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Gene expression of klotho & antioxidative enzymes in peripheral blood mononuclear cells of essential hypertension patients in Indian population

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***Background & objectives*: Oxidative stress is known to have a causal role in hypertension. Klotho has emerged as a novel anti-aging molecule to inhibit oxidative stress at cellular level. This study aimed at evaluating the gene expression of klotho and antioxidative enzymes, manganese superoxide dismutase (Mn-SOD) and catalase, in peripheral blood mononuclear cells of essential hypertensive patients as compared to normotensive healthy controls.**

***Methods*: Ninety-nine newly diagnosed hypertensives and 103 age- and BMI-matched controls were recruited. The participants were non-diabetic and not on any medication. Soluble α-klotho levels were detected using enzyme-linked immunosorbent assay. Gene expression was evaluated by quantitative real-time polymerase chain reaction.**

***Results*: Soluble α-klotho levels were significantly lower (27%, *P*=0.001) in patients as compared to controls. The trend remained same when compared against 44 out of 103 controls considered for gene expression analysis. Relative gene expression of klotho and catalase were 3-fold and 1.25-fold lower in patients as compared to controls, respectively. ΔCt value-based gene expression were also significantly lower for both genes (*P*=0.001). A decreasing but non-significant trend was observed for Mn-SOD gene expression. ΔCt value-based gene expression of catalase positively correlated with that of Mn-SOD in patient (rs=0.448) and control (rs=0.547) groups (*P*<0.001). In patients, the gene expression of Klotho positively correlated with that of catalase (rs=0.498, *P*=0.001), but not Mn-SOD (rs=0.155, *P*=0.126).**

***Interpretation & conclusions*: In the present study on newly diagnosed hypertensives, klotho and catalase gene expression were found to be significantly lower as compared to controls, indicating the role of oxidative stress in this patient group. In addition, a significant correlation between Klotho and catalase gene expression suggests a role for klotho in essential hypertension with respect to antioxidant defence.**

Key words 8-iso-prostaglandin F2α - cardiovascular disease - catalase - insulin/insulin-like growth factor-1 - manganese superoxide dismutase - oxidative stress - α-klotho

Hypertension is considered one of the most important risk factors for the occurrence of cardiovascular disease (CVD)[1]. Essential, primary or idiopathic hypertension is a heterogeneous disorder, defined as high blood pressure (BP) in the absence of secondary causes such as renovascular disease, renal failure, pheochromocytoma, aldosteronism, or other causes of secondary hypertension. It accounts for 95 per cent of all cases of hypertension[2]. Although the pathophysiology of essential hypertension remains an enigma, several factors have been implicated in its genesis. Recently, oxidative stress has gained attention as one of the fundamental mechanisms responsible for the development of essential hypertension. Elevation of BP by oxidants and its amelioration by antioxidants strongly suggests a causal role of reactive oxygen species (ROS) in hypertension, and is supported by several animal and clinical studies[1].

In recent years, klotho has emerged as a novel molecule to inhibit ROS and oxidative stress[3,4]. The klotho gene – originally identified as an ageing-suppressor gene in mice – extends the lifespan when overexpressed and induces complex phenotypes resembling human premature ageing syndromes when disrupted[5]. It encodes a single-pass transmembrane protein consisting of intracellular, transmembrane and extracellular domains and a circulating klotho protein. This circulating klotho arises from the cleavage of the extracellular domain of the transmembrane protein as well as alternative RNA splicing[5]. It acts as a peptide hormone and influences various signalling pathways including p53/p21, cAMP, protein kinase C, Wnt pathways as well as the insulin-like growth factor-1 (IGF-1) signalling cascade[6].

The anti-ageing effect of klotho is partly attributed to increased resistance to oxidative stress at the cellular level via inhibition of insulin/IGF-1 signalling pathway: an evolutionarily conserved mechanism for extending the lifespan[6]. The role of insulin/IGF-1 signalling pathway in hypertension has been well documented[7]. IGF-1 plays a role in the regulation of blood flow and pressure via endothelial nitric oxide synthase activity and nitric oxide production in vasculature as well as via non-endothelium-dependent coronary vasorelaxation[7]. Serum IGF-1 levels are high in hypertensive patients[8]. In animal models, klotho has been shown to inhibit the insulin and IGF-1 pathways. Its hormonal effects, mediated through a yet unidentified klotho receptor on the cell membrane, involve inhibiting the tyrosine kinase activity of the insulin/IGF-1 receptor[9] and subsequently the insulin receptor substrates. The Forkhead box O (FoxO) transcription factors, which are downregulated by insulin/IGF-1 signalling, are thereby activated by this klotho-mediated inhibition. There exists an established link between FoxO transcription factors and cellular antioxidant defence[10]. The activation of FoxO transcription factors induces the expression of manganese superoxide dismutase (Mn-SOD), which, in turn, removes the ROS and reduces oxidative stress at the cellular level[3]. In an *in vitro* study on human umbilical vein endothelial cells, Cui *et al*[11] found that klotho protein reduced ROS-induced oxidative stress, endoplasmic reticulum stress-mediated apoptosis and the release of inflammatory mediators through activation of the PI3K/AKT pathway.

Peripheral blood mononuclear cells (PBMC) are emerging as novel models to explore gene expression in CVDs[12,13], morevover, there is an increasing interest in exploring the klotho gene expression in PBMCs. The present study aimed at evaluating the expression of klotho and antioxidative enzymes, Mn-SOD and catalase, in PBMCs of patients with essential hypertension as compared to normotensive healthy individuals. The circulating levels of soluble α-klotho were also evaluated in the two study groups.

Material & Methods

Study subjects: The experimental work of this case–control study was conducted at the Department of Biochemistry, Sir H.N. Medical Research Society, Sir H.N. Hospital and Research Centre, Mumbai, between July 2015 and June 2018. The study protocol was approved by the Institutional Ethics Committee. Ninety-nine patients diagnosed with essential hypertension [systolic blood pressure (SBP)/ diastolic blood pressure (DBP) ≥140/90 mmHg] and 103 age- and body mass index (BMI)-matched healthy normotensive (SBP/DBP ≤120/80 mmHg) controls were recruited for the study. Recruitment of hypertensive individuals was in accordance with the Seventh Joint National Committee report guidelines[14]. Newly diagnosed individuals (age >18 yr) with persistent high BP and normal fasting blood glucose were enrolled. These patients were monitored (twice daily) for BP and referred to a consultant immediately if SBP/DBP was >160/100 mmHg and after monitoring for eight days if SBP/DBP was <160/100 mmHg, prior to recruitment. On confirmation of essential hypertension, these individuals were recruited under

hypertension group after obtaining written informed consent. Detailed information regarding demographic status, clinical and family history and medication was obtained. Individuals detected with high BP, were not on any medication and their blood sample collected on the day of the health check-up was used for analyzing all the study parameters. The normotensive individuals were recruited as controls only if the measurements for fasting blood glucose, lipid profile, serum transaminases, blood urea nitrogen (BUN) and creatinine levels were within the normal range. The recruited controls did not show clinical symptoms or history of any other organic disease.

Biochemical and Experimental analysis: Fasting venous blood samples were collected in plain and K_2-EDTA (dipotassium ethylenediaminetetraacetic acid) vacutainers. Serum was used to analyze routine biochemical parameters, and the remaining sample was stored at −80°C till its further use to detect soluble α-klotho and 8-iso-prostaglandin F2α levels. The latter was measured to analyze oxidative stress levels in the sera of hypertensive patients and controls. Routine biochemical investigations (lipid profile, renal profile and liver profile tests) were performed for both the groups, and normotensive healthy individuals with test levels beyond the normal range were excluded from control group. Enzyme-linked immunosorbent assay was carried out to estimate soluble α-klotho (Immuno-Biological Laboratories Co., Ltd., Hamburg, Germany) and 8-iso-prostaglandin F2α (Cell Biolabs, Inc., San Diego, CA, USA) in serum samples.

RNA extraction from PBMCs and complementary DNA synthesis: PBMCs were isolated from K_2-EDTA anticoagulated blood. Ficoll–Histopaque®-1077 (Sigma-Aldrich, St Louis. MO, USA) gradient separation method was used for isolation. RNA was extracted from PBMCs by NucleoSpin RNA isolation kit (Macherey-Nagel, Düren, Germany). The quality and quantity of RNA were assessed using agarose gel electrophoresis and Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA), respectively. One microgram RNA was reverse transcribed to complementary DNA (cDNA) for each sample using The PrimeScript 1st strand cDNA Synthesis Kit (Takara Bio USA, Inc., CA, USA) as per the manufacturer's instructions.

Gene expression studies: The gene expression was evaluated using quantitative real-time PCR (qRT-PCR) in StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using TaqMan gene expression assays for klotho (Hs00183100_m1), Mn-SOD (Hs00167309_m1) and catalase (Hs00156308_m1). The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Hs03929097_g1) was used as an internal control. Relative gene expression was calculated according to the comparative C_t method ($2^{-\Delta\Delta Ct}$)[15]. All experiments were performed in duplicates according to the manufacturer's protocol.

Statistical analysis: The results were expressed as frequency and percentage and mean ± standard deviation for parametric variables and median with interquartile ($25^{th}/75^{th}$) ranges for non-parametric variables. Student's unpaired *t*-test and Mann–Whitney U-test were used to determine the significance of differences between the two study groups for parametric and non-parametric variables, respectively. Correlations were evaluated by Spearman's rank correlation test. For all tests, $P<0.05$ was considered statistically significant. Analyses were performed using statistical software IBM SPSS version 21.0 (IBM Corp., Armonk, NY, USA). Although all 103 controls were gender, age, and BMI matched to the patients, those of the same gender, age, and BMI were grouped and matched against a single control for expression studies. Therefore, all correlations between the groups conformed to a total of 99 patients and corresponding 44 controls.

Results

Demographic and clinical characteristics: The baseline demographic characteristics of patients and controls are depicted in Table I. The comparison of lipid profile is given in Table II. The trend of increase or decrease as well as significance observed between hypertensives and 103 controls or 44 controls out of the 103 considered for gene expression study, remained the same for demographics as well as lipid profile. The levels of total cholesterol, triglycerides and very-low density lipoprotein (VLDL) cholesterol were significantly high as compared to controls. The median levels ($25^{th}/75^{th}$ quartiles) of 8-iso-prostaglandin F2α levels were significantly higher (P=0.001) in the sera of patients (1557 pg/ml, 643/4216) as compared to controls (271 pg/ml, 186/452). Similar trend was also

Table I. Comparison of demographic parameters between patient and control groups

Parameters	Total recruited individuals		Included for gene expression analysis from total recruited individuals	
	Controls (n=103)	Patients (n=99)	Controls (n=44)	Patients (n=99)
Age (yr)	47±7.3	47.3±6.5	47.1±7.1	47.3±6.5
Male/female	79/24	92/7	36/8	92/7
BMI (kg/m^2)	26.1±3.8	26.6±3.3	26.7±3.9	26.6±3.3
SBP (mmHg)	124.5±11.6	165.4±16.2***	126.5±10.6	165.4±16.2***
DBP (mmHg)	78.8±7.5	102.4±8.4***	79.4±7.4	102.4±8.4***

***P<0.001. Values are expressed as mean±SD. BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; SD, standard deviation

Table II. Comparison of lipid profile between patient and control groups

Parameters	Total recruited individuals			Included for gene expression analysis from total recruited individuals		
	Controls (n=103)	Patients (n=99)	Increase (%)	Controls (n=44)	Patients (n=99)	Increase (%)
TC (mg/dl)	171.3±30.7	184.5±36.1	7.7 (0.004)	163.1±29.7	184.5±36.1	13.1 (0.001)
HDL-C (mg/dl)	44.7±13.9	47.1±18.4	5.4 (0.589)	45.1±14.4	47.1±18.4	4.4 (0.936)
TC/HDL-C	4.1±1.3	4.3±1.4	4.9 (0.229)	3.9±1.2	4.3±1.4	10.2 (0.051)
Triglycerides (mg/dl)	105.2±41.4	140.3±80.7	33.4 (0.001)	101.0±41.6	140.3±80.7	38.9 (0.006)
VLDL-C (mg/dl)	21.0±8.3	28.1±16.1	33.8 (0.001)	20.2±8.3	28.1±16.1	39.1 (0.006)
LDL-C (mg/dl)	105.6±28.1	109.3±31.9	3.5 (0.126)	97.8±26.5	109.3±31.9	11.7 (0.022)
LDL-C/HDL-C	2.6±1.1	2.6±1.1	0	2.4±0.9	2.6±1.1	8.3 (0.11)

Values are expressed as mean±SD. TC, total cholesterol; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; VLDL-C, very low-density lipoprotein-cholesterol

observed for hypertensive patients with respect to 44 controls (253 pg/ml, 181/398).

Soluble α-klotho levels: The levels of soluble α-klotho in the sera of hypertensive patients were significantly lower (27%, P=0.001) as compared to controls (Fig. 1). On comparing the levels between patients and 44 controls considered for gene expression analysis, same trend was observed (28.7% decrease, P=0.001). Soluble α-klotho levels showed a positive correlation with high-density lipoprotein-cholesterol (HDL-C) (rs=0.349, P=0.015) and an inverse correlation with serum triglycerides (rs= −0.430, P=0.002) in control group. In the patient group, soluble α-klotho levels were negatively correlated with BMI (rs= −0.288, P=0.004).

Gene expression studies: The relative gene expression of klotho, Mn-SOD and catalase in hypertensive patients is depicted in Fig. 2. As compared to controls, Klotho gene expression was found to be 3-fold lower in hypertensive patients. Based on ΔCt values of klotho as well, a similar trend was observed (P=0.001). Similarly, mRNA levels of catalase were 1.25-fold lower in patients and a similar trend was observed based on ΔCt values (P=0.001) as compared to controls. Although the median of Mn-SOD gene expression was low based on relative gene expression and ΔCt values as compared to controls, it was not significant (P=0.055).

Correlation studies for gene expression: Based on ΔCt values, the correlations of gene expression with different parameters were analyzed. Catalase gene expression showed a positive correlation with that of Mn-SOD in patient (rs=0.448, P=0.001) as well as control (rs=0.547, P=0.001) groups. In patient group, the gene expression of klotho showed a significant positive correlation with that of catalase (rs=0.498, P=0.001), but not Mn-SOD (rs=0.155, P=0.126). With respect to the lipid profile, a positive correlation was observed between HDL-C and the relative gene expression of klotho (rs=0.296, P=0.003) and Mn-SOD (rs=0.260, P=0.009).

Discussion

Significantly low soluble α-klotho levels as well as gene expression of klotho and catalase in hypertension were the major finding of the present study. In patient group, the gene expression of klotho showed a significant positive correlation with that of catalase. In addition, soluble α-klotho levels showed a positive correlation with HDL-C and an inverse correlation with triglycerides in controls and with BMI in patients.

The measurement of 8-iso-prostaglandin F2α, the most abundant F2-isoprostane produced *in vivo*, is one of the most sensitive and reliable markers for oxidative stress in animal models and human diseases including essential hypertension[16]. The significantly high 8-iso-prostaglandin F2α levels in hypertensive patients substantiate the role of oxidative stress in the disease.

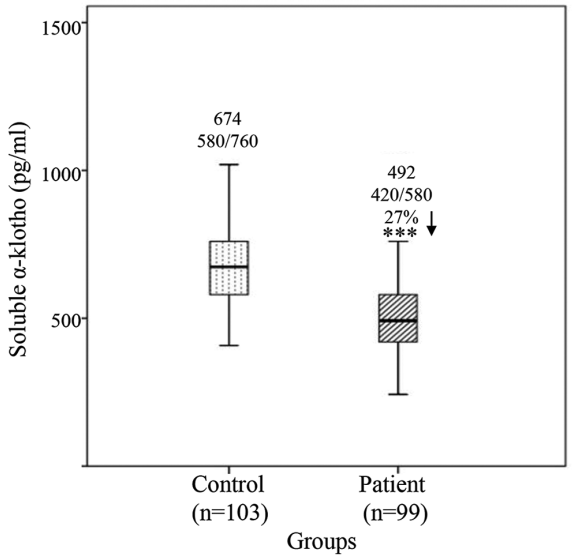

Fig. 1. Soluble α-klotho levels in patient and control groups. ***P<0.001.

The significant association of soluble α-klotho levels in the current study with HDL-C and triglycerides, supports studies where reduced circulating α-klotho levels have been associated with the presence and severity of coronary artery disease[17] and CVD[18] (coronary heart disease, heart failure, stroke and peripheral artery disease). Kitagawa *et al*[19] have found serum soluble klotho to be an independent marker of arterial stiffness in Japanese patients with chronic kidney disease. Su and Yang[20] have suggested that reduction in serum α-klotho levels may be responsible for systolic or elderly hypertension in Chinese population. Martín-Núñez *et al*[21] have found lower serum concentrations of klotho in patients with atherosclerotic vascular disease. The present study also reports depleted soluble α-klotho levels in essential hypertension patients.

In mouse model of hypertensive disease, Tang *et al*[22] have reported significantly decreased klotho gene and protein expression in spontaneously hypertensive rats (SHR) as compared to the control Wistar-Kyoto (WKY) rats. Zhou *et al*[23] have reported similar finding in klotho mutant mice, wherein the deficiency of klotho caused significant and persistent elevation of SBP, and thus suggested it to be essential for the maintenance of normal BP. In humans, Xia *et al*[24] have also found lower gene expression of klotho in PBMCs of atherosclerotic patients as compared to healthy controls, whereas Martín-Núñez *et al*[21] have reported lower vascular expression of klotho gene in atherosclerotic disease.

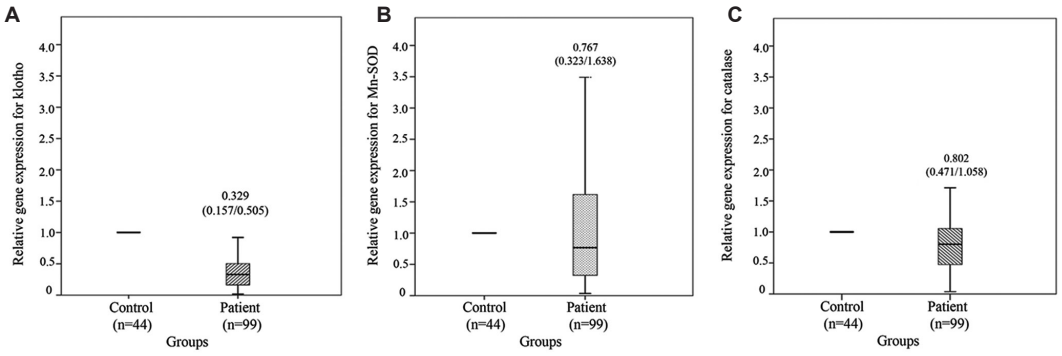

Fig. 2. (**A**) Relative gene expression of klotho – values are expressed as median with interquartile (25^{th}/75^{th}) ranges. (**B**) Relative gene expression of manganese superoxide dismutase (Mn-SOD)– values are expressed as median with interquartile (25^{th}/75^{th}) ranges. (**C**) Relative gene expression of catalase, values are expressed as median with interquartile (25^{th}/75^{th}) ranges.

The present study is the first to report a three-fold lower klotho gene expression in PBMCs of hypertensive patients as compared to age-, gender- and BMI-matched controls. The present study demonstrated a significant fall in catalase gene expression akin to that of klotho. Lower activity of catalase has also been reported in the aorta of high BP mouse model by Uddin *et al*[25], similar to findings in the present study. However, contrary to this, Godin *et al*[26], have reported that overexpression of catalase in angiotensinogen-overexpressing transgenic mice was associated with the prevention of hypertension. Dieterich *et al*[27] have also reported increased catalase gene expression, while SOD and glutathione peroxidase gene expression remained unchanged in human end-stage heart failure. They further suggested that this increase in catalase expression could be a compensatory mechanism in response to increased oxidative stress observed in the disease.

In the present study, the expression of Mn-SOD was unchanged in the patient group as compared to controls. Similar to our findings, Tang *et al*[22] have reported that Mn-SOD activity did not differ in SHR group, age-matched WKY rat group (control) and angiotensin-converting enzyme inhibitor-treated groups. Yamamoto *et al*[3] have proposed that klotho confers resistance to oxidative stress by inducing the expression of Mn-SOD via inhibition of insulin/IGF-1 signalling pathway. Their study reported that the gene expression of Mn-SOD positively correlated with circulating klotho levels in cultured cells, Klotho-deficient wild-type and klotho-overexpressing mice. However, this relation was not observed in the present study population. The complex nature of a biological system indicates that there may be factors, other than the insulin/IGF-1 signalling pathway, which may be responsible for regulating the expression of Mn-SOD for survival. One such mechanism, documented by Bai *et al*[28], is an increase in superoxide anions that leads to a complementary increase in the expression of Mn-SOD, as demonstrated in the brain of rats with neurogenic hypertension. This may also explain the present study findings, wherein essential hypertension patients showed no significant difference in the expression of Mn-SOD gene as compared to healthy controls in spite of lower gene expression and serum levels of klotho.

In the light of the relationship between klotho and catalase observed in the present study, and the regulatory role of klotho in insulin/IGF-1 signalling, klotho appears to have an influence on the gene expression of catalase. Thus, the application of klotho against oxidative stress-related diseases, such as hypertension, assumes great significance. To our knowledge, this is the first study to evaluate the gene expression of klotho and antioxidant enzymes Mn-SOD and catalase in PBMCs of newly diagnosed human hypertensive patients. The limitation of the present study is the fewer matched controls included for expression studies against hypertensives and fewer females enrolled in the study. However, the fact that both gene expression and soluble klotho levels were significantly lowered in hypertensive patients and a strong association with catalase suggests a role for klotho in essential hypertension.

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***Conflicts of Interest*:** None.

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