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Association between KLOTHO Gene G395A Polymorphism and Carotid Artery Calcification in regular Hemodialysis Patients

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Abstract

BACKGROUND: The vascular calcification process in chronic kidney disease (CKD) is a complication caused by mineral and bone abnormalities and becomes the risk of cardiovascular disease and mortality in CKD patients. KLOTHO is an inhibitor of calcification and its expression has been observed to decrease in CKD patients. The KLOTHO gene G395A polymorphism is a genetic variation that is common in Asian populations and is associated with vascular dysfunction in hemodialysis patients.

OBJECTIVE: The objective of the study was to determine the association between the KLOTHO gene G395A polymorphism and carotid artery calcification in regular hemodialysis patients.

METHODS: This study was an analytical study with a cross-sectional design and was carried out at Rasyida Kidney Hospital Medan. Venous blood sample was taken from the patients who met the inclusion criteria for examination of the KLOTHO gene -395 polymorphism and carotid ultrasonography was assessed to evaluate the thickness of the tunica media-intima as a marker of vascular calcification.

RESULTS: The majority of the study subjects were men, as many as 35 patients (50.7%). From the results of the KLOTHO gene -395 polymorphism, it was found that the majority of subjects had GG genotypes as many as 36 people (52.2%) followed by GA genotypes as many as 30 people (43.5%) and AA genotypes as many as 3 (4.3%). There was a statistically significant of the association between KLOTHO gene -395 polymorphism and the incidence of carotid artery calcification ($p = 0,015$).

CONCLUSION: There was an association between the KLOTHO gene G395A polymorphism and carotid artery calcification in regular hemodialysis patients.

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Introduction

The incidence of chronic kidney disease (CKD) in the world remains high and becomes a major global health burden. CKD can develop into end-stage renal disease (ESRD) which requires renal replacement therapy such as hemodialysis [1]. In Norway, the incidence of CKD is 10,2% of total population. This result is similar in China where the incidence is 7,2–13,7% [2]. There are so many complications of CKD, one of them is a mineral abnormality in the form of vascular classification [3]. This situation is an important contributor to the incidence of cardiovascular disease that determines the mortality of patients with CKD [4].

In our body, there are some promoter and inhibitor of vascular classification to maintain the calcification process in vascular. KLOTHO, a transmembrane protein expressed in kidney, is one of the classified inhibitors [5]. KLOTHO gene has been reported to be related to bone density, coronary artery disease, and cardiovascular risk factors such as the homeostasis calcium and phosphate in the kidney.

The defect in this gene shows the acceleration of vascular atherosclerosis along with classification [6]. All experiments to date have shown the protective effect of KLOTHO on the incidence of vascular classification [5]. Single-nucleotide polymorphisms (SNPs) are inherited human gene variations when a single nucleotide changes in the genome sequence [7]. In the KLOTHO gene, SNPs on G395A are very common in Asian populations [8]. So far, there are no data on whether the G395A KLOTHO gene polymorphism is related to carotid artery calcification in regular hemodialysis patients in Indonesia, so the authors are interested in conducting this study.

Methods

Study design

This study was an analytical research method with cross-sectional design in June–August 2018

at Rasyida Kidney Hospital, Medan. The Faculty of Medicine, Universitas Sumatera Utara Ethics Committee approved this study. The main objective of this research was to determine whether there was an association between KLOTHO gene G395A polymorphism and carotid artery classification in regular hemodialysis patients.

Selection of participants

Subjects over the age of 18, CKD patients who have undergone regular hemodialysis (HD) ≥ 3 months, cooperative patients, and willing to participate by signed an informed consent form were served as research inclusion criteria. We determined the comorbid diseases, duration of hemodialysis from history taking and medical record, and calculated the body mass index (BMI). Then, the research subjects were taken a blood sample to evaluate the calcium and phosphate serum levels, the KLOTHO gene G395A polymorphisms and carotid ultrasonography were done to evaluate the carotid intima-media thickness (CIMT).

The KLOTHO gene G395A polymorphism was examined by polymerase chain reaction with confronting two-pair primers method (PCR-CTTP). In this genotyping of G395A in the promoter region procedure, as described previously by Shimoyama *et al.* (2009), the confronting pairs of primers are as follows: Forward primer 1: GTTCGTGGACGCTCAGGTTTCATTCTC, forward primer 2: GAGAAAAGGCGCCGACCAACTTTC, reverse primer 1: GATCCCGCCCC- CAAGTCGGGA, and reverse primer 2: GTCCCTCTAGGATTTTCGCCAG [9]. These polymorphism regions were amplified by PCR for 10 min with the initial denature at 95°C followed for 1 min at 95°C (35 cycles), for 1 min at 65°C, for 1 min at 72°C, and additionally for 5 min at 72°C and the products were visualized with 3% agarose gel and ethidium bromide staining. The GG genotype is located at 252 bp (base pair) and 175 bp, the GA genotype at 252, 175, and 121 bp, and the AA genotype at 252 and 121 bp (Figure 1). Research subjects were also done the carotid ultrasonography examination to evaluate the carotid intima-media thickness (CIMT). CIMT is defined as the measurable distance between the intima lumen and the media-adventitia boundary [10]. A positive result is if the CIMT > 1 mm.

Statistical analysis

We performed the univariate, bivariate, and multivariate analysis. The distribution of subject characteristics was analyzed by univariate analysis and displayed as a percentage. The association between independent and dependent variables was analyzed by bivariate analysis. For categorical variables, we performed Chi-squared test or Fisher's exact test as an alternative while numerical variables were analyzed by independent t-test or Mann-Whitney U-test as an

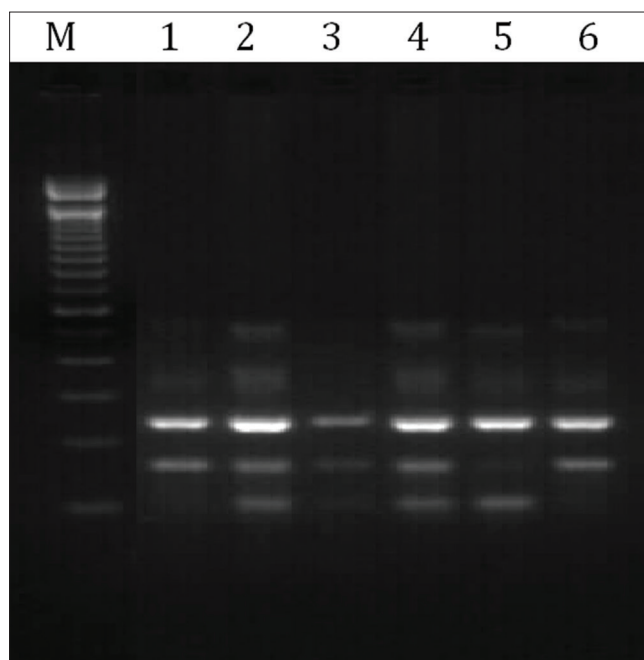


Figure 1: The PCR-CTTP analysis for genotype of KLOTHO gene G395A polymorphisms. Lanes 1, 3, and 6 indicate the GG genotype, lanes 2 and 4 indicate GA genotype, lane 5 indicates the AA genotype

alternative. Multivariate analysis was performed by regression logistic test. The significant results was considered if $p < 0,05$ ($p < 0,05$).

Results

Characteristics of research subjects

From a total of 69 research subjects, the majority of subjects were men as many as 35 (50,7%) subjects with median age was 55 (26–78) years old. Hypertension was the most comorbid disease as many as 53 (76,8%) subjects followed by diabetes mellitus as many as 16 (23,3%) subjects. The normal BMI group was the most in this study as many as 26 (18,8%) subjects (Table 1).

From the KLOTHO gene G395A polymorphisms examination, we found that the majority of subjects had GG genotype as many as 36 (52,2%) subjects followed by GA genotype as many as 30 (43,5%) subjects and AA genotype as many as 3 (4,3%) subjects. Based on carotid ultrasonography examination, it was found that the majority of subjects had CIMT ≤ 1 mm, as many as 41 (59,4%) subjects (Table 1).

Association between research subjects characteristics and KLOTHO gene -395 polymorphisms with carotid artery calcification

We performed a bivariate analysis to determine the association of research subjects characteristics

Table 1: Baseline characteristics

Characteristics	Frequency (n=69)
Sex, n (%)	
Men	35 (50.7%)
Women	34 (49.3%)
Age, median (min-max), years old	55 (26–78)
Comorbid disease, n (%)	
Hypertension	53 (76.8%)
Diabetes mellitus	16 (23.2%)
Duration of HD, median (min-max), months	60 (33-135)
BMI, median (min-max), kg/m ²	23.6 (16.7-42.6)
BMI categories, n (%)	
Type 2 obesity	2 (1.4%)
Type 1 obesity	5 (3.6%)
Pre-obesity	18 (13%)
Overweight	14 (10.1%)
Normal	26 (18.8%)
Underweight	4 (2.9%)
Calcium levels (Ca), median (min-max), mg/dL	9.8 (8.0-10.9)
Phosphate levels (P), mean (± SD), mg/dL	5.5 (0.63)
KLOTHO gene -395 polymorphisms, n (%)	
AA genotype	3 (4.3%)
GA genotype	30 (43.5%)
GG genotype	36 (52.2%)
Carotid ultrasonography interpretation, n (%)	
Calcification	28 (40.6%)
No calcification	41 (59.4%)

to the incidence of carotid artery calcification. Based on statistical test, hypertension as comorbid disease ($p = 0,009$) and the categories of BMI ($p = 0,039$) are associated with the incidence of carotid artery calcification. Both of these risk factors along with other risk factors that have $p < 0,25$, such as diabetes as comorbid disease and calcium levels, will be included in the multivariate analysis (Table 2).

Table 2. Association between research subjects characteristics with carotid artery calcification

Risk factors	Calcification	No calcification	p value
Sex, n (%)			0.378*
Men	16 (57.1)	19 (46.3)	
Women	12 (42.9)	22 (53.7)	
Diabetes, n (%)			0.145*
Yes	9 (32.1)	7 (17.1)	
No	19 (67.9)	34 (82.9)	
Hypertension, n (%)			0.009*
Yes	26 (92.9)	27 (65.9)	
No	2 (7.1)	14 (34.1)	
BMI categories, n (%)			0.039*
Overweight – obesity	20 (71.4)	19 (46.3)	
Underweight – normal	8 (28.6)	22 (53.7)	
Mean calcium levels, mg/dL	39.5	31.8	0.119 [§]
Mean phosphate levels, mg/dL	5.5	5.4	0.862 [^]
Median of age, year (min-max)	54.5 (26.0–69.0)	57.0 (31.0–78.0)	0.437 [§]
Median duration of HD, month (min-max)	53.5 (33.0–131.0)	63.0 (35.0–135.0)	0.353 [§]

*Chi-squared test, [^]Mann-Whitney U-test, [§]Independent t-test

A bivariate analysis was performed to determine the association between the KLOTHO gene -395 polymorphisms on the incidence of carotid artery calcification. Based on statistical tests, a statistically significant association was found between the KLOTHO -395 gene polymorphisms and the incidence of carotid artery calcification with $p = 0,015$. Furthermore, groups with GA and AA genotypes were known to increase risk factors by 4,071 times for carotid artery calcification compared to the GG genotype group ($p = 0,006$; CI 95% = 1,464–11.320). The presence of A alleles in a person is also known to increase the risk of carotid artery calcification by 2,292 times compared to individuals with G alleles ($p = 0,033$; CI 95% = 1,058–4,963) (Table 3).

We determined risk factors that influence the incidence of carotid artery calcification by multivariate analysis with logistic regression tests. The variables with $p \leq 0,25$ in the bivariate tests such as KLOTHO gene -395 polymorphisms GA and AA genotypes, history of diabetes, history of hypertension, calcium levels, and BMI categories will be included in the test.

Based on this analysis, risk factors that influence the incidence of carotid artery calcification were KLOTHO gene -395 polymorphisms GA and AA genotype, history of diabetes, and history of hypertension ($p = 0,002$; $p = 0,022$; and $p = 0,006$) (Table 4). These three risk factors will increase the likelihood of carotid artery calcification. Based on these statistical tests, a formulation can be made in predicting the occurrence of carotid artery calcification as follows:

Possible calcification events = $(-2,479) + (1,996 \text{ GA and AA KLOTHO gene polymorphisms}) + (1,829 \text{ history of diabetes}) + (2,336 \text{ history of hypertension})$

Discussion

CKD is an increasing public health problem where the prevalence of CKD in various regional countries varies with gender. Epidemiological studies conducted by Lebov *et al.* in the Pacific coastal regions of Nicaragua and El Salvador, in 2015, showed an increased in the incidence of CKD among young adult men and the average age of CKD occurrence is 51 (± 12) years [11]. Our study showed the similar results, from 69 research subjects, the majority of subjects were men, as many as 35 people (50.7%) with a median age of 55 years. Different results are shown as in the study of Carrero *et al.*, in 2018, on gender differences in the epidemiology of CKD in some countries that the prevalence of CKD in the regional majority is more common in female as in Finland with a ratio of women to men which is 3,1:1,9 [12]. Testosterone has a bad effect on the kidneys because it induces apoptosis of podocyte cells which can develop into glomerulosclerosis and increases TGF- $\beta 1$ expression associated with the incidence of tissue fibrosis, while estradiol inhibits this process. Meanwhile, decreased estrogen levels have been linked to decrease nitric oxide (NO) synthesis in the renal medulla which is generally associated with kidney injury [13], [14], [15].

Various etiologies that cause kidney damage include primary glomerulopathy, hypertension, diabetes, and the presence of post-renal obstruction and others. Data from the Indonesian Renal Registry in 2017 show that hypertension is still the most common comorbid disease in CKD patients undergoing HD in Indonesia as many as 36% and followed by diabetes as much as 29% [16]. Our findings are consistent with the previous

Table 3: Association between KLOTHO gene -395 polymorphisms with carotid artery calcification

KLOTHO gene -395 polymorphisms	Calcification	No calcification	p value*	PR	CI 95%
AA	1 (3.6)	2 (4.9)	0.015	-	-
GA	18 (64.3)	12 (29.3)			
GG	9 (32.1)	27 (65.3)			
GA+AA	19 (67.9)	14 (34.1)	0.006	4.071	1.464–11.320
GG	9 (32.1)	27 (65.9)			
A allele	20 (35.7)	16 (19.5)	0.033	2.292	1.058–4.963
G allele	36 (64.3)	66 (80.5)			

*Chi-squared test

studies where the majority of subjects had comorbid hypertension in 53 people (76.8%) and followed by diabetes mellitus in 16 people (23.3%). Zhang *et al.*, in 2012, conducted a study on the prevalence of CKD in China, out of 1185 subjects with a decrease in GFR <60 ml/min/1.73 m², as many as 60.5% of the subjects had comorbid hypertension and 19.1% of the subjects had comorbid diabetes [17]. Hypertensive nephropathy in patients with pathological renal changes is indicated by the presence of vascular changes, glomerular ischemia, and acute tubular interstitial injury. Increased intraglomerular pressure caused by hypertension will lead to glomerulosclerosis [18].

Table 4: Risk factors that associated with carotid artery calcification (final stage of multivariate analysis)

Risk factors	B	Wald	P*	PR	CI 95%
KLOTHO gene -395 polymorphisms GA and AA genotype	1.996	9.796	0.002	7.363	2.109-25.704
Diabetes	1.829	5.264	0.022	6.230	1.306-29.731
Hypertension	2.336	7.620	0.006	10.343	1.969-54.337
Constanta	-2.479				

*Logistic regression test

Genetic and environmental factors are two factors that make variations in human phenotypes. When the DNA genome sequence on the same chromosomes of two individuals is compared, there is substantial variation in the order at many points throughout the genome. Single-nucleotide polymorphisms (SNPs) are a genetic variation that occurs when a single-nucleotide base changes in genome sequence and is inherited, which is the most common form of genetic variation. This genetic variation is believed to influence the tendency of people to suffer from certain diseases [7], [19], [20]. KLOTHO, a gene related to the aging process, has been known to have a role in atherosclerosis and cardiovascular disease. KLOTHO mRNA is mainly expressed in the kidneys (especially in the distal tubule) and it has been shown that serum KLOTHO levels begin to decrease in mild CKD patients (stage ≤ 2) and this decrease occurs before the onset of increased serum fibroblast growth factor-23 (FGF23), parathyroid hormone (PTH), and phosphate [21], [22]. KLOTHO expression in humans can vary with single-nucleotide polymorphisms (SNPs). Several studies have identified polymorphisms in KLOTHO and are associated with various phenotypes. Recent research has focused on G-395A polymorphisms in the KLOTHO promoter area [6], [20].

From the results of the KLOTHO gene -395 polymorphism, we found that the majority of research subjects had GG genotype as many as 36 people

(52.2%) followed by GA genotype as many as 30 people (43.5%) and AA genotype as many as 3 people (4.3%). This finding was consistent with a study of 478 HD patients in Korea which conducted by Ko *et al.*, in 2013. They found that the majority of research subjects had KLOTHO gene -395 polymorphisms with GG genotype as many as 302 people (63.2%) followed by GA genotype as many as 158 people (33%) and AA genotype as many as 18 people (3.8%) [6]. Nazarian *et al.*, in Iran, with 60 HD patients and 60 patients control group found that the majority of HD patients had KLOTHO gene -395 polymorphism of GG genotype as many as 42 people (70%) [8].

Existing studies show the important role of KLOTHO in vascular calcification. The G395A polymorphism in the KLOTHO gene promoter region modifies bone mineral density, which appears to be related to atherosclerosis and arterial calcification, both of which have the potential to influence coronary heart disease [20]. Endothelial dysfunction is a systemic pathological condition caused by an imbalance between vasorelaxation and vasoconstriction of blood vessels. Nitric oxide is a vasodilator that is oxidized by acetylcholine and is a major endogenous vasodilator. Several studies have shown that KLOTHO can induce NO production, the expression of mitochondria superoxide dismutase (MnSOD), and suppression of NADPH oxidase to protect from oxidative stress or show an anti-inflammatory action in protecting endothelium [19], [23], [24], [25]. In animal subjects, namely, mutant rats or mice performed by nephrectomy, it can be seen the acceleration of the development of atherosclerosis due to the effects of this gene in production of NO [26]. Furthermore, KLOTHO deficiency in mice produces increase phosphate and calcium levels, hypervitaminosis D, arteriosclerosis, and ectopic calcifications including vascular calcification [25]. KLOTHO interacts with the FGF23 receptor to take sodium-dependent type IIa phosphate transporters from the proximal renal tubular cell membrane, which causes phosphaturia. The effect of KLOTHO on urinary phosphate excretion may contribute to its inhibitory effect of ectopic calcification [24], [25].

This study shows that there is a significant relationship between the KLOTHO gene -395 polymorphisms and the incidence of carotid artery calcification with $p = 0,015$, where patients with the GA and AA genotype are 4,071 times more likely to develop carotid artery calcification than patients with GG genotype ($p = 0,006$). The KLOTHO gene G-395A

polymorphisms, which is located in the region of the KLOTHO gene promoter, has never been reported to be related to the incidence of coronary artery disease in Europe. However, in Asian countries, for example, in Korea and Japan, the G-395A gene is considered as a risk factor for coronary artery disease [27].

In the research of Donate-Correa *et al.*, in Spain, the KLOTHO gene -396 polymorphisms showed no significant results on the appearance of cardiovascular diseases such as vascular calcification. However, they found that KLOTHO mRNA expression was lower in subjects with coronary artery disease and the presence of A allele variant of KLOTHO gene G-395A polymorphism was associated with lower KLOTHO expression in vascular so the risk of causing vascular calcification and coronary artery disease [28]. But, studies of Rhee *et al* in Korea and Imamura *et al* in Japan showed that there was a statistically significant association between klotho gene G395A polymorphisms with the incidence of coronary artery disease and could be an independent risk factor for the incidence of coronary artery disease [29], [30].

Ko *et al.* have shown that the presence of A allele carriers from the KLOTHO -395 gene is associated with accelerated atherosclerosis manifested as coronary artery disease and hypertension [6]. This study shows that the presence of KLOTHO -395 gene A allele in a person can increase the risk of carotid artery calcification by 2,292 times compared to individuals with G allele ($p = 0,033$; CI 95% = 1,058–4,963). The KLOTHO gene G395A polymorphism has been reported to be related in influencing the promoter function of the gene, where G/A substitution has an effect on KLOTHO expression affecting the bone density (A allele forms fewer DNA-protein complexes compared to G allele) [31]. Our finding was similar to those of Imamura *et al.*, in which A allele carrier had a significantly higher incidence of coronary artery disease than controls [30].

Many clinical studies have tried to find an association between risk factors for coronary artery calcification or peripheral arterial calcification. Jensky *et al.*, in California, showed that hypertension and diabetes had a significant risk of coronary atherosclerotic calcification characterized by calcium deposits in coronary arteries with $p < 0.01$ and $p < 0.01$ [32]. In addition to KLOTHO gene -395 polymorphisms, diabetes and hypertension also have an association with risk factors for calcification in this study ($p = 0,022$ and $p = 0,006$). Diabetes mellitus has a risk of increasing the incidence of coronary calcification, especially medial calcification, and the incidence of diabetic nephropathy increases that risk [33]. High blood sugar levels trigger the expression of osteoblast and osteocalcin transcription factors, thereby triggering calcification of vascular smooth muscle cells (VSMC) [34], [35]. Angiotensin renin system in hypertension plays an important role in the incidence

of apoptosis, growth, and differentiation of VSMC, and therefore, these changes are likely to play a role in the occurrence of vascular calcification. [34], [36].

Conclusion

There was an association between the KLOTHO gene G395A polymorphism and carotid artery calcification in regular hemodialysis patients and the presence of KLOTHO -395 gene A allele can increase the risk of carotid artery calcification.

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