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Original Article

Reduced klotho is one risk factor for the occurrence and development of coronary artery disease in elderly people

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Abstract: Background: S-Klotho has been deemed as a protein related to human longevity. However no studies have specially examined the relationship between serum levels of Klotho and coronary artery disease (CAD) in the people more than 70 years old. Hypothesis: We proposed that low plasma klotho concentrations would be independently associated with CAD in elderly people. Methods: 315 consecutive patients who had at least one coronary angiography examination were included in our study. All the patients were older than 65 years old. Their coronary artery condition, blood pressures, s-Klotho concentration, liver function, renal function and thyroid function were evaluated at their first visits. Their readmissions because of CAD were recorded during their follow-up. Logistic regression analysis was used to select the risk factors for CAD readmission. Results: The 315 consecutive patients were divided into primary cohort (200 cases) and validation cohort (115 cases) randomly. The final model included 6 significant variables, which were a-klotho, CAD grade, creatinine, HDL, hemoglobin and uricacid. All these variables were then used to build a risk score in terms of the prediction of incidence of CAD. The ROC curve for the weighted score showed good discriminant power with a c statistic of 0.894 (95% CI: 0.8237-0.9637). Conclusions: Our study is the first time reporting that the elderly patients with lower level of s-klotho concentrations have a higher risk of readmission because of CAD.

Keywords: Klotho, risk factor, coronary artery disease, elderly people

Introduction

Coronary artery disease (CAD) remains the leading cause of death in the world. The risk of CAD can be quantified by associations among classical risk factors, however the susceptibility, occurrence, and progression of CAD is not completely explained by these factors. Thus, new biological systems could provide important additional information to improve our understanding of atherosclerotic disease biology and the assessment of cardiovascular risk.

klotho, a gene originally identified in 1997 which encodes a antiaging protein, has been postulated as a regulator of human aging process [1, 2], including arteriosclerosis, decreased bone mineral density, sarcopenia, skin atrophy, and impaired cognition. Overexpression of *klotho* in transgenic mice resulted in a significant extension of life span compared with wild-type mice [3].

The klotho protein which is encoded by *klotho* is a single-pass transmembrane protein with a large extracellular domain. It is primarily produced in the kidney [4]. The extracellular domain of klotho can be cleaved and released into extracellular fluids [5], where soluble klotho (s-klotho) could be detected [6]. The function of s-klotho is different from that of membrane form. Membrane klotho acts as an obligate co-receptor for fibroblast growth factor (FGF)-23, a bone-derived hormone that induces phosphate excretion into urine [7]. While s-klotho is involved in the endothelium calcium homeostasis in the kidney by regulating nitric oxide production and inhibiting intracellular insulin and insulinlike growth factor-1 signaling [8]. Therefore, klotho has been suggested as a mas-

ter regulator of cardiovascular disease, with a potential role in the pathogenesis of atherosclerosis [6, 9].

In recent years, more and more attention has been paid on the function of klotho. Many studies have shown that variants of the *Klotho* are associated with atherosclerotic disease [10]. A sensitive and specific assay was also developed for the measurement of s-klotho in humans [11]. Some studies have shown that s-klotho is related with the vascular calcification in patients with renal failure, hypertension (HT), and diabetes mellitus (DM) [12], Levey AS et al [13] and Maekawa Y et al [14] have revealed a significant association between higher plasma klotho and lower risk of CAD. However no studies have specially examined the relationship between serum levels of klotho and CAD in the people more than 70 years old. Besides there were no studies clarifying the different levels of s-klotho in the acute and chronic CAD. So we proposed the hypothesis that low plasma klotho concentrations would be independently associated with CAD in elderly people and we have conducted a cross-sectional study to test this hypothesis.

Methods

Study population

315 patients who visited the department of cardiology at the Huashan Hospital, Shanghai Fu Dan University School of Medicine, from January 2011 to December 2012, were included in our study. All the patients were older than 65 years old with at least one coronary angiography examination using standard technique. By the coronary angiography examination, coronary stenosis were diagnosed according to the following four major epicardial arteries: left main coronary artery, left anterior descending artery, circumflex artery, and right coronary artery. All the patients have normal (close to normal) kidney function. Those who had the following diseases were excluded in our study: haemodynamic instability, cardiac arrhythmia, immunologic or inflammatory diseases (such as rheumatoid arthritis, systemic lupus erythematosus, or inflammatory bowel disease). According to the coronary angiography, clinical manifestations and history of the ischemic heart disease, these patients were divided into three groups: ① Control group (No significant CAD: grade 0): There are 0-49% stenosis in any epicardial coronary artery. ② Stable-CAD (grade 1): The presence of at least one lesion leading to ≥50% lumen diameter stenosis of these arteries without obvious clinical symptoms recently (such as Chest pain and stuffiness, palpitation and dyspnea). ③ Acute-CAD (grade 2): the presence of at least one lesion leading to ≥50% lumen diameter stenosis of these arteries, and at the same time they have a typical unstable angina or acute myocardial Infarction.

The study protocol was approved by the local ethics committee, and written informed consent was obtained from all the participants.

Clinical evaluation

Demographic information and medical history were collected using standardized questionnaires. Systolic and diastolic blood pressures were measured for three times using a standard mercury sphygmomanometer and the mean value of measurements were calculated as the blood pressure.

Laboratory studies

Venous blood samples of all the patients were collected in the morning before coronary angiography. Serum and plasma were isolated and at -80°C immediately. Soluble a-klotho in the plasma was detected using a solid-phase sandwich enzyme-linked immunosorbent assay (ELISA) (Immuno-Biological Laboratories, Takasaki, Japan). The assay revealed an intra- and interassay coefficient of variation of 3.4 and 4.9%, respectively. The serum levels of hemoglobin, albumin, renal function, uric acid, creatinine, calcium and phosphate, glucose, total cholesterol, triglycerides, high density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), thyroid-stimulating hormone (TSH), free triiodothyronine (FT3), FT4 and N-terminal pro-B-type natriuretic peptide (NT-pro-BNP) were measured in all the patients using an automated biochemical analyzer (RX Daytona+ Benchtop Clinical Chemistry Analyzer, Randox Laboratories US Limited). The left ventricular ejection fraction the (LVEF) was also detected by the echocardiography (ACUSON SC2000 Ultrasound System, SIEMENS Heathineers).

Table 1. Clinical characteristics of patients in the training and test datasets

Demographic or Characteristic	Primary Cohort (n = 200)	Validation Cohort (n = 115)
	No. of Patients	No. of Patients
Age		
Median	70	70
Range	46-94	47-97
Albumin		
Median	41	41
Range	27.00-49.28	29-49.76
s-klotho		
Median	540.19	586.44
Range	54.28-1282.17	12.27-1107.33
ca		
Median	2.227	2.205
Range	1.792-2.670	1.82-2.840
CAD		
0	67	48
1	80	42
2	53	25
Cholesterol		
Median	4.179	4.36
Range	2.101-7.365	2.298-7.010
Clinics		
0	102	60
1	98	55
Creatinine		
Median	87.5	89
Range	29.49-400.54	31.48-309.27
Diabetes		
0	139	85
1	61	30
HDL		
Median	1.1025	1.124
Range	0.64-2.0300	0.610-2.133
Hemoglobin		
Median	128.83	133
Range	14.67-180.68	24.0-170.2
Hyperten		
0	99	50
1	101	65
LDL		
Median	2.287	2.366
Range	1.11-4.826	1.110-4.765
LVEF		
Median	60.23	58
Range	19.9-95.16	28.09-77.00
Phos		
Median	1.1044	1.1818

Outcome assessment during follow-up

The patients were required to follow for two years. The incidence of readmission to the hospital because of cardiovascular disease (including myocardial infarction, myocardial ischemia, decompensated heart failure) were recorded during the following years.

Statistical analysis

The dataset was randomly divided into 2 datasets using a computer-generated random number: the training dataset for model development and the test dataset for validation of the developed nomogram. In the training dataset, associations between each variable and readmission were first tested using univariate analysis. Clinical variables considered for entry into the multivariate predictive model included alphaklotho, creatinine, HDL, hemoglobin, ureaN, Uricacid. Multiple backward stepwise logistic regression was performed to identify the significant predictors with regard to readmission by the Akaike information criterion [15]. Variables with a significant association ($P < 0.1$) with readmission based on univariate analyses were entered into a multivariate model. All the significant variables were then used to establish nomogram, which was formulated based on the results of multivariate analysis and by using the package of rms26 in R version 3.3.1 (http://www.r-project.org/).

Validation of the nomogram was performed using the test dataset. The discriminating properties of the readmission prediction model were investigated by calculating the area under a

Range	0.34-1.7784	0.5326-1.8700
proBNP		
Median	234.55	220.14
Range	1.11-6097.00	3.72-3159.02
Triglyceride		
Median	1.4054	1.3175
Range	0.5588-6.3600	0.3955-4.1005
ureaN		
Median	6.229	6.879
Range	2.347-44.670	2.253-41.000
Uricacid		
Median	0.3754	0.3802
Range	0.1752-0.7598	0.2004-1.4990

Table 2. Multivariate logistic regression analysis of the association between CAD readmissionand multiple parameters

Variables	P	Odds	95% CI
s-klotho	5.93E-04	9.95E-01	9.91E-01 to 9.98E-01
CAD1:0	5.81E-02	3.79E+00	9.55E-01 to 1.50E+01
CAD2:0	1.17E-01	3.10E+00	7.53E-01 to 2.80E+00
Creatinine	3.00E-03	9.78E-01	9.64E-01 to 9.92E-01
HDL	1.67E-04	1.30E-03	4.10E-05 to 4.14E-02
Hemoglobin	1.04E-05	9.46E-01	9.23E-01 to 9.70E-01
Uricacid	1.86E-06	6.47E+08	1.54E+05 to 2.71E+12

ROC curve. The ROC curve reflects the relationship between the sensitivity and the specificity. Based on the ROC curve, the following values were calculated: the sensitivity, specificity, positive predictive value, and negative predictive value.

Statistical analyses were performed using R version 3.3.1. All *P* values reported are 2-tailed, and P < 0.05 was considered to be statistically significant.

Results

Clinical biochemical index characteristics of patients

In the primary cohort, of 315 patients with CAD who received coronary angiography during the study period, 200 met the inclusion criteria be entered onto this study. For the validation cohort, we studied 115 consecutive patients. The clinical biochemical index characteristics of patients in the primary and validation cohorts are listed in **Table 1**.

Independent prognostic factors in the primary cohort

The results of multiple stepwise logistic regression analysis showed that 6 major predictors highly significantly correlated with the incidence of CAD: s-klotho (odds ratio [OR]: 0.99, 95% confidence interval [CI]: 0.9914-0.9976, P < 0.001), CAD grade (OR: 3.79, 95% CI: 0.96-15.02, P = 0.05), creatinine (OR: 0.98, 95% CI: 0.96-0.99, P = 0.002), HDL (OR: 0.001, 95% CI: 4E-5-0.04, P < 0.001), hemoglobin (OR: 0.95, 95% CI: 0.92-0.97, P < 0.001), and uric acid (OR: 6.47E+8, 95% CI: 1.54E+5-2.71E+12, P < 0.001). These identified predictors were then used to build a clinical score system regarding the prediction of post procedural CAD (**Table 2**).

Prognostic nomogram for readmission

The prognostic nomogram that integrated all significant independent factors for readmission in the primary cohort is shown in **Figure 1**. The validity of the nomogram score was then tested in the other half of the study population (n = 115). The ROC curve for the weighted score showed good discriminant power with a c statistic of 0.894 (95% CI: 0.8237-0.9637) (**Figure 2**).

Discussion

The protein s-klotho has opened an extraordinarily wide research area because of its relationship with human longevity. The absence of klothoin murine models can result in accelerated aging symptoms, including atherosclerosis, vascular calcifications, defects in angiogenesis, endothelial dysfunction and vascular calcifications [16-19], which can be reversed by regulating the *klotho* gene. Although an increasing studies have reported that variants of the *klotho* gene are associated with carotid atherosclerosis, stroke, and CAD [20, 21], most of them are studies on the gene level. In recent years, Semba et al [22] reported that the risk of

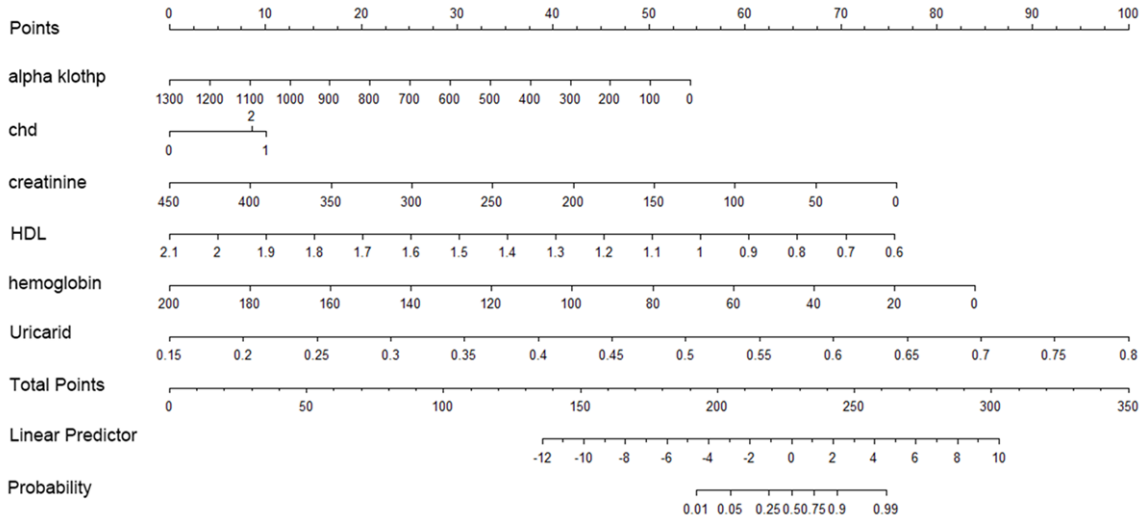

Figure 1. Pronostic nomogram predicting two year readmission probability of CAD patients.

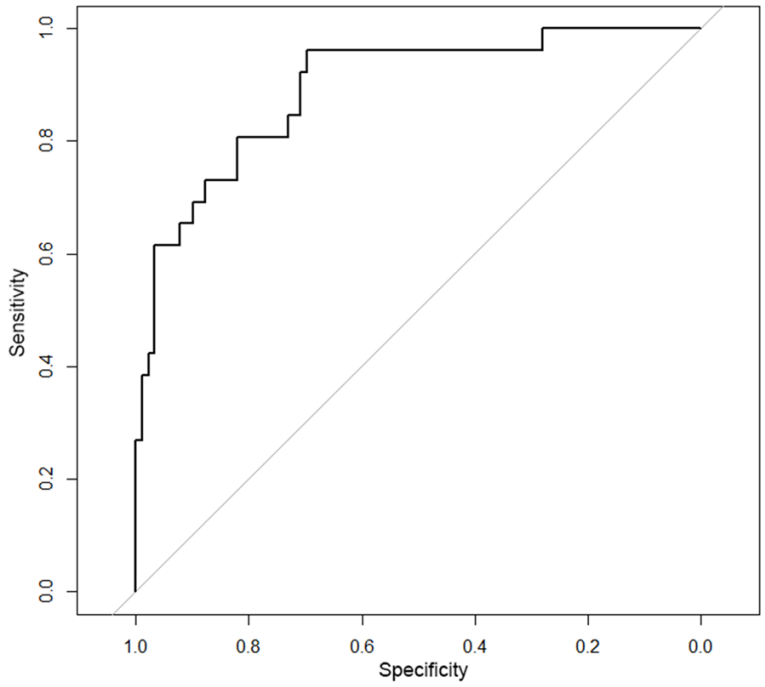

Figure 2. ROC curve for the Nomogram Model in the test dataset.

CAD was lower in subjects with higher klotho concentrations. This result was also confirmed by Navarro-González JF [23], whose study concluded that reduced levels of *klohto* gene expression in the vascular with CAD. However all these previous studies focused on the general population with CAD.

With the trend of aging, CAD of elderly people received more and more attention. In general, CAD in the elderly manifests a gradual increasing incidence. Thus in the present study, we only focused on the elderly people who were older than 65 years old (with a mean age of 76.7±7.3 years old). Besides, we divided all the patients into three grades according to their CAD conditions for the first time, which made our study much closer to the clinic. Based on multiple logistic regression, 6 significant indicators were located and then nomogram was built which could be used to identify patients at high risk of CAD. According the nomogram, clinical staff would be able to readily and rapidly identify those who might require further care. We also tested the accuracy of this newly built nomogram in testing group and found that, with clinical and procedural variables, its efficacy is good in predicting post procedural NRF (AUC = 0.894).

In the present study, in terms of the significance in the predication, the order of the 6 indicators are the level of s-klohto, CAD grades, creatinine, HDL, hemoglobin and Uricacid. In agreement with Jo et al [24], who reported that overexpression of the *Klotho* gene extends lon-

gevity, while defective Klotho proteins are associated with premature death, we found that low level of s-klohto in blood had a strong relevance to the incidence of CAD readmission. That is the lower s-klotho level, the higher incidence of readmission in CAD population. These result suggested that the level of s-klohto had a strong independently predictive effect of readmission in the elderly patients.

In conclusion, our study is the first time reporting that the elderly patients with lower level of s-klotho concentrations have a higher risk of readmission because of CAD. So klotho might be a marker representing the happening and prognosis of CAD. It also suggests that Klotho might be a potential target for new therapeutic interventions aimed at the prevention of CAD.

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Disclosure of conflict of interest

None.

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