The association of the Klotho polymorphism rs9536314 with parameters of calcium-phosphate metabolism in patients on long-term hemodialysis

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ABSTRACT

Background: Patients on long-term hemodialysis frequently suffer from complications, such as secondary hyperparathyroidism, bone fractures, and arteriosclerosis. The process of regulating Ca/P metabolism depends on factors, such as FGF23 and Klotho. This study aimed to answer the question of whether the Klotho polymorphism rs9536314 is associated with FGF23 plasma concentration.

Methods: In 118 patients undergoing hemodialysis, blood was collected before and after hemodialysis. The following parameters were measured in plasma: FGF23, serum: Ca, P, PTH, HGB, and iron concentrations. The KL gene polymorphism rs9536314 was identified by PCR-RFLP.

Results: The KL polymorphism rs9536314 was not associated with Ca, P, PTH, or FGF23. There was a negative correlation between FGF23 and blood HGB levels and positive correlation between FGF23 and ESA dose.

Conclusions: The results obtained may indicate that there is no association between the KL polymorphism and FGF23 concentration in patients undergoing long-term.

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Introduction

Patients with chronic kidney disease (CKD) present high annual mortality rates with cardiovascular diseases – uremic cardiomyopathy or arteriosclerotic vasculopathy being a major cause of death. Disorders of calcium/phosphate metabolism in patients with CKD are present in the initial stages of the disease and increase with the disease progression. Patients on long-term hemodialysis frequently suffer from complications, such as secondary hyperparathyroidism, bone pain, bone fractures, or arteriosclerosis.

More recent studies have shown that the regulation of calcium/phosphate metabolism depends on other factors, such as fibroblast growth factor 23 (FGF23) and its cofactor – the Klotho (KL) protein. FGF23 is a valuable biomarker in CKD. FGF23 is a member of the FGF family which promotes negative external phosphate balance through inhibition of intestinal absorption and enhanced renal excretion. FGF23 is primarily released by osteocytes, regulates phosphate, and vitamin D metabolism and regulates parathyroid hormone synthesis and secretion. Recently, it was reported that the FGF23 signaling requires Klotho protein in addition to the FGF-receptor. KL, discovered in 1997 by Kuro-o, proved to be an important factor responsible for ageing. Mice without this gene (Klotho –/–) die as early as 2 months after birth. Alpha-Klotho-null mice have elevated levels of FGF23, suggesting that Klotho protein is downstream of FGF23 and there is a failure of feedback inhibition in the alpha-Klotho(−/−) mouse. The correlation between high FGF23 levels and mortality in hemodialysis patients has been reported by Gutierrez et al.
complex is to inhibit phosphate re-absorption by the kidneys under physiological conditions. If renal function is impaired, this axis becomes disrupted and phosphate accumulation occurs. As kidney function declines, levels of serum FGF23 increase gradually and can reach more than 200-fold above normal in advanced renal failure.\(^\text{15}\) In addition to hyperphosphatemia in \(\text{KL} \ (-/-)\) animals, calcium and 1,25-dihydroxyvitamin D concentrations are also elevated. The same abnormalities occur in animals with \(\text{KL}\) gene intact but without FGF23 encoding gene.\(^\text{16}\) An injection of FGF23 reduces serum 1,25-dihydroxyvitamin D levels.\(^\text{15}\) Despite the lack of evidence for a direct association of \(\text{KL}\) polymorphisms with non-diabetic ESRD, it is possible that polymorphism in the \(\text{KL}\) gene may affect the severity of ESRD.\(^\text{17}\) An allele of the Klotho termed KL-VS is prevalent in the general population and in homozygosity is associated with reduced human longevity. Of the three mutations in exon 2, one is silent and two code for amino acid substitutions F352V and C370S that influence Klotho metabolism.\(^\text{18}\) The objective of this study was to answer the question whether the \(\text{KL}\) polymorphism rs9536314 is associated with the plasma FGF23 concentration and selected parameters of calcium/phosphate metabolism: serum calcium (Ca), phosphorus (P), and parathyroid hormone (PTH) concentration.

**Materials and methods**

Totally, 118 patients undergoing long-term hemodialysis (HD) participated in this study (Female – 49, Male – 69). The study protocol was approved by the medical ethics committee of Pomeranian Medical University. Inclusion criteria: age 18–80 years, hemodialyzed more than 6 months, no changes in pharmacological treatment since 3 months, stable dose ESA and orally given calcium. Exclusion criteria: pregnancy, any type of cancer in the past 5 years, any signs of inflammation during collection samples, episode of acute cardio-vascular diseases or stroke in past 3 months. The patients were undergoing four-hour HD sessions three times per week. The study subjects had the following conditions: diabetes mellitus (DM) \(n = 32\), hypertension (HA) \(n = 117\), and parathyroidectomy \(n = 15\) individuals. All participating patients were treated with erythropoietin and calcium carbonium. Characteristics of the study group shown in Table 1.

The tests were performed before the start of HD session and after its completion, and the following parameters were measured plasma FGF23, serum Ca, P, PTH, hemoglobin (HGB), and iron concentrations (Table 2). Prior to the procedure start, blood was collected to identify the KL protein polymorphism.

**Table 1. Antrophometric characteristics of the study group.**

| Parameter | Patients, \(n = 118\) | Mean ± SD |
|-----------|----------------------|------------|
| Age (years) | 62.75 ± 16.37 | |
| BMI (kg/m\(^2\)) | 26.36 ± 4.50 | |
| DBW (kg) | 74.98 ± 14.66 | |
| ∆BW (kg) | 2.10 ± 1.0 | |
| Dialysis duration (months) | 31.54 ± 28.73 | |
| Residual diuresis (ml/24h) | 614.4 ± 727.98 | |

**Table 2. Basic laboratory tests in the study group.**

| Parameter | Mean ± SD |
|-----------|------------|
| ESA U/week | 3966 ± 3119 |
| HGB (g/dl) | 11.18 ± 1.29 |
| PTH (pg/ml) | 406.6 ± 314.6 |
| FE (µg/dl) | 64.09 ± 32.93 |
| FGF 23 pre-HD (pg/ml) | 580.5 ± 679.6 |
| FGF 23 post-HD (pg/ml) | 625 ± 673.5 |
| Ca (mmol/l) | 2.18 ± 0.25 |
| P (mmol/l) | 1.70 ± 0.56 |

**Genetic studies**

**Klotho**

Blood from the vena basilica was collected in tubes containing EDTA and without anticoagulant. The blood was centrifuged at 20 °C for 20 min at 3000 g. The serum and plasma obtained were frozen and stored at −80 °C until the time of the assay.

Genomic DNA from peripheral blood leukocytes was isolated using a QiAamp DNA Mini Kit (QIAGEN). Polymorphism rs9536314 (T1054G; F352V) of the \(\text{KL}\) (Klotho) gene was identified by PCR-RFLP using a pair of specific oligonucleotide primers:

\[ 5'\text{-CTTTCATCTATTCGCGGAT-3'}; 5'\text{-AACCAAGCCATT TTCACAA-3'} \]

The PCR products were 221bp long DNA fragments, which were analyzed using the restriction enzyme BseGI.

Plasma FGF23 was measured using a Human Intact FGF23 Reagent Kit (Immunotopics, Inc.; USA). Ca, P, and PTH serum levels, complete blood counts, and serum iron concentration were determined using ready to use reagent kits.

**Statistics**

The statistical analysis of quantitative variables was performed using non-parametric tests – Mann–Whitney U test for comparisons between groups and Spearman’s rank correlation coefficient (\(R_S\)) for correlation analysis. Qualitative variables were compared between groups with Fisher’s exact test. The conformity of the distribution for the rs9536314 \(\text{KL}\) polymorphisms with the Hardy–Weinberg equilibrium (HWE) was analyzed by exact test. The threshold for statistical significance was \(p < 0.05\). The calculations were performed using the Statistica 10 program (StatSoft, Inc., Tulsa, OK).
Results

The distribution of the KL polymorphism in the study group is shown in Table 3. The TT genotype was present in 92 individuals (77.97%), TG in 24 individuals (20.34%), and GG in two individuals (1.7%).

Based on the results, the rs953614 polymorphism did not correlate with age, BMI, length of dialysis therapy, or Ca, P, or PTH concentration. The clinical characteristics of the genotype groups are shown in Table 4.

No association was observed between the KL polymorphism and sex, HA, parathyroid resection, or ischemic heart disease.

Next, the correlation between FGF23 concentration and selected clinical and biochemical parameters was analyzed. The FGF23 concentrations measured pre- and post-HD correlated with age. In addition, the FGF23 concentrations in blood collected pre- and post-HD positively correlated with PTH and phosphate levels. The FGF23 concentration measured after HD positively correlated with the Ca concentration.

The correlation between FGF23 and HGB concentration and iron metabolism parameters and erythropoietin (ESA) dose was also studied. A negative correlation between HGB and FGF23 concentrations pre- and post-HD was demonstrated. A positive correlation between the weekly ESA dose and FGF23 concentration in the HD was demonstrated. A positive correlation between HGB and FGF23 concentrations pre- and post-HD correlated with age. In addition, the FGF23 concentration measured pre- and post-HD correlated with age. In addition, the FGF23 concentration measured pre- and post-HD correlated with age.

No association was observed between the FGF23 concentration pre- or post-HD and the following parameters: DBW, BMI, dialysis duration, and history of parathyroidectomy. However, a negative correlation was detected between residual diuresis and the FGF23 concentration pre- and post-HD.

Discussion

The KL protein is found in two forms: transmembrane and secreted. The transmembrane form is found in the kidneys and forms a complex with FGF23, which is responsible for maintaining phosphate homeostasis. The secreted form of the protein has a different function. This form regulates the activity of ion channels, membrane transporters, and growth factor receptors. Thus, the secreted form maintains calcium homeostasis because the secreted KL protein causes N-glycan modification of TRPV5 channels and inhibits the insulin and insulin-like growth factor pathway. Moreover, reduced expression of the KL gene was demonstrated in neoplastic diseases, including breast, pancreas, stomach, and colon carcinomas. This protein is believed to have an anti-neoplastic effect by suppressing neoplastic cells.

This study aimed to determine the association between the rs953614 polymorphism and the concentration of FGF23, calcium, phosphate, and parathyroid hormone levels in patients undergoing long-term hemodialysis.

The analysis compared two genotype groups: TT (n = 92) and GG + TG (n = 26). There was no association between the rs95612 polymorphism and FGF23 concentration, measured pre-, and post-HD. Studies of the KL gene polymorphism today have yielded inconsistent information regarding its correlation with individual parameters of calcium/phosphate metabolism in CKD patients. In the Friedman study, the hypothesis was that the KL gene polymorphism may correlate with the survival of patients on hemodialysis. The analysis was performed on a group of 1307 individuals, and the 12 most

Table 3. Distribution of the rs953614 genotype in the study population (n = 118).

| Genotype | Number of patients | Percent (%) | HWE p |
|----------|--------------------|-------------|-------|
| TT       | 92                 | 77.97       | 0.66  |
| TG       | 24                 | 20.34       |       |
| GG       | 2                  | 1.7         |       |

Table 4. The selected anthropometric, clinical, and biochemical characteristics in patients with the Klotho polymorphism rs953614.

| Parameter                  | TT polymorphism (number of patients) | Mean ± SD | TG + GG polymorphism (number of patients) | Mean ± SD | p    |
|----------------------------|--------------------------------------|-----------|------------------------------------------|-----------|------|
| Age (years)                | 92                                   | 63.3 ± 15.9 | 26                                      | 60.7 ± 18.2 | 0.813 |
| DBW (kg)                   | 92                                   | 74.7 ± 14.4 | 26                                      | 75.8 ± 15.6 | 0.859 |
| Height (m)                 | 92                                   | 1.68 ± 0.1  | 26                                      | 1.69 ± 0.07 | 0.889 |
| BMI (kg/m²)                | 92                                   | 26.4 ± 4.5  | 26                                      | 26.3 ± 4.4  | 0.869 |
| Dialysis duration (months) | 92                                   | 33.9 ± 30.8 | 26                                      | 23.08 ± 17.5 | 0.267 |
| Ca (mmol/l)                | 92                                   | 2.19 ± 0.23 | 26                                      | 2.11 ± 0.30 | 0.267 |
| P (mmol/l)                 | 92                                   | 1.72 ± 0.59 | 26                                      | 1.66 ± 0.44 | 0.769 |
| PTH (ng/ml)                | 92                                   | 406.6 ± 314.6 | 26                                 | 382.4 ± 342.2 | 0.635 |
| HGB (g/dl)                 | 92                                   | 11.12 ± 13.3 | 26                                      | 11.37 ± 11.9 | 0.33  |
| FE (µg/dl)                 | 92                                   | 62.0 ± 31.1  | 26                                      | 71.7 ± 38.4  | 0.34  |
| ESA U/week                 | 92                                   | 4141 ± 3227 | 26                                      | 3346 ± 2667 | 0.261 |
| Residual diuresis (ml/24 h)| 92                                   | 591 ± 725   | 26                                      | 696 ± 745   | 0.475 |
| FGF23 pg/ml pre HD         | 92                                   | 536.8 ± 873.8 | 26                                 | 742.4 ± 797.8 | 0.82  |
| FGF23 pg/ml post HD        | 92                                   | 584.1 ± 620.1 | 26                                 | 776.7 ± 840.5 | 0.68  |
| ΔFGF23                    | 92                                   | 47.3 ± 322.6 | 26                                      | 34.3 ± 222.3 | 0.219 |
common KL gene polymorphisms were studied. Only the rs577912 polymorphism correlated with an increased mortality risk ($p = 0.005$). In another study conducted among patients on long-term HD, two polymorphisms in KL gene, occurring with the highest frequency in the Korean population, were selected: in the promoter region (G-395A) and in exon 4 (C1818T). The following conclusions can be made based on the results obtained. In females, the uric acid concentration was significantly higher in individuals with the A allele (G-395A) compared to those with the GG genotype. In males, the LDL cholesterol concentration was significantly lower in individuals with the T allele (C1818T) compared to individuals with the CC genotype. The question remains whether this difference correlates with survival in these patients.

The major role of the FGF23 is to regulate the phosphate concentration; therefore, the FGF23 concentration in patients with impaired kidney function undergoing long-term hemodialysis, is higher compared to the healthy population. The recently conducted studies have led to the discovery of the consequences of increased FGF23 concentration in patients undergoing long-term dialysis. The main finding is that there is a toxic effect of FGF23 itself on the vascular endothelium. A high FGF23 level was demonstrated to directly correlate with vascular calcification in patients undergoing long-term hemodialysis. Other studies conducted in animals showed that treatment of calcium/phosphate metabolism disorders and an attempt to restore normal calcium and phosphate values do not significantly affect the reduction in FGF23 expression. FGF23 is also known to reduce the synthesis of the active form of vitamin D and in this way can increase mortality due to cardiovascular causes in patients undergoing long-term dialysis. Moreover, observations to date indicate that FGF23 can serve as a predictor of worsening renal function and is a better indicator compared to the concentration of calcium or phosphate.

In the present study, we demonstrated that FGF23 correlates with the level of HGB and weekly ESA dose. Based on the studies to date, in the general population, there is an inverse correlation between FGF23 and hemoglobin concentrations, and this correlation is more pronounced when calcium is deficient. Observations from Takeda indicate that intravenous administration of ferrous preparations to patients on long-term dialysis causes a significant increase in already high FGF23 values. Yamamoto demonstrated that administration of iron preparations induces hypophosphatemia.

In this study performed by Yamamoto, intravenous supplementation had no effect on the level of phosphate or vitamin D. A similar treatment administered to the individuals with anemia and abnormal glomerular filtration caused a significant increase in FGF23 concentration, hypophosphatemia, and vitamin D deficiency. The mechanism of the influence of intravenous iron supplementation on the FGF23 concentration remains still unknown. This mechanism may be related to the oxidative stress that develops as a result of intravenous injection.

In patients with chronic kidney disease, KL gene and protein expression are significantly reduced. This reduced expression has been suggested to cause further complications in this group of patients. The FGF23 concentration increases with disease progression and constitutes a repair mechanism for correcting hyperphosphatemia, an abnormality that appears in the early stages of kidney disease. Changes in the concentrations of the analyzed substances could be independent risk factors for mortality in CKD patients due to their effect on the axial symptoms of CKD.

To summarize, FGF23 and the KL protein are not only involved in regulating calcium/phosphate metabolism but may be important factors in regulating a whole range of metabolic processes. Mice with the KL gene inactivated demonstrate early symptoms of ageing, and mice with hyperactive gene live significantly longer. In our study no evidence of an association of polymorphism rs9536314 Klotho gene with calcium/phosphorous metabolism was revealed.

Disclosure statement
The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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