Original Paper

The Association of *Klotho* Polymorphism with Disease Progression and Mortality in IgA Nephropathy

Gang Jee Ko^a Eun Ah Lee^a Un Sil Jeon^a Heui Jung Pyo^a Ho Jun Chin^b
Dong Wan Chae^b Suhnggwon Kim^b Young Joo Kwon^a

^aKorea University Medical school, Department of Internal Medicine, ^bSeoul National University Bundang Hospital

Key Words

IgA nephropathy • Polymorphism • *Klotho* gene

Abstract

Backgrounds: IgA nephropathy (IgAN) is the most common primary glomerulonephritis causing end stage renal disease (ESRD), and vasculopathy is known to involve disease progression. *Klotho*, a gene related to aging, has been reported to play a role in atherosclerosis and endothelial dysfunction. We investigated whether *klotho* gene polymorphism affect clinical course of IgAN. **Methods:** The data registered for PREMIER study which enrolled the patients with biopsy proven IgAN were analyzed. Two single nucleotide polymorphisms for *klotho* gene, G395A of promoter region and C1818T of exon 4, were examined, and investigated the association *klotho* genotypes with the progression of IgAN and patient survival. **Results:** Clinical data from 973 patients confirmed about survival were analyzed. The allele frequency was 0.830 and 0.170 for allele G and A, and 0.816 and 0.184 for allele C and T, which were complied with Hardy-Weinberg equilibrium ($p=0.996$ and 0.531 respectively). Death was observed more frequently in A-allele carriers of G395A polymorphism (0.7 vs 2.6 %, GG vs GA+AA, $p=0.022$). Renal survival in Kaplan-Meier survival curve was also worse in same group ($p=0.04$). **Conclusion:** *Klotho* gene polymorphism was associated with patient survival and disease progression of IgAN.

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Introduction

Klotho gene was discovered by Kuro-o et al as a gene related to suppression of aging process. It was demonstrated to have pleiotropic functions that *klotho* mutant mice showed shortened life span, growth retardation, infertility, senile atrophy of the gonads,

arteriosclerosis, skin and muscle atrophy, osteoporosis, and emphysema resembling human aging process [1]. *Klotho* is predominantly expressed in distal tubule of kidney and choroid plexus of brain, and to lesser extent in reproductive and endocrine organ [1-2]. *Klotho* or its metabolites are considered to function as humoral factors because it shows multiple functions affecting various systems in spite of relatively limited distribution [3-4]. However, dominant expression of *klotho* in kidney suggested that *klotho* may have an important role in the pathogenesis of renal diseases, and the relationship of *klotho* with various renal diseases has been reported [5-7].

IgA nephropathy (IgAN) is the most common glomerulonephritis throughout the world especially in Far East and Southeast Asia comprising nearly half of all the patients with glomerular disease [8]. Up to 40% of patients with IgAN progress to end stage renal disease (ESRD) within 25 years, and IgAN accounts for 10%-30% of all cases of ESRD [9-10]. Although IgAN is considered as an immune complex disease with complement activation in mesangial cells due to IgA deposition [11], little has been known about the exact mechanisms. Endothelial dysfunction was known involve with the glomerular injury in IgAN [12-14], and the genetic factors were also suggested to contribute to the pathogenesis of IgAN that some genetic variations affected on the incidence and progression of IgAN [15-16]. Regarding the facts that *klotho* has been considered to implicate in atherosclerosis and endothelial dysfunction [17-20], and genetic variation of *klotho* has been reported to modulate the expression of *klotho* in tissue [21-22], we aimed to investigate the association of *klotho* gene polymorphisms with the progression and survival of IgAN patients in Korea.

Materials and Methods

Study subjects

We used the database of the Progressive Renal disease and Medical Informatics and gEnomics Research (PREMIER) program sponsored by the Korean Society of Nephrology (KSN) which enrolled the subjects aged 18yr or more with primary and secondary glomerulonephritis diagnosed by renal biopsy from 34 hospitals and clinics in Korea since August 2003. The clinical data and genomic DNA extracted from the peripheral blood of the patients were collected and shared. In 2010, the genomic data of 1,080 patients who were diagnosed with IgAN by renal biopsy from April 1988 to May 2007 were analyzed for our study. Patients with evidence of systemic disease such as chronic liver disease and systemic lupus erythematosus were excluded, and data of patients who were not confirmed about survival were also dropped. This study was approved by the Institutional Review Board in Korea University Kuro Hospital and other participated hospitals.

Clinical data

Database was made for the candidate patients by a qualified nurse who visited every participated institution to collect the clinical data at the time of renal biopsy and during follow-up visits. Clinical data were gathered such as age, gender, body mass index (BMI), history of diabetes, blood pressure, serum protein, albumin, cholesterol, uric acid, creatinine, hemoglobin, proteinuria by dipstick test, urine RBC measured by microscopic examination, and medication history related to hypertension including angiotensin II type I receptor blockers, HMG-CoA reductase inhibitors, and steroids. We calculated the estimated glomerular filtration rate (eGFR) by the modified modification of diet in renal disease (MDRD) equation. ESRD patients were defined as the patients who should start dialysis treatment, and follow-up duration for renal survival was defined as the time period between diagnosis with renal biopsy and initiation of renal replacement therapy or last follow-up date. ESRD data were obtained from the Korean ESRD registry, "Insan Memorial Dialysis Registry" of KSN. The registry contained the data of patients treated with renal replacement therapy from 1985 to 2008. The mortality data were obtained from the database of the Korean National Statistical Office (<http://www.nso.go.kr>). The mortality data available until December 2008 were collected.

Klotho genotype assessment

The buffy coat was obtained from blood samples and then refrigerated at -70°C, and genomic DNA was extracted using QIAamp blood kits (Kyoto, Japan). The genotypings of G395A in the promoter region and C1818T in exon 4 were performed via an allelic discrimination assay with TaqMan probes (SCG, Seoul, Korea). The detector used in this experiment was an ABI Prism 7200 sequence detection platform (Perkin Elmer Applied Biosystems, Foster City, CA). The quality control of the machine was performed with regular background calibration and pure dye calibration. The primers and probes were used as follows.

- (1) G-395A: Forward primer, TAGGGCCCGGCAGGAT; Reverse primer, CCTGGAGCGGCTTCGTC
- (2) C1818T: Forward primer, CCAGCCCCAGATCGCTTTA; Reverse primer, GGCCAGTCCAGGGAGAA

Statistical assessment

The statistical analysis was done by SPSS program (ver 12.0, Chicago, IL, USA). All results are expressed as means±standard deviation, and significance was defined as a *p* value < 0.05. Distribution of allele frequency was evaluated with Hardy-Weinberg equilibrium using the χ^2 test. Independent t-test and chi-square test were used to compare differences between group according to genotypes or survival. Logistic regression analysis was performed to adjust for age, gender, and univariate risk factors for survival. Renal survival curves were prepared using the Kaplan-Meier method, and compared the cumulative incidence of case which required dialysis treatment by log-rank test.

Results

Clinical characteristics of study subjects

Nine hundred seventy three people were included in total. The general characteristics of the study population are shown in Table 1. The mean age was 37.0±13.7 years, and the proportion of male patients was slightly larger than female (55.5%). The mean BMI was 23.5±4.0, and patients who were diagnosed diabetes were 31 (3.2%). Mean follow-up period was 50.0±27.8 months. In G395A single nucleotide polymorphism (SNP) of promoter region for *klotho* gene, 671 (68.9%) had GG genotype, 274 (28.2%) the GA genotype, and 28 (2.9%) the AA genotype. In C1818T SNP, 645 (66.3%) had CC genotype, 298 (30.6%) the CT genotype, and 30 (3.1%) the TT genotype. The allele frequency was 0.830 and 0.170 for allele G and A, and 0.816 and 0.184 for allele C and T, which were complied with Hardy-Weinberg equilibrium (*p*=0.996 and 0.531 respectively). For comparison between genotypes with G395A and C1818T polymorphisms, patients were divided by A allele into A allele carriers vs non-carriers (GA+AA vs GG), and by T allele into T allele carriers vs non-carriers (CT+TT vs CC). A and T allele carriers of both polymorphisms have been reported as genotypes with lower *klotho* expression. The proportion of patients who have history of hypertension at diagnosis was higher in A allele carriers (A allele carriers vs non-carriers 43.9 vs 35.8%, *p*<0.05). However, systolic and diastolic blood pressure, and the number of medication for hypertension were not different between A allele carriers and non-carriers. Except that, there were no statistically significant differences in general characteristics among groups according to genotype.

Association of genotypes with patient survival

Thirteen patients were dead during follow-up period (1.3%). The association of both SNPs with survival was analyzed (Table 2). There were no differences in survival between the different genotypes of C1818T polymorphism. As for G395A polymorphism, the A allele carriers showed higher mortality compared to non-carriers (A allele carriers vs non-carriers 2.6 vs 0.7%, *p*=0.022).

Association of genotypes with renal survival

The proportion of patients who should be treated with dialysis was not significantly different between genotypes of both polymorphisms (Table 3). However, upon the

Table 1: Baseline characteristics of the study population (n=973) and comparison according to G395A and C1818T polymorphism genotypes

	Total (n=973)	GG (n=671)	GA+AA (n=302)	CC (n=645)	CT+TT (n=328)
		671 (70%)	302 (30%)	645 (66.3%)	328 (33.2%)
Age (yrs)	37.0±13.7	36.5±13.5	37.7±15.1	36.9±13.7	37.1±14.6
Sex (male %)	540 (55.5%)	367 (54.6%)	173 (57.1%)	351 (54.2%)	189 (57.7%)
BMI	23.5±4.0	23.3±4.0	23.7±3.9	23.5±4.1	23.4±3.9
Follow-up periods (m)	50.0±27.8	51.1±28.0	48.2±26.8	50.2±26.8	50.3±29.3
History of DM (%)	31 (3.2)	21 (3.1)	10 (3.2)	23 (3.6)	8 (2.5)
History of HTN (%)	373 (38.4)	240 (35.8)	133 (43.9)*	245 (37.9)	128 (39.2)
Medication (%)					
ACEi or ARB	545 (56.0)	388 (57.8)	157 (51.9)	369 (57.1)	176 (53.8)
Steroid	112 (11.5)	78 (11.6)	34 (11.1)	73 (11.2)	39 (11.9)
HMG-CoA reductase	160 (16.4)	106 (15.7)	54 (17.8)	100 (15.4)	60 (18.3)
Systolic / diastolic blood pressure (mmHg)	125.0±17.7/ 78.0±12.2	124.3±18.4/ 77.5±12.7	126.7±16.7/ 79.2±11.3	125.1±18.5/ 77.9±12.9	124.9±16.7/ 78.3±10.9
Hgb (g/dL)	13.9±2.0	13.0±2.0	12.9±1.9	13.0±2.0	12.9±1.9
Albumin (g/dL)	3.8±0.6	3.8±0.6	3.8±0.7	3.8±0.6	3.8±0.7
Total cholesterol (mg/dL)	190.1±16.0	188.9±62.6	193.4±73.6	189.4±60.0	192.4±78.0
Uric acid (mg/dL)	6.1±1.9	6.0±1.8	6.3±2.1	6.1±1.9	6.1±2.0
Creatinine (mg/dL)	1.34±1.36	1.35±1.48	1.34±1.08	1.38±1.40	1.31±1.29
eGFR (ml/min/1.73m ²)	78.2±48.7	76.5±32.6	81.2±70.9	79.1±54.2	75.7±34.6
Proteinuria>3+ (%)	173 (17.8)	115 (17.1)	58 (18.9)	111 (17.1)	62 (18.8)
Hematuria (%)	729 (74.9)	513 (76.5)	216 (71.4)	493 (76.4)	236 (71.9)

Data are expressed as number of cases (percentage, %) or mean ± standard deviation (SD). P-values were obtained by the Pearson Chi-squared test for categorical variables, and the independent two-sample t-test for numerical variables.*p<0.05 compared to GG group

Table 2: The association of *klotho* genotypes with patient survival

	Promoter G395A		Exon 4 C1818T	
	GG	GA+AA	CC	CT+TT
Survivor (%)	666 (99.3)	294 (97.4)	635 (98.4)	325 (99.1)
Non-survivor (%)	5 (0.7)	8 (2.6)	10 (1.6)	3 (0.9)
<i>p</i> value	0.022		0.371	
<i>p</i> value was obtained by the Pearson Chi-squared test				

comparison of renal survival, which was calculated by Kaplan-Meier methods with follow-up periods and cumulative incidence of case treated with dialysis, A allele carriers at G395A showed significant worse renal outcome compared to non-carriers (p=0.04, Figure 1).

Predictors of ESRD progression in IgA nephropathy

In the univariate analysis using binary logistic regression about the factors affecting ESRD progression defined as entrance into dialysis treatment, older age, history of HTN, higher diastolic pressure, HDL, uric acid, creatinine and proteinuria increased the risk. On the other hand, higher weight, hemoglobin, albumin, and bilirubin were related to decrease the risk (data were not shown). Multivariate analysis with significant factors of renal survival in univariate analysis was shown in Table 4 with adjusted odd ratios with their 95% CI values.

Fig. 1. Kaplan-Meier survival curves for renal survival of IgAN patients according to genotypes of G395A.

Table 3: The association of disease progression between genotypes

	Promoter G395A		Exon 4 C1818T	
	GG	GA+AA	CC	CT+TT
Non-ESRD (%)	633 (95.3)	277 (92.6)	600 (94.2)	310 (94.8)
ESRD (%)	32 (4.7)	22 (7.4)	37 (5.8)	17 (3.2)
<i>p</i> value	0.07		0.41	
<i>p</i> value was obtained by the Pearson Chi-squared test				

Table 4: Multivariate analysis about the factors affecting renal survival

Variables	Multivariate		
	Odd ratio	[95% C.I.]	<i>p</i> value
Age	1.035	[0.985, 1.088]	0.177
HTN	4.395	[0.714, 27.009]	0.110
Weight	0.890	[0.830, 0.955]	0.052
Diastolic BP	0.973	[0.911, 1.040]	0.425
Hemoglobin	1.208	[0.755, 1.931]	0.430
Albumin	0.299	[0.097, 0.922]	0.036
Bilirubin	0.542	[0.057, 4.563]	0.548
HDL	1.017	[0.985, 1.051]	0.302
Uric acid	1.296	[1.014, 1.658]	0.039
Creatinine	1.720	[1.172, 2.525]	0.006

Data are expressed as the Odds ratio (OR) and its 95% confidence interval for each factor

It revealed that lower albumin and higher creatinine and uric acid were associated with ESRD progression treated with dialysis.

Discussion

We have demonstrated that genetic variation of single nucleotide polymorphism in *klotho* which was known to modulate the expression of *klotho* protein was associated with renal and patient survival of IgAN. It suggested that *klotho* might implicate the pathogenesis of progression of IgAN.

Although IgAN has been a concern with potential for ESRD, little has been known about influential factors on the prognosis of IgAN. Recently, genetic factor gained interest having prognostic value in IgAN, because

genetic variations for vasomotor activity and oxidative stress were reported the association with the prognosis of IgAN [15-16]. We hypothesized that *klotho* could be considered as one of candidate genes implicating in the pathogenesis of IgAN regarding the role of *klotho* on endothelial dysfunction and atherosclerosis [17-19]. *Klotho* was discovered as an anti-

aging factor. *Klotho* mutant mice developed premature aging phenotype [1], whereas overexpression of *klotho* extended life span conversely [2]. Predominant expression of *klotho* in the kidney [23] was decreased in patients with chronic kidney injury and in the kidneys of diabetic nephropathy of rat [7, 24], which suggested that *klotho* may be associated with the progression of chronic kidney disease. The role of *klotho* in kidney diseases was also demonstrated in recent studies that overexpression of *klotho* ameliorated endothelial dysfunction in diabetic nephropathy of rat [24] and conferred renal protection in glomerulonephritis and acute kidney injury of mice [5, 25].

Among more than 10 mutations with single nucleotide polymorphism (SNP) of *klotho*, genotypings of G395A in the promoter region and C1818T in exon 4 were conducted in this study, because other SNPs including KL-VS have been reported rare in asian people [26-27]. The allele frequencies of G and A allele of G395A and C and T allele of C1818T were not different with the results of previous studies done in Korean population with normal renal function [27-28], which might suggest that SNPs of *klotho* in G395A and C1818T less likely affected the incidence of IgAN. However, the effect of *klotho* polymorphism on the incidence of IgAN should be demonstrated later by direct comparison with control group. Upon the comparison of baseline characteristics between genotypes, history of hypertension was higher in A allele carriers of G395A. It was consistent with some previous reports that SNPs of *klotho* affected the incidence of hypertension [22, 29]. The researchers assumed that endothelial dysfunction might induce the hypertension in patients with genotypes expressing *klotho* less such as A allele in G395A. But, the mechanism is not clear yet, and we don't believe that the history of hypertension itself influenced over the renal and patient survival of IgAN in our study, because measured blood pressure was not different between groups with similar amount of anti-hypertensive medication, and hypertension was not the significant factor for ESRD in multivariate analysis.

Despite relatively small number of mortality, there was a significant difference in patient survival between genotypes, and A allele carriers showed higher mortality. The role of genetic variation of *klotho* on mortality was also demonstrated in hemodialysis patients [21], which manifested similar result that the genotype with lower *klotho* expression showed higher mortality. Renal survival of IgAN was also different between genotypes. The probability of renal survival defined as expected duration from diagnosis of IgAN to entering into dialysis was significantly worse in A allele carriers at G395A. All these results indicated that *klotho* polymorphism played a role in renal and patients outcome of IgAN.

The role of *klotho* in chronic kidney disease has been explained at first related to the activity of fibroblast growth factor-23 (Fgf23) and *klotho* regulating calcium and phosphate homeostasis [30]. Recent studies demonstrated that *klotho* was associated with various kidney diseases including glomerulonephritis, ischemic kidney disease, hypertensive and diabetic nephropathy, and even polycystic kidney disease [5-7, 24, 31]. The underlying mechanisms are not clear, however, it has been shown that *klotho* involved with wide-ranging phenomena such as oxidative stress, apoptosis, inflammation, and fibrosis implicated in renal pathology [25, 32-34], and the interaction of *klotho* with renin-angiotensin system or mTOR signaling was suggested to modulate those processes [35-36]. Genetic variations of *klotho* could influence on the progression of chronic kidney disease through these mechanisms, and it was reflected in a report that SNPs of *klotho* affected the severity of non-diabetic ESRD [37] as in our study.

Beyond the effect on renal function, the extensive function of *klotho* also could be associated with the progression of cardiovascular disease [38] and uremic atherosclerosis [19] in CKD patients. Although the cause of death was not checked exactly, it might explain the higher mortality in less active *klotho* carriers in our study and previous study done in hemodialysis patients [21].

Though this is the first remark on the association between *klotho* polymorphism and the renal and patient survival of IgAN to our knowledge, there were several limitations. First, the exact causes of death were not specified, and it hindered the speculation of the role of *klotho* in patient survival of IgAN. Second, the level of *klotho* was not measured so that the inferior

genotypes for *klotho* expression were not verified directly. Third, all the genotypes of *klotho* polymorphisms were not examined in this study, because the amount of available samples was not enough. Fourth, genotypes of *klotho* were not included as a significant predictor for ESRD in multivariate analysis. However, the total number of ESRD patients entering into dialysis was relatively small for the conclusion, and the effect of genotypes on renal survival was demonstrated with other method.

Conclusion

The genotypes expressing *klotho* less may be associated with poor patient survival and renal outcome in IgAN.

Conflict of Interests

The authors of this manuscript state that they have no conflict of interests.

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