and exclusion criteria set for the study. On the basis of the renal profile carried out during enrollment, patients were classified into CKD stages as per estimated glomerular filtration rate (eGFR) calculated using "Modification of Diet in Renal Disease" (MDRD) method and presence of microalbumin creatinine ratio (ACR) more than 30mg/gm creatinine in urine. (Eknoyan, *et al*., 2002) CKD Stage-1 patients were with eGFR≥ 90 ml/min/1.73 m^2, CKD Stage-2 were with 60–89 ml/min/1.73 m^2, CKD Stage-3 were with 30–59 ml/min/1.73 m^2 and CKD Stage-4 were with 15–29 ml/min/1.73 m^2. Healthy individuals recruited were volunteers in the study with no CKD or any other organic disease and normal eGFR (eGFR ≥ 90 ml/min/1.73m^2) and ACR less than 30mg/gm creatinine. They were confirmed healthy on the basis of their clinical history and routine biochemical investigations. The study protocol was approved by the Institutional Ethics Committee which follows the ethical standards laid down by the ICMR's ethical guidelines for biomedical research on human participants. Patient Information Sheet (PIS) was explained to each patient in the language understood by them in the presence of the house doctor and patient's relatives. On agreeing to participate in the study, signature on PIS and Informed Consent (IC) was obtained from all patients before the blood collection. After the enrolment of patients in the study, blood samples were obtained just before starting hemodialysis sessions for renal profile analysis.Aliquots of serum were immediately obtained from the blood sample and then stored at -80 °C until further use. From these 100 CKD patients, samples of pre-dialytic CKD patients of CKD stage 1 & 2 (N=10), CKD Stage 3 & 4 (N=14) and dialytic CKD stage 5 (N=48) were also tested for Klotho levels after six month.

Soluble levels of alpha-Klotho were assayed with a novel enzyme-linked immunosorbent assay (ELISA) method (Immuno-Biological Laboratories Co., Ltd., Hamburg, Germany) which specifically detected and measured the 130kDa circulating soluble alpha-Klotho protein in the serum. DNA was obtained from peripheral blood cells from K-EDTA anti-coagulated blood using Purelink Genomic DNA kit (ThermoFisher Scientific, #: K182002). Genotyping was performed for T42568G SNP (rs9536314, F352V) of the KL-VS variant and G-395A SNP (rs1207568) of the promoter polymorphism of Klotho gene using Real Time PCR (RT PCR; Step One Plus Applied Biosystems) with Taqman genotyping assays (Assay numbers: For rs9536314-C_2983037_20; for rs1207568 - C_7604792_10).

Statistical Analysis

Measured variables as Mean ± SD or median (25^{th}/75^{th} quartiles) were compared between patient groups and controls with the use of unpaired student's t test or Mann–Whitney U test respectively. Genotype frequencies were estimated by the gene-counting method. Allelic frequencies were calculated from genotype frequencies. Genotypes were tested for deviations from Hardy–Weinberg equilibrium. Difference in the distribution of genotypes between patients and controls were calculated by chi square statistics. Analyses were performed using statistical software SPSS (version 21.0, Chicago, IL).

RESULTS

The baseline demographic of 97 controls and 100 CKD patients are depicted in Table 1 while Table 2 represents demographic and clinical data of pre-dialytic and dialytic CKD patients whose serum samples were assayed for alpha-Klotho levels. There was significant difference in the age between CKD stage 3 and 4 patients and controls as very few healthy individuals in corresponding age group who were not associated with any comorbidity could be enrolled. For CKD stage 5 patients, healthy age matched individuals were enrolled but there was significant reduction in the weight and BMI of these CKD patients as compared to the controls due to their debilitating condition.

Table 1. Demographicand Biochemical Data of CKD patients

	Control N=97	CKD Patients N=100
M/F	60/40	61/39
Age (years)	36.7± 11.4	50.1***± 13.7
Weight (kg)	64.0 ± 16.4	58.9*** ± 15.1
BMI (kg/m^2)	24.5 ± 5.32	23.7± 5.1^{NS}
Smoking	-	29(29%)
Alcohol	-	50(50%)
Diabetes	-	57(57%)
Hypertension	-	70(70%)
GFR(ml/min/1.73m^2)	104.5± 27.4	25.8*** ± 24.9(4.0 fold ↓)
BUN (mg/dl)	9.2± 2.68	39.9*** ± 25.3 (4.0 fold ↑)
Creatinine (mg/dl)	1.06 ± 0.32	5.66*** ± 5.87 (5.3 fold ↑)

P<0.05, ** p<0.01, *** p<0.001, NS non significant

Table 2. Demographic and Biochemical Data of PredialyticCKD and Dialytic CKD patients

	Control (N=14)	CKD Stage 1&2(N = 10)	CKD Stage 3 &4(N = 14)	Control (N=66)	CKD Stage 5 (N = 48)
		Pre-dialytic CKD Patients			Dialytic CKD patients
M/F	4/10	6/4	7/7	32/34	32/16
Age (years)	55.3 ± 6.62	56.5^{NS} ± 11.3	63.5** ± 6.68	41.39 ± 10.49	42.6 ^{NS} ±12.43
Weight(kg)	59.8 ± 18.2	60.8 ^{NS} ± 12.2	63.1 ^{NS} ± 17.6	65.16 ± 15.35	55.4*** ± 13.93(14.96% ↓)
BMI (kg/m^2)	23.8 ± 6.2	24.5 ^{NS} ± 3.69	24.32 ^{NS} ± 4.92	25.21 ± 5.16	22.3*** ± 4.72(11.58%↓)
Smoking	-	2(20%)	3(21%)	-	13(27%)
Alcohol	-	3(30%)	7(50%)	-	21(44%)
Diabetes	-	1(10%)	10(71%)	-	27(56%)
Hypertension	-	8(80%)	9(64%)	-	26(54%)
eGFR(ml/min/1.73m2)	90.81 ± 13.55	73.01*** ± 10.48 (19.6%↓)	45.02 *** ± 12.76 (50.4% ↓)	101.7 ± 26.21	9.24*** ±3.38 (11.0 fold↓)
BUN (mg/dl)	8.0 (7.75/9.3)	11.5** (10.5/14.8) (43.8% ↑)	16*** (13.8/24.5) (100.0%, 2.0 fold↑)	9.50 (8/12)	49.5*** (38/58.8) (421.0%,5.21 fold↑)
Creatinine (mg/dl)	0.85 (0.70/1.0)	1.0* (0.9/1.15) (17.6 % ↑) ~~17.6 % ↑~~	1.60*** (1.37/1.75) (88.2%↑)	1.0 (1.0/1.0)	7.0*** (6.0/8.0) (600%, 7.0 fold ↑)

P<0.05, ** p<0.01, *** p<0.001, NS non significant

Table 3. Alpha-Klotho levels in CKD patients after 6 months follow up

	Recruitment	At 6 months
Stage 1 and 2 (n=10)	319 (96.6/550)	280 (165/412) NS
Stage 3 and 4 (N=14)	142.5 (52.5/264)	176 (31.8/249) NS
Stage 5 (N=48)	133 (96.5/177)	122 (31.3/158.5) NS

NS non significant

Table 4. Genotype and allele Frequencies of T42568G (rs9536314, F352V) of Klotho Gene

	Genotype Frequencies			Allele frequencies	
	Wild Type Genotype (TT)	Heterozygous Genotype(TG)	Homozygous Genotype (GG)	Wild Type Allele(T)	Variant Allele(G)
	Number(%)				
CasesN=87	70 (80.5)	15(17.2)	2 (2.3)	0.89	0.11
ControlsN=79	65 (82.3)	11 (13.9)	3 (3.8)	0.89	0.11
	Chi Square (χ^2) =0.62, p= 0.73., NS			Chi Square (χ^2) =0.0022, p=0.96, NS	

NS non-significant

Table 5. Genotype and Allele Frequencies of G-395A (rs 1207568) of Klotho Gene

	Genotype Frequencies			Allele frequencies	
	Wild Type Genotype (GG) Number (%)	Heterozygous Genotype (GA)	Homozygous Genotype (AA)	Wild Type Allele (G)	Variant Allele (A)
Cases N=94	66 (70.2)	27 (28.7)	01 (1.1)	0.85	0.15
Controls N=86	57 (66.3)	26 (30.2)	03 (3.48)	0.81	0.19
	Chi Square (χ^2) =1.26, p=0.531, NS			Chi Square (χ^2) =0.645, p=0.42, NS	

NS non-significant

Table 6. Alpha-KlothoLevels According to KL-VS and G-395A Genotypes

Genotype (F352V)	Controls (N=40)	All Stages (N=72)	Genotype (G-395A)	Controls (N=49)	All CKD Stages (N=78)
TT	611(530/767) (N=34)	143.5(98/189.8) (N=60)	GG	637(532/750) (N=32)	150(100/286) (N=56)
GT	697(566/748) (N=5) NS	135(15/224.5) (N=9) NS	AG	615(517/800) (N=15) NS	135(96.4/241) (N=21) NS
GG	577.5 (N=1)	50/84(N=3)	AA	637(735/-) (N=2)	152 (N=1)

NS Non-significant

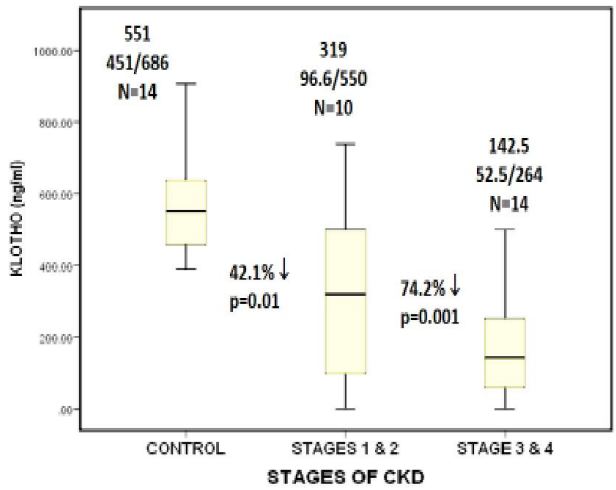

Figure 1. Alpha-Klotho Levels in Pre-dialyticCKD stage (1 – 4) patients

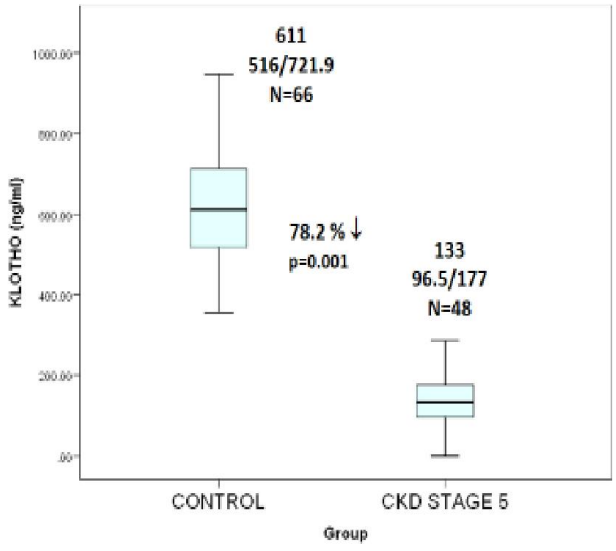

Figure 2. Alpha-Klotho Levels in Dialytic CKD stage 5 patients

rs9536314,

F352F, T42568T

Wild Type Homozygous

Genotype

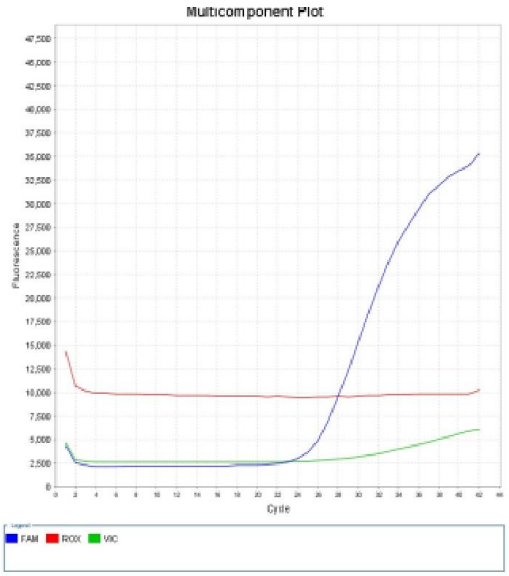

Figure 3a. Wild type Genotype of KL-VS polymorphism

rs9536314

F352V,

T 42568 G

Heterozygous

Genotype

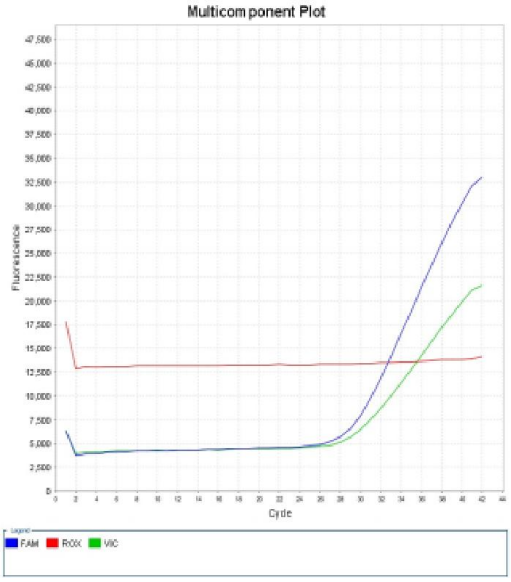

Figure 3b. Heterozygous Genotype of KL-VS polymorphism

rs9536314

V352V, G 42568 G

Mutant Homozygous

Genotype

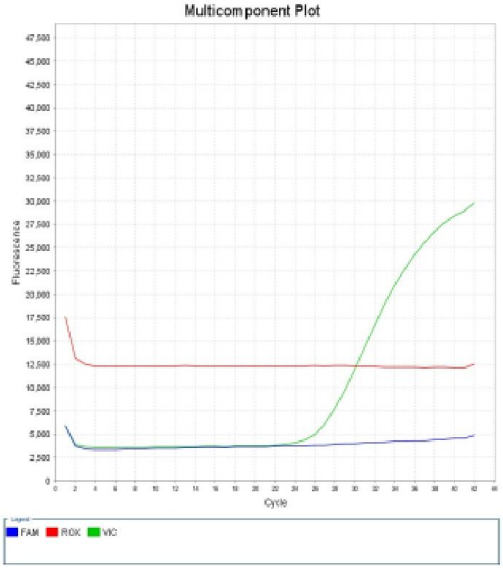

Figure 3c. Homozygous Genotype of KL-VS polymorphism

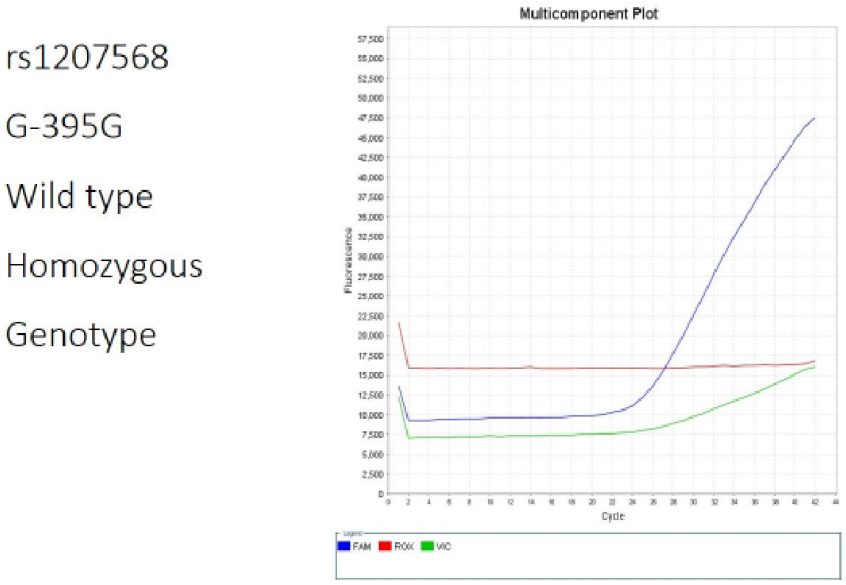

Figure 4a. Wild type Genotype of G-395A polymorphism

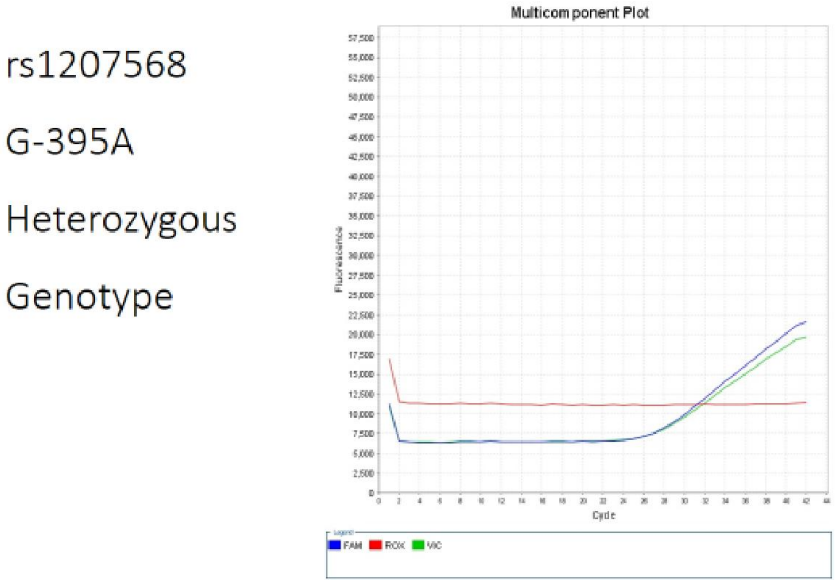

Figure 4b. Heterozygous Genotype of G-395A polymorphism

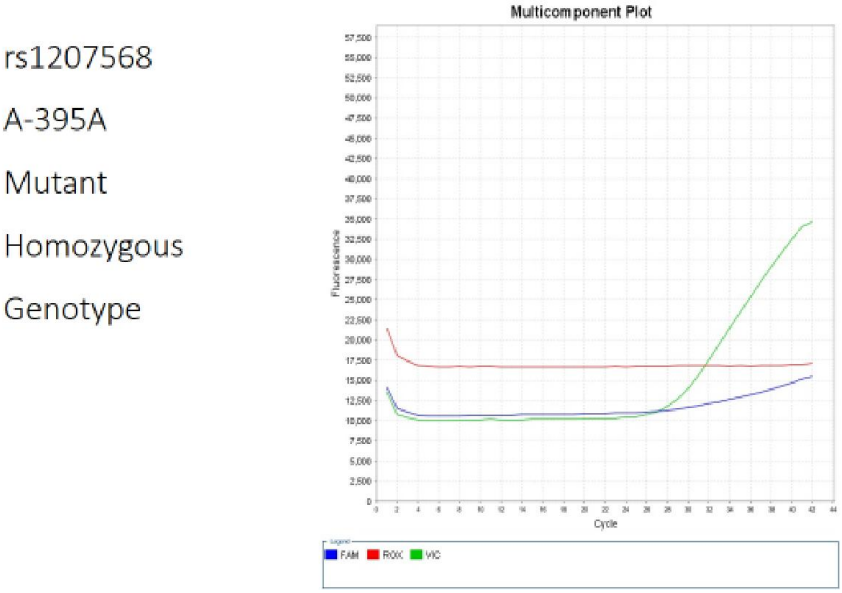

Figure 4c. Homozygous mutant Genotype of G-395A polymorphism

Soluble alpha-Klotho levels

A significant decrease in solublealpha-Klotho levels in CKD stage 1 & 2 (42.1%, p=0.01), stage 3 & 4 (74.2%, p=0.001) (Figure 1) and in stage 5 (78.2%, p=0.001) (Figure 2) was observed as compared with respective age matched controls. However, no significant change was seen in alpha-Klotho levels in pre-dialytic (CKD stage 1 and 2 as well as CKD stage 3 and 4) and in dialytic (CKD stage 5) patients after a follow up of six months as compared with the corresponding baseline levels (Table 3).

Klotho gene polymorphisms

Wild type, heterozygous and homozygous genotypes of T42568G (F352V) and G-395A polymorphisms is presented in Figure 3a, 3b and 3c and Figure 4a, 4b and 4c respectively as obtained by Real Time PCR. The prevalence of T42568G (F352V) variants of KL-VS and G-395A polymorphisms in CKD group and in controls is shown in Table 4 and 5 respectively. The genotype distribution did not deviate from the Hardy-Weinberg equilibrium for both the SNPs. The distribution of the genotypes did not show any statistical significant difference between CKD and Control group. Within the patient group, both the polymorphisms did not show any significant association with risk factors like smoking, hypertension or diabetes (Data not shown).

Distribution of Klotho gene polymorphisms and soluble alpha-Klotho levels

Table 6 shows the distribution of genotypes of G-395A and T42568G (F352V) and corresponding soluble alpha-Klotho levels among the CKD patients and controls. Alpha-Klotho levels did not differ as per genotypes in both the groups.

DISCUSSION

In the present study soluble levels of anti-aging protein alpha-Klotho were analysed in different stages of CKD which decreased with increased CKD stages. The decrease in the earlier stages (CKD-1 and 2) was remarkable as compared to controls. The alpha-Klotho levels did not differ as per genotypes of T42568G (F352V, variants of KL-VS polymorphism) and G-395A in both the groups. The prevalence of heterozygous and homozygous variants for both these SNPs was low and the overall genotype distribution did not differ between CKD patients and controls. Studies (Weinstein *et al*., 2010, Zhou *et al*., 2008, Fred *et al*., 2005) have universally reported decrease in renal function along with the increase in age. Masuda *et al*., (2005) in-vivo studies have demonstrated that expression of Klotho is down-regulated during aging. Additionally studies conducted (Xiao *et al*., 2004 and Shimamura *et al*., 2012) in different populations confirmed the finding that Klotho levels decrease with reduction in renal Klotho. Shimamura *et al*. (2012) have demonstrated that soluble Klotho levels were low in CKD stage 2-5 patients as compared with CKD stage 1 patients. In accordance to these results, present study showed an inverse relationship between soluble levels of alpha-Klotho protein and progress of CKD. However, the kidneys are primary site but not the only site for Klotho production (Aizawa *et al*., 1998, Kato *et al*., 2000, Hofman-Bang *et al*., 2010, Krajisnik *et al*., 2010, Li *et al*., 2004). In our study it was observed that soluble Klotho levels dropped consistently from stage 2 – 4 while Klotho level of stage 5 was similar to that of CKD stage 4; indicating minor contribution of Klotho from other organs as well. A study carried out in elderly (>79 years) populations of Ashkenazi Jewish and Bohemian Czech have observed that after adjustment for traditional risk factors, heterozygous individuals (F352V) of KL-VS polymorphism were at significantly lower risk for stroke than wild-type individuals (F352F), whereas homozygous KL-VS individuals (V352V) had the highest risk, suggesting that the heterozygous genotype of KL-VS polymorphism (F352V) had survival advantage at later ages in conjunction with a marked homozygous KL-VS(V352V) disadvantage (Arking *et al*., 2005). However, the same was not observed in Caucasian and African-American population of Baltimore (Novelli *et al*., 2008). Xu *et al*., (2015) in their study have further demonstrated that serum creatinine was significantly high in the Kazak group compared with the Uygur group in TT (wild type) genotype of rs9536314 (F352V, variants of KL-VS polymorphism) ($P<0.05$). Between Kazak and Uygur populations of Xinjiang China, longevity in the Uygurs was considerably greater than in Kazaks; thus suggesting that wild type genotype of KL-VS polymorphism (F352F) was associated with susceptibility towards CKD. However, an Indian study, by Mazumdar *et al*. (2011), have demonstrated association of the variant of KL-VS with increase in blood pressure, insulin resistance and triglyceride levels and thus with metabolic syndrome. Our data is in concordance with study by Ceppiuglu *et al*. (2011) from Turkey, wherein no association of KL-VS with CKD has been reported. Literature documents contradictory reports of the association of promoter (G-395A) polymorphism with risk factors or CKD in different populations. In Chinese population –395A allele has been reported to be protective factor for hypertension (Gao *et al*., 2015) and metabolic syndrome (Luo *et al*., 2016). Contrary to these reports, in Korean population -395A allele is demonstrated to be associated with increased mortality with hemodialysis patients (Kim *et al*., 2008 and Ko *et al*., 2013) and IgA nephropathy (Ko *et al*., 2012). Further in Japanese population-395 A allele was significantly associated with low density lipoprotein and uric acid levels in hemodialysis patients (Shimoyama *et al*., 2009) Thus, these studies underline the fact that the effect of Klotho gene variation differ with difference in ethnicity. In the present study, G-395A polymorphism was not found to be associated either as protective or increasing risk of CKD.

Small sample size of the present study is acknowledged. Loss of patients during follow-up left only few samples for analysis at six month. Remarkable finding was that as compared to controls, soluble alpha-Klotho levels were found to decrease significantly in earlier CKD stages. This highlights the role of soluble alpha-Klotho, an anti-aging protein as a potential indicator for early detection of CKD, much before the severity sets in. KL-VS and G-395A genotypic association were not observed of Klotho gene with CKD in our Indian population. Further studies in larger population may unfold many mysteries of this anti-aging protein.

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