KLOTHO and VDR Gene Polymorphisms and Clinical Phenotype in Chronic Kidney Disease Patients

Kronik Böbrek Hastalarında KLOTHO ve VDR Gen Polimofizm ve Klinik Fenotip

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ABSTRACT

Objective: With an increasing incidence and prevalence, Chronic Kidney Disease (CKD), is a bad prognosed and high cost common public health problem. KLOTHO gene which is defined as aging suppressor gene is highly expressed in kidneys. Decreased Vitamin D receptor (VDR) activation plays a role in CKD morbidity and mortality. The aim of this study is to examine the association between CKD and polymorphisms of KLOTHO (G395A and C1818T) and VDR (ApaI and TaqI) genes as well as investigating the effects of these polymorphisms on different clinical phenotype of the disease.

Methods: In 104 cases diagnosed to have CKD and 104 healthy controls, KLOTHO gene G395A, C1818T polymorphisms were studied by DNA sequence analysis and VDR gene ApaI and TaqI polymorphisms were studied by polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) method.

Results: No statistical difference was found in genotype and allele frequencies for KLOTHO gene G395A, C1818T and VDR gene TaqI polymorphisms between the groups. VDR gene ApaI polymorphism was found to be significantly higher in the patient group than in the control group (p=0.018).

Conclusion: Our results demonstrated that VDR gene ApaI polymorphism may be related to CKD and may be a risk factor for the development of the disease.

Key Words: Chronic Kidney Disease, KLOTHO, VDR, Polymorphism.

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ÖZET

Amaç: Kronik böbrek hastalığı (KBH), insidansı ve prevalansı giderek artan, kötü prognozu ve yüksek maliyetli yaygın bir halk sağlığı problemidir. Yaşılamayı baskılayıcı gen olarak tanımlanan KLOTHO geni, böbreklere fazla miktarla eksprese olmaktadır. Azalmış vitamin D reseptör (VDR) aktivasyonu, KBH’nın morbidite ve mortalitesinde rol oynamaktadır. Bu çalışmada, KBH ile KLOTHO geni G395A ve C1818T polimorfizm ve VDR geni ApaI ve TaqI polimorfizm arası ilişki ortaya koymak amaçlanmıştır. Ayrıca, kronik böbrek hastalığının farklı klinik fenotipleri üzerine bu polimorfizmelerin potansiyel etkisini araştırarak amaçlanmıştır.

Yöntem: KBH tanısı almış 104 hasta ve 104 sağlıklı kontrol grubunda KLOTHO geni G395A, C1818T polimorfizm ve VDR geni ApaI ve TaqI polimorfizm genotipleri ve alel frekansları DNA dizi analizi ve PCR–RFLP yöntemiyle çalışılmıştır.

Bulgular: KLOTHO geni G395A, C1818T ve VDR geni TaqI polimorfizm arası ilişki ortaya koymak amaçlanmıştır.

Sonuç: Bulgularımız, VDR geni ApaI polimorfizminin KBH ile ilişkili olabileceği ve KBH’nin gelişmesi için bir risk faktörü olarak davranışa yardımcı olabileceği göstermiştir.

Anahtar Sözcükler: Kronik Böbrek Hastalığı, KLOTHO, VDR, Polimorfizm.
INTRODUCTION

Chronic kidney disease (CKD) can be defined as the chronic and progressive deterioration of metabolic-endocrine functions and renal fluid-solute balance functions depending on the decrease in the glomerular filtration rate (GFR) (1). In many studies it is reported that, chronic kidney disease susceptibility genes affect prognosis of the disease together with the environmental and hereditary factors (2).

KLOTHO is defined as the ‘aging suppressor gene’ which fastens the aging when it is deteriorated and which increases the life spans when it is excessively expressed in mice (3). Klotho protein is expressed mostly in spiral distal tubular cells of the kidney and choroid plexus of the brain. Moreover, it is also expressed in various endocrine organs such as pituitary gland, parathyroid, pancreas, ovary, testes and placenta (4). There are more than 10 mutations or SNPs of the human KLOTHO gene which were reported by population-based studies (4). It has been stated that G395A (rs1207568), which is localized in the promoter, is associated with bone mineral density, osteoarthritis, systolic blood pressure, atherosclerotic coronary artery disease, early vascular dysfunction and cardiac embolic episodes (4). Other SNP C1818T (rs564481), which is localized in the exon 4, is associated with bone mineral density, fasting glucose, atherosclerotic coronary artery disease (5, 6).

Vitamin D receptor (VDR) protein is one of the members of the nuclear hormone receptor family. VDR protein regulates the vitamin D hormone which has the central role in the endocrine system and bone-calcium balance (7). It has been specified that vitamin D and vitamin D analogues have kidney protective roles in CKD (8). Meanwhile, they also lead to positive effects via decreasing the risks of diabetes, autoimmune diseases, cancer, and cardiovascular morbidity and mortality (9). In various studies which have been performed until now, three adjacent polymorphisms such as RFLPs BsmI (rs1544410), Apal (rs7975232) and TaqI (rs731236) have been emphasized which are located respectively in the 3’ end of the VDR gene, intron 8 and exon 9 (10). Some researchers found that VDR gene TaqI polymorphism was associated with T1DM (Type 1 Diabetes Mellitus) whereas others did not detect any relationship with VDR gene and osteoporosis, T2DM (Type 2 Diabetes Mellitus) and breast cancer (11, 12, 13, 14). Similarly, there is an association with Apal polymorphism and epithelial ovarian cancer, however there is no relationship with osteoporosis, T1DM and T2DM (11, 12, 13, 15).

Furthermore, it was also stated that there was a relationship between the Apal polymorphism, the aa genotype and the elevated PTH, osteocalcin levels, changes in calcium level, regulation of the PTH secretion in predialysis patients (16).

The aim of this study is to determine the incidence of G395A and C1818T polymorphisms of KLOTHO gene and Apal and TaqI polymorphisms of Vitamin D gene between case and control groups and to establish the relationship between corresponding polymorphisms and chronic kidney disease. Moreover, this study aims to investigate the potential effects of these SNPs on different clinical phenotype of chronic renal disease.

METHODS

Study subjects

The study protocol was in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of the Kirikkale University Faculty of Medicine. During the study period we enrolled 104 predialysis chronic renal disease patients (49 female and 55 male; mean age: 49.13±1.00) from the Department of Internal Medicine, Afyon Kocatepe University Research and Application Hospital. Written informed consent was obtained from all participants. Subjects were evaluated for complete blood count, sedimentation, CRP, renal function test, fasting glycose, HbA1c, total cholesterol levels, HDL cholesterol, LDL cholesterol, VLDL, triglyceride, TSH, Parathyroid hormon besides to age, gender, BMI, systolic and diastolic blood pressure. BMI of left forearm was measured using dual-energy X-ray absorptiometry.

DNA extraction and genotyping

Genomic DNA was isolated from whole blood samples using a commercially available DNA extraction kit (Roche, High Pure PCR Template Preparation Kit) according to the manufacturer’s instructions. Analysis of KLOTHO gene G395A and C1818T polymorphism were performed by DNA sequence analysis. The following primer sets were used for the SNPs of the KLOTHO gene; C1818T F: 5’-TTACCTGGAGGTCATGACCA-3’, C1818T R: 5’-GATGCGCCAGCTTTGGAAG-3’ and G395A F: 5’-CAGTCCTCAGGCACCTCATA-3’, G395A R: 5’- TCAGGTTCATCCTTCTGCTGTA-3’. The length of the PCR products for C1818T and G395A polymorphisms were 401 bp and 421 bp. The region containing these polymorphisms was amplified by PCR with initial denaturation at 95°C for 5 min and followed by 30 cycles at 95°C for 1 min, 55°C for 1 min, 72°C for 1 min and additionally at 72°C for 7 min. Sequence PCR for KLOTHO C1818T and G395A was carried out following PCR cleaning by the same forward primers using Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Inc, Foster City, CA, USA). For KLOTHO C1818T and G395A sequence analysis, capillary electrophoresis was performed on an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems).

VDR gene Apal and TaqI polymorphisms were determined by PCR-RFLP analysis. The following primer sets were used for the SNPs of the VDR gene Apal F: 5’-CAGACGACTGGACAGGGACAG-3’ and Apal R: 5’-GCAATCGACGTGGACAGAAGG-3’. PCR products and restriction fragments were separated in 2% agarose gel with ethidium bromide staining by electrophoresis and visualized by BioDoc-it® Imaging System-UVP. Genotyping for Apal polymorphism was performed as follows; 740 bp for AA genotype, 470, 515, 225 bp for Aa genotype and 515, 225 bp for aa genotype. Genotyping for TaqI polymorphism was performed as follows; 495, 245 bp for TT genotype, 495, 290, 245, 205 bp for Tt genotype and 290, 245, 205 bp for tt genotype.

Statistical analysis

Genotype distribution within the groups of the cases and controls was compared with values predicted by Hardy-Weinberg equilibrium (HWE) using the chi-square test. Continuous variables were expressed as mean ±SD, and the significance level was defined as p<0.05. Test for normality was conducted with Kolmogorov-Smirnov test. Demographic and clinical data were compared using Mann–Whitney U test. The difference of allelic and genotypic frequencies between the case and control groups was also estimated by the Pearson’s chi-square test and Fisher’s exact test. The odds ratios (ORs) and 95% CI for all potential risk factors were calculated by multivariate logistic regression analyses to evaluate the effects of genetic factors and various environmental factors on the presence of CKD. For the multivariate model, hypertension, diabetes, gender, along with C1818T, G395A, Apal, TaqI genotype were incorporated into the model as categorical variables, whereas age and BMI were expressed as a continuous variable. SPSS 18.0 software was used to evaluate all statistical analyses.

RESULTS

Clinical characteristics of the study subjects

Demographics and clinical characteristics of subjects are shown in Table 1. Compared to controls, BMI, serum creatinine, serum phosphate and PTH were significantly higher; GFR and BMD-Radius were significantly lower in the patient group. Difference in the ratio of insulin resistance between the groups was not statistically significant.
Genotype and allele frequencies of KLOTHO and VDR SNPs

Genotype and allele frequencies of KLOTHO SNPs in the case and the control groups are shown in Table 2. As for CKD patients, the allele frequencies of the C1818T polymorphism of the KLOTHO gene were 0.649 for the C allele and 0.351 for the T allele, and those for the G395A polymorphism were 0.683 for the G allele and 0.317 for the A allele. While the genotype distributions for C1818T and G395A polymorphisms on risk of developing CKD and found a 2.32 fold increased risk in patients having Aa genotype of ApaI polymorphism. On the other hand, there was no association between Taq I polymorphism and the disease risk (Table 3).

Genotype and allele frequencies of VDR SNPs in the case and the control groups are shown in Table 3. As for CKD patients, the allele frequencies of the Apal polymorphism of the VDR gene were 0.678 for the A allele and 0.322 for the a allele; and those for the TaqI polymorphism were 0.697 for the T allele and 0.303 for the t allele. While the genotype distributions for Taq I SNPs were in HWE (p=0.005), ApaI SNPs were not (p=0.000). The Aa genotype frequency was higher in the case group than those of control group while the genotype frequencies of TaqI polymorphism were not different between case and control groups (p=0.018 and p=0.902, respectively). We performed multivariate logistic regression analysis to evaluate effects of C1818T and G395A polymorphisms on risk of developing CKD. There was no association detected between C1818T ve G395A polymorphisms and the CKD risk (Table 2).

The allele frequencies of C1818T and those of the G395A were not significantly different from CKD patients (p=0.683 and p=0.832, respectively). The genotype distributions for C1818T and G395A SNPs were not HWE (p=0.008, p=0.03). There was no statistically significant difference between the groups in terms of genotype and allele frequencies. We performed multivariate logistic regression analysis to evaluate effects of C1818T and G395A polymorphisms on risk of developing CKD. There was no association detected between C1818T ve G395A polymorphisms and the CKD risk (Table 2).

Table 2: Genotype and Allele Distributions of C1818T and G395A Polymorphisms

| Genotypes | Controls (n=104) | Cases (n=104) | p-value | OR (95% CI) | p-value |
|-----------|-----------------|---------------|---------|-------------|---------|
|           |                  |               |         | Adjusted<sup>a</sup> |         |
| C1818T    |                  |               |         |             |         |
| CC        | 35(33.65%)       | 43(41.3%)     | 0.231   | 1<sup>c</sup> |         |
| CT        | 61(58.65%)       | 49(47.1%)     |         | 0.64(0.33-1.24) | 0.187 |
| TT        | 8(7.7%)          | 12(11.5%)     |         | 0.94(0.28-3.09) | 0.912 |
| C+T+TT    | 69(66.35%)       | 61(58.6%)     |         | 0.69(0.36-1.29) | 0.244 |
| C         | 131(62.9%)       | 135(59.6%)    | 0.683   | 1<sup>c</sup> |         |
| T         | 77(37.1%)        | 73(40.4%)     |         | 1.08(0.72-1.62) | 0.759 |
| G395A     |                  |               |         |             |         |
| GG        | 36(34.6%)        | 42(40.4%)     | 0.302   | 1<sup>c</sup> |         |
| GA        | 59(56.7%)        | 58(55.8%)     |         | 0.69(0.36-1.34) | 0.277 |
| AA        | 9(8.7%)          | 11(10.6%)     |         | 0.22(0.05-0.98) | 0.046 |
| GA+AA     | 68(65.4%)        | 41(38.6%)     |         | 0.65(0.34-1.23) | 0.188 |
| G         | 131(62.9%)       | 142(68.3%)    | 0.832   | 1<sup>c</sup> |         |
| A         | 77(37.1%)        | 80(31.7%)     |         | 1.04(0.70-1.54) | 0.842 |

<sup>a</sup>Compared with CC versus CT, TT, and CT+TT.
<sup>b</sup>Compared with GG versus GA, AA, and GA+AA.
<sup>c</sup>Reference genotype/allele. OR adjusted for age, gender and BMI.
Kim et al. indicated that genotype frequencies of C1818T and those of the G395A were not significantly different compared to the ones who did not carry the A allele (30).

We did not find a significant association between polymorphisms of VDR (ApaI, TaqI) and KLOTHO (C1818T, G395A) gene and hypertension and diabetes risk in CKD patients. The comparison of subgroups with normotensive/hypertensive and non-diabetic/diabetic kidney disease did not indicate a difference in genotype distribution.

Genotype distribution of KLOTHO and VDR gene in CKD patients with different clinical phenotypes.

Genotype distributions were compared in subgroups of CKD patients with different clinical phenotypes. The results are summarized in Table 4.

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Genotype distribution of KLOTHO and VDR SNPs in CKD patients classified according to hypertension and diabetes status.

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Recently, the number of patients who have renal failure due to the diabetes is increasing throughout the world. Hyperglycemia which is developed in diabetic patients deteriorates the vessels and leads to the renal damage (20). The roles of Klotho in the glucose control, diabetes and phosphate mechanism have not yet been investigated (21). In a study which was performed with 804 adults living in a nursing home, it was observed that the levels of serum Klotho which is a negatively regulated hormone in renal and cardiovascular systems.

Klotho is a member gene for T2DM due to its importance in the insulin signalization as shown in murine models and it plays a potential role during the life (23). Shimoyama et al. performed a study in which they included 476 healthy Japanese in order to determine the association between the KLOTHO gene C1818T and G395A polymorphisms and fasting glucose levels (4). According to their findings, they showed that fasting glucose levels were higher in individuals who were carrying G395A polymorphism A allele compared to ones who were carrying GG genotype. It is also higher in individuals who were carrying C1818T polymorphisms T allele compared to ones who were carrying CC genotype. Unlike the related literature findings, we did not detect any relationship between C1818T and G395A polymorphisms and diabetes development.

Progression of the CKD is shown to be associated with Vitamin D deficiency (24). In studies, it has been stated that Vitamin D is an important regulator in immune, renal and cardiovascular systems. Furthermore, it has been specified that Vitamin D has regulatory roles in the Renin Angiotension System (RAS) in CKD patients (24). According to our findings, in terms of VDR gene Apa polymorphisms genotype distribution, Aa genotype was significantly higher in case group compared to control group (p=0.018). Even though A allele frequency was higher in control group compared to the patient group (OD = 0.701 CI=0.469-1.048), this difference was not statistically significant (p=0.083). There was no difference between the groups in terms of VDR gene TaqI polymorphism allele frequency distributions (p=0.902).

VDR gene has an important role in the regulation of the RAS which is crucial in blood pressure regulation (25). Vural et al. performed a study with 100 Turkish patients with hypertension and diabetes and 100 Turkish healthy controls in order to evaluate the VDR gene TaqI polymorphism, and they showed that the genotype distributions were 51% TT, 46% Tt and 3% tt in the patient group and 35% TT, 49% Tt and 16% tt in the control group (26). Even though they found the T allele frequency in the patient group was higher than the control group (p=0.05), there was no difference between hypertension and diabetes patients and control individuals in terms of the VDR gene TaqI polymorphism allele frequency. In line with the related literature, we did not detect any association between Apal and TaqI polymorphisms and hypertension development risk.

Vitamin D has an important immune regulatory feature and it can play a role in the T1DM and T2DM etiology via affecting the insulin secretion. VDR polymorphisms can have roles in the T2DM pathogenesis by influencing the secretion capacity of β-cells (10). In studies, a relationship between T1DM, T2DM and some allelic variants of VDR gene has been identified (11, 27). In French population, TaqI genotype is associated with T2DM, obesity and early onset of type II diabetes mellitus (28). A European population study showed an association between glucose intolerance and Apal polymorphisms GG genotype was detected in patients with T2DM (13). These findings show that VDR gene is a member gene for both of the two diabetes types. Dilmec et al. (2010) found that allele frequencies were 9.7% GG, 52.8% GT and 37.5% TT for Apal (g.59979G>T) SNP and 45.8% TT, 34.7% Tt and 19.4% CC for TaqI (g.6008T>C) in 72 Turkish T2DM patients. They did not detect a significant difference between allele and genotype frequencies of both groups. Even though the relationship between the diabetes and VDR polymorphisms was investigated in some population studies, we did not detect any association between Apal and TaqI polymorphisms and diabetes development risk which was also shown in the study performed by Dilmec et al(10).

According to our knowledge, this study is the first one which has been performed on KLOTHO and VDR gene polymorphisms and CKD in Turkish population. In our study, we did not support the idea that there is a relationship between CKD and KLOTHO gene polymorphisms in the Turkish population. Additionally, in our study, VDR gene Apal polymorphisms Aa genotype was found higher in case group compared to control group, and individuals who were carrying this genotype were thought to have higher risks in terms of CKD development. Although our study is a comprehensive study, there are also some limitations. The first limitation is that we had a correlation study, mostly due to the relatively small number of patients and control groups. Secondly, CKD is a heterogeneous disease which is affected by both genetic and environmental factors. Therefore, we believe that our findings should be supported by further studies investigating more polymorphisms and having well matched and large number of cases and controls.

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