

TITLE PAGE

α-KLOTTHO EXPRESSISON IN HUMAN TISSUES

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ABSTRACT

Context: α -Klotho has emerged as a powerful regulator of the aging process. To-date, the expression profile of α -Klotho in human tissues is unknown and its existence in some human tissue types is subject to much controversy. **Objective:** This is the first study to characterize system-wide tissue expression of transmembrane α -Klotho in humans. We have employed next generation targeted proteomic analysis using Parallel Reaction Monitoring (PRM) in parallel with conventional antibody-based methods to determine the expression and spatial distribution of human α -Klotho expression in health. **Results:** The distribution of α -Klotho in human tissues from various organ systems, including arterial, epithelial, endocrine, reproductive and neuronal tissues was first identified by immunohistochemistry. Kidney tissues showed strong α -Klotho expression, while liver did not reveal a detectable signal. These results were next confirmed by western blotting of both whole tissues and primary cells. To validate our antibody-based results, α -Klotho expressing tissues were subjected to PRM mass spectrometry identifying peptides specific for the full length, transmembrane α -Klotho isoform. **Conclusions:** The data presented confirms α -Klotho expression in the kidney tubule and in artery, and provides evidence of α -Klotho expression across organ systems and cell-types that have not previously been described in humans.

Abstract Word Count: 195

INTRODUCTION

The identification of the novel anti-aging protein α -Klotho in 1997 (1) first challenged the long-held paradigm of aging as a passive, inevitable process of deteriorating organ function and declining health. Since α -Klotho knock-out mice exhibited a shortened lifespan and transgenic mice that overexpress α -Klotho live 30% longer (2), aging has instead emerged as a regulated and potentially modifiable process. α -Klotho deficiency results in a variety of features characteristic of mammalian aging including organ atrophy, infertility, vascular calcification, atherosclerosis, osteomalacia, osteoporosis, peripheral insulin sensitivity, metabolic derangements, and cerebral changes (1), all of which occur in “normal” aging.

In humans, emerging data indicate that aging is also modifiable, and subject to regulation by complex genomic, proteomic and environmental interactions (3). Premature or accelerated aging occurs in a number of human genetic disorders such as Werner syndrome and Hutchinson-Gilford Progeria Syndrome (HGPS), conditions that recapitulate many or all of the features of normal aging. Further, features of aging and a reduced life span accompany a number of chronic disease states in humans, including chronic kidney disease (CKD), cancer, diabetes, HIV and inflammatory arthropathies (4). Only one case of a human α -Klotho mutation has been described to date, but polymorphisms in the α -Klotho gene (KL-VS variant) are associated with normal human aging (5). Given the implications of these discoveries for human health, there has been much interest in molecules such as α -Klotho, as potential longevity-modulating therapeutic targets.

α -Klotho has two known human isoforms: the full length protein is a 130kDa, 1012 amino acid (AA), single pass transmembrane protein which contains a signal sequence, two homologous domains (denoted KL1 and KL2), a transmembrane domain and a short cytoplasmic tail. The second isoform (62kDa, 549AA) arises from alternative splicing and is a secreted soluble protein (hereafter referred to as ^S α -Klotho) which contains only the SS domain and KL1, with the terminal 15 residues replaced by SQLTKPISSLTKPYH (Figure 1A)(5). Although the secreted isoform predominates and circulates in plasma, its function is largely unknown. In contrast, full-length α -Klotho is involved in aging and in phosphate homeostasis. α -Klotho functions as a co-receptor with the FGF receptor for the phosphatonin FGF23. Additional pleiotropic functions have been ascribed to tissue α -Klotho including protection against oxidative stress (6), inhibition of apoptosis (7) and fibrogenesis (8), promotion of angiogenesis and vascularization (9), vasculo-protective properties (10) (11) and regulation of stem cell proliferation through modulation of Wnt signaling (12), all of which may protect against ageing.

α -Klotho protein expression has been found in mice, rats and humans predominantly in the renal distal convoluted tubular (DTC) cells (1) and to a lesser extent in proximal convoluted tubular epithelial cells (PTEC) (13) and parathyroid gland (14). We recently reported α -Klotho expression in human vasculature (11). While α -Klotho expression has been described in other rodent tissue-types, including the pituitary gland, pancreas, ovary, testis, placenta, choroid plexus of the brain (1) and most recently in rat aorta (15), no study has to-date systematically investigated α -Klotho expression in humans. Expression in some tissues, particularly the cardiovascular

system has been controversial (16), partly due to limitations and variability of antibody-based techniques employed to-date which may not distinguish between α -Klotho and $^S\alpha$ -Klotho. Given its implications for human health, data on the expression of α -Klotho in human tissues are urgently needed.

To overcome the above limitations, we employed state-of-the-art targeted proteomic analysis using Parallel Reaction Monitoring (PRM) in parallel with conventional antibody-based methods to determine the distribution and precise nature of human α -Klotho expression in health.

METHODS

Tissue Sample and Preparation

Cell lysates were obtained from commercially available primary cell cultures (SciencCell, USA; Human Epidermal Keratinocyte Lysate-adult (Product Code: HEKL-a, Catalog No: 2116), Human Prostate Epithelial Cell Lysate (Product Code: HPrEpiCL, Catalog No: 4416), Human Mammary Epithelial Cell Lysate (Product Code: HMEpiCL, Catalog No: 7616), Human Renal Proximal Tubular Epithelial Cell Lysate (Product Code: HRPTEpiCL, Catalog No: 4106), Human Neuron Lysate (Product Code: HNL, Catalog No: 1526). Human hepatocytes (HepG2, SIGMA) were kindly gifted by Dr Graeme Alexander, University of Cambridge. Human aortic smooth muscle cells (HA-SMCs; Caltag (Sciencell) catalogue number SC-6110).

Human tissues for immunohistochemistry and tissue lysate preparation were obtained with local ethical approval and informed written consent from four sources:

Surgical specimens from the human tissue bank, Department of Pathology, University Hospital Coventry and Warwickshire NHS Trust, UK (Ethics approval 13WM\0072), artery and kidney tissue from Warwick Medical School, UK (Ethics approval 05/Q2802/26 and 10/H12111/36), aorta from the Department of Medicine, University of Cambridge (Ethics approval 05/MRE04/7), and neuronal tissue was kindly donated by the New York Brain Bank (NYBB) at Columbia University, New York. Human parathyroid whole cell lysate was obtained from Abcam, MA, USA (Catalogue Number: ab29792).

Immunohistochemistry for α -Klotho Protein

The rabbit polyclonal α -Klotho antibody (Catalog No. Ab69208 and Ab181373; Abcam,) and rabbit polyclonal isotype antibody (Catalog No. Ab27478) were used at a concentration of 1:100 to 1:250 for immunohistochemistry (IHC) staining and control experiments on formalin fixed and paraffin embedded, glass mounted sections. Please see supplementary methods for details of IHC protocol.

Assessment of α -Klotho distribution along the nephron was done morphologically. Determination was limited to proximal tubular and distal tubular (distal tubule and collecting duct). Consecutive section staining with hematoxylin and eosin was also used.

Tissue Preparation for Western Blot and Mass Spectrometry

Human tissues were homogenised in liquid nitrogen and resuspended in standard RIPA buffer supplemented with protease inhibitors cocktail (Sigma) and 1% Triton-X-100. The lysates were clarified by microcentrifugation at 10,000g for 10 min at 4°C and protein concentration assayed by Lowry assay. As control, we used recombinant human full-length α -Klotho protein (rh- α -Klotho) (R&D Systems, 5334-KL-025).

Western Blot for α -Klotho Protein

Aliquots of cell lysates containing 20 μ g protein were separated by SDS-PAGE and Western blotted with anti-human α -Klotho (Catalog No. Ab69208; Abcam, MA, USA) at a concentration of 1:1000. 10 μ g of rh- α -Klotho was used as control (see supplementary material). The expected protein size for the full length α -Klotho protein was 116kDa.

Sample Preparation for Mass Spectrometry

All protein samples (50 μ g) were fractionated on pre-cast SDS PAGE gels (Biorad, 456-1083, 4-15%, 10 well) and stained with Coomassie blue stain. All samples were either run with an empty lane in between or on separate gels to avoid sample-to-sample contamination.

In order to restrict analyses to the full-length protein, only the gel band between 75kDa and 150kDa was resected for analysis, thus excluding $\text{S}\alpha$ -Klotho (Figure 1A). Gel fractions were macerated with a sterile blade and subjected to in-gel digestion. Gel fractions were de-stained by 3 washes with 80 μ l of 50% acetonitrile (ACN)/50mM ammonium hydrogen carbonate, and washed with 100% ACN. The alkylation step was

omitted, given the absence of Cysteine residues from target α -Klotho peptides. Tryptic digestion was carried out overnight at 37°C with 60 μ l of trypsin (Promega, sequencing grade modified, V511A) in 50mM ammonium hydrogen carbonate (0.005 μ g/ μ l). This process yielded 25 μ l, of which 5 μ l (1 μ l for rh- α -Klotho) was subjected to nano-LC ESI-MS/MS and Parallel Reaction Monitoring (PRM) analysis in an Orbitrap nano-ESI Q-Exactive mass spectrometer (Thermo Scientific), coupled to a nanoLC (Dionex Ultimate 3000 UHPLC).

Data Dependent Acquisition (DDA) and Parallel Reaction Monitoring (PRM)

A top10 DDA MS/MS analysis was performed first to assess sample quality. Samples were trapped on a 100 μ m \times 2cm, C18, 5 μ m, 100Å trapping column (Acclaim PepMap 100) in ulPickUp Injection mode at 4 μ L/min flow rate for 10 minutes. Samples were then loaded on a RSLC, 75 μ m \times 50cm, nanoViper, C18, 3 μ m, 100Å column (Acclaim, PepMap) retrofitted to an EASY-Spray source with a flow rate of 350nl/min (buffer A: HPLC H₂O, 0.1% formic acid, buffer B: 100% ACN, 0.1% formic acid. 60 minutes gradient; 0-5 min: 5% buffer B, 5-45 min: 5->56% buffer B, 45.1-50 min: 56%->95% buffer B, 50.1-60 min: 5% buffer B). Peptides were transferred to the gaseous phase with positive ion electrospray ionization at 1.8kV. In DDA the top10 precursors were acquired between 400 and 1600 m/z with a 2Th (Thomson) selection window, dynamic exclusion of 30 seconds, normalised collision energy (NCE) of 25 and resolution of 70,000. For PRM, precursors were targeted in a 2Th selection window around the m/z of interest. Precursors were fragmented in HCD mode with NCE energy dependent on

the target peptide. MS1 was performed at a 70,000 resolution, an AGC target of $3e^6$ and a maximum C-trap fill time of 200ms; MS/MS was performed at 35,000 resolution, an AGC target of $5e^4$ and a maximum C-trap fill time of 100ms. Spectra were analysed using Skyline and/or MASCOT, with manual validation. A peptide was considered present when at least one fragment ion was detected by Skyline, in addition to the identification of a clear isotope pattern of the fragment ion in the raw data, at the expected retention time for the parent peptide.

Data processing and statistical analysis

Skyline analysis utilised .raw Thermo files. For MASCOT searches, .mgf files were generated from .raw Thermo files using MSconvert, uploaded in MASCOT and searched against a human database (Uniprot 2013) using the following settings: carbamidomethyl as fixed modification, methionine oxidation as variable modification; 25 ppm peptide tolerance, 0.8 Da MS/MS tolerance; maximum of 2 missed cleavages, peptide selection charge of +2, +3 or +4 and selection of a decoy database.

RESULTS

Spatial distribution of α -Klotho protein in human tissues

Consistent with previous reports (13), we confirmed expression of α -Klotho in both the proximal and distal tubular epithelial cells of the kidney by IHC (Figure 2A, 2B). In subsequent experiments, kidney tissue served as a positive control.

α -Klotho is expressed throughout the arterial tree

Expression of α -Klotho was identified in smooth muscle cells throughout the arterial tree (Figure 2C-H). In proximal vessels, α -Klotho was present in elastic (aorta, Figure 2C) and muscular artery (renal, Figure 2D). Aorta α -Klotho was expressed to a similar level in intima and media layer (Figure 2: C1) but much stronger in vasa vasorum of the adventitia (Figure 2: C2). More distally, α -Klotho was also identified in smaller arteries within vascular beds in kidney, thyroid, prostate and testis (Figure 2E-H). We next sought α -Klotho in the liver, but α -Klotho could not be identified in hepatocytes (Figure 2I).

 α -Klotho is present in epithelia

We next examined α -Klotho protein expression in epithelial tissues. α -Klotho was highly expressed throughout the epidermis (Figure 3A) and skin appendages such as hair follicles and sebaceous glands (Figure 3B). In addition, epithelial cells of both the small bowel (jejunum, Figure 3C) and colon (Figure 3D) also demonstrated strong expression of α -Klotho.

In the male reproductive system, α -Klotho was expressed in sertoli cells (Figure 3E, 3F) of the testis, as well as epithelial cells of the prostate gland (Figure 3G). In the female reproductive system, α -Klotho expression was present in mammary epithelium (Figure 3H), endometrium of the uterus (Figure 3I) and salpynx (Figure 3K).

 α -Klotho is present in endocrine tissues

Examination of endocrine tissue revealed α -Klotho expression in follicular epithelial cells of the thyroid (Figure 4A), in the insulin producing islet cells of the pancreas (Figure 4B), in medullary cells of the adrenal gland (Figure 4C) and testosterone-producing Leydig cells (Figure 3F) of the testis.

α -Klotho is present in neuronal cells

We next examined α -Klotho expression in neural tissues. Expression was found in neuronal cells of the cerebral cortex (Figure 4D). In the cerebellum, α -Klotho was expressed primarily in purkinje cells between the molecular and granular layer (Figure 4E). In the spinal cord, α -Klotho was present in the motor neurons in the grey matter of the ventral horn (Figure 4F). In the myenteric plexus, α -Klotho protein was found in ganglial neuronal cell bodies (Figure 4E).

Analysis of α -Klotho expression by western blotting

In order to confirm the presence and molecular weight of α -Klotho for those tissues where it was identified by immunohistochemistry experiments, we next performed western blotting on both human tissue lysates and, where available, on human primary cells. For these experiments, we used full-length rh- α -Klotho protein as positive control. Western blotting demonstrated the presence of full-length (116kDa) α -Klotho in lysates from human renal PTEC, HA-SMCs, neuronal cells, keratinocytes and mammary epithelial cells (Figure 1C). Consistent with IHC (Figure 2I), α -Klotho could not be identified in hepatocytes. In human tissue lysates, full-length α -Klotho was identified at

116kDa in kidney, renal artery, aorta, epigastric artery, cerebral cortex, spinal cord and cerebellum (Figure 1D).

Mass spectrometry analysis of human samples

The data described above provide strong support for the presence of full-length α -Klotho in the tissues shown. However, since these data are antibody-dependent, we next sought to corroborate these findings using a targeted proteomics approach, PRM. PRM is able to robustly and reproducibly identify a peptide signature for a protein of interest with precision and specificity and, importantly, can yield isoform-specific data. This methodology is therefore able to distinguish $^S\alpha$ -Klotho from α -Klotho. To further mitigate against the potential for contamination with $^S\alpha$ -Klotho, samples were first fractionated by 1 dimensional (1D) gel electrophoresis. Only gel fragments between 75 and 150kDa were excised for analysis, thus excluding $^S\alpha$ -Klotho isoform (62kDa). Full-length transmembrane rh- α -Klotho protein served as control and allowed generation of a reference PRM signature.

First, spectra for sixteen candidate peptides unique to α -Klotho were generated by subjecting purified rh- α -Klotho to tandem mass spectrometry (LC-MS/MS) (supplementary Table 1). We next sought the presence of these peptide spectra in positive control samples (human kidney and PTEC lysates) by PRM, aiming to identify the most reliable spectra and seeking in particular peptide spectra present in α -Klotho but absent in $^S\alpha$ -Klotho. The most robust signatures were yielded by GLFYVDFLSQDK (α -Klotho and $^S\alpha$ -Klotho) and QGAWENPYTALAFAEYAR, NNFLLPYFTEDEK, VYYMQNYINEALK, LWITMNEPYTR (all absent from $^S\alpha$ -Klotho). Given that

GLFYVDFLSQDK is also present in ^S α -Klotho, it does not distinguish between isoforms; in contrast, QGAWENPYTALAFAYAR, NNFLLPYFTEDEK, VYYMQNYINEALK and LWITMNEPYTR are not present in ^S α -Klotho and can only be identified if full-length α -Klotho is present. We therefore restricted subsequent PRM analyses to GLFYVDFLSQDK and the four full-length-specific peptides described above. Figures 5A to 5H show representative spectra for GLFYVDFLSQDK and LWITMNEPYTR from rh- α -Klotho, human kidney lysate and human PTEC lysate.

We next sought the presence of these peptide signatures in a variety of human tissue and cell lysates. α -Klotho extracellular domain peptide GLFYVDFLSQDK was present in whole kidney lysate, kidney cortex, kidney medulla, proximal tubular epithelial cells, parathyroid, pancreas, keratinocytes, mammary epithelial cells, prostate epithelial cells, neuronal cells, cerebral cortex, cerebellum and artery (aorta, renal and epigastric artery) (Table 1A and B). The spectrum for GLFYVDFLSQDK in human renal artery is shown (Figure 5D) as representative example.

Peptides present only in the full-length α -Klotho and absent from ^S α -Klotho were identified in whole kidney lysate, kidney cortex, kidney medulla, proximal tubular epithelial cells, parathyroid, pancreas, keratinocytes, neuronal cells, cerebral cortex, cerebellum and artery (aorta, renal and epigastric artery), indicating the presence of full-length α -Klotho in these tissues (Table 1A and B). Representative spectra for LWITMNEPYTR in human renal artery and human neuronal cells are shown in figure 5G and 5H. Detailed peptide evidence for full-length α -Klotho in each sample is shown in supplementary table 2. All proteomic data have been deposited with the

ProteomeXchange consortium open access repository, and can be accessed at <http://proteomecentral.proteomexchange.org>.

DISCUSSION

We report the identification of full-length α -Klotho protein in a wide variety of human tissues including the arterial tree, epithelia, endocrine and neuronal tissues. Previously, α -Klotho protein expression in humans had only been described in the kidney (13) and parathyroid glands (14), along with our own report of α -Klotho in human muscular artery (11). Our findings are consistent with data from rodent studies demonstrating α -Klotho expression in pituitary gland, pancreas, ovary, testis, placenta and choroid plexus of the brain (1), and suggest tissue-specific roles for α -Klotho at sites not involved in phosphate transport signalling.

The wide tissue distribution of α -Klotho is consistent with its known role in aging. Rodent models of α -Klotho deficiency demonstrate reduced life span with a wide range of tissue phenotypes including gonadal failure, arteriosclerosis, emphysema, impaired cognition, hearing loss, vascular calcification, cardiac hypertrophy, osteopaenia, and atrophy of skin, adipose tissue, thymus and skeletal muscle (1). In humans α -Klotho deficiency or functional variants of α -Klotho are associated with the development of vascular calcification (11,17), atherosclerosis (18), diabetes (19), hypertension (20), CKD (21), osteoporosis (22), anaemia (23) as well as various cancers such as hepatocellular carcinoma (24), breast cancer (25) gastric cancer (26) and renal cell carcinoma (27).

Our data show full-length α -Klotho in elastic and muscular artery, consistent with our previous report (11). Other investigators have failed to demonstrate the presence of α -Klotho in vascular tissue (16). This apparent conflict is most likely due to differences in sample preparation, experimental conditions and antibodies employed, and highlights the difficulties inherent in antibody-based methods. Further, given the hydrophobic nature of full-length α -Klotho, its isolation from experimental samples is not straightforward. Here, data derived from PRM experiments demonstrate the presence of full-length α -Klotho in human artery. Given that α -Klotho deficiency results in a striking arterial phenotype (1) and is associated with an increased risk of coronary artery disease (28) and stroke (29), our data confirming the presence of α -Klotho in the human artery tree is of high clinical importance.

Expression of α -Klotho in human skin has not previously been reported. α -Klotho deficiency results in skin atrophy, a hallmark of the ageing process. The mechanisms by which α -Klotho maintains healthy skin are not known. We also identified α -Klotho in other epithelia including the intestine. Though it is possible that α -Klotho may similarly maintain intestinal epithelial health, it may equally be involved in phosphate and calcium transport and homeostasis. Indeed, α -Klotho may regulate expression of intestinal TRPV5 and TRPV6 as observed in kidney (30). Evidence for this has been shown in animal models examining the role of Klotho-FGF-23 in regulating phosphate concentration via NaPi-II transporters in intestinal epithelial cells (31) and renal distal tubules (32).

We found strong expression of α -Klotho in thyroid follicular cells suggesting that it may be involved in regulation of thyroid hormone production. Given that clinical and

subclinical hypothyroidism increases in prevalence with increasing age, α -Klotho may be involved in regulating synthesis of thyroid hormone (33). This may similarly be true for testosterone synthesis from Leydig cells, which also declines with age. Indeed, Hsu and colleagues have proposed the existence of a testosterone - α -Klotho feedback loop (34). In our study, α -Klotho expression was also found in insulin producing islet cells of the pancreas. This finding assumes considerable significance given emerging evidence that demonstrates a role for α -Klotho in regulating the insulin/IGF-1 pathway, a highly conserved mechanism that itself impacts on lifespan (2,35). Finally, α -Klotho was also found in the adrenal medulla, the site of synthesis of catecholamines, hormones that regulate the immediate stress response, with a significant role in the cardiovascular system.

We identified α -Klotho at various sites throughout the central nervous system (CNS). Whereas previous reports have demonstrated α -Klotho in the rodent brain choroid plexus, purkinje cells of the cerebellum (36), and studies in humans confirm the presence of α -Klotho in cerebrospinal fluid in health, its CNS tissue distribution has not previously been described in humans (37,38). The functional significance of α -Klotho in the CNS remains largely unknown. However, reduced cerebrospinal fluid klotho was found to be associated with age, and the development of Alzheimer disease, in one small cohort of 70 patients (38). Intriguingly, one recent report by Dubal et al demonstrated that the KL-VS functional variant of the α -Klotho gene was associated with enhanced cognition in three independent cohorts of healthy older humans (39). These observations suggest that α -Klotho may modulate human learning and memory, and may be protective against degenerative disease within the CNS.

Although we identified α -Klotho in a wide variety of organs and tissues, it could not be identified in hepatocytes. This is consistent with a recent report by Chen and colleagues, showing that although α -Klotho is increasingly expressed with progressive dedifferentiation in hepatoma cells, normal liver was negative for α -Klotho staining on immunohistochemistry (40). In addition, hepatocytes (including HepG2 cells used in our experiments) did not show α -Klotho in Western blot experiments. Although α -Klotho appears to be absent, an analogous system is operational in hepatocytes: the α -Klotho homologue, β -klotho, is expressed in hepatocytes and adipose tissue where it appears to have a role in glucose metabolism. Similar to α -Klotho which forms a coreceptor with the FGF receptor for FGF23 in phosphate signalling, β -klotho complexes with FGF receptors in hepatocytes to form a coreceptor for FGF19 and FGF21 (41).

Our study has several important strengths. First, our report is the first to systematically evaluate α -Klotho expression in a wide range of human tissue samples and cell types. Second, we have confirmed our previous observation of α -Klotho in the vascular tree. Third, in parallel to IHC and Western blotting, we have employed state-of-the-art PRM to provide antibody-independent data confirming the presence of α -Klotho within samples. All mass spectrometry data are publically accessible. Furthermore, we were able to identify peptide signatures specific for isoform 1 of α -Klotho (Figure 1), allowing us to demonstrate with a high degree of certainty the presence of full-length transmembrane α -Klotho. This finding cannot be explained by tissue contamination with soluble exogenous α -Klotho.

Our findings should be interpreted against the limitations of our study. First, it has proven very challenging to liberate the hydrophobic full-length α -Klotho protein

sufficiently to allow detection. Although evidence of presence is robust, the absence of peptides derived exclusively from full-length α -Klotho (isoform 1) should be interpreted with caution, for example in the case of prostate and mammary epithelial cell lines (Table 1A). Secondly, any study of human tissues is limited by the availability of experimental material, and our panel of samples and cell lines was not exhaustive.

In summary, this is the first study to systematically characterise tissue expression of full-length α -Klotho in humans. Given the influence of α -Klotho on longevity and the impact of its deficiency on health and aging across multiple organ systems, our data provides an important first step towards elucidating the role of α -Klotho in health and disease.

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