

## Review Article

# $\alpha$ -klotho: a novel regulator in female reproductive outcomes and hormone-related cancer

Cuifang Fan<sup>1\*</sup>, Chang Tan<sup>2\*</sup>, Suqing Wang<sup>2,3</sup>

<sup>1</sup>Department of Obstetrics and Gynecology, Renmin Hospital, Wuhan University, China; <sup>2</sup>Department of Nutrition and Food Hygiene, School of Health Sciences, Wuhan University, China; <sup>3</sup>Hubei Provincial Key Laboratory for Applied Toxicology, Hubei Provincial Academy for Preventive Medicine, China. \*Equal contributors.

Received March 9, 2017; Accepted March 10, 2017; Epub May 15, 2017; Published May 30, 2017

**Abstract:**  $\alpha$ -klotho, as an anti-aging protein, has been involved in a wide range of biological functions, especially in calcium, phosphate and vitamin D metabolism. Recent studies suggested that  $\alpha$ -klotho plays important roles in female reproductive development, pregnancy, infant health and hormone related cancers. Effects of  $\alpha$ -klotho include anti-oxidation, promoter methylation, histone deacetylation and IGF, FGF, Wnt signaling pathways transduction. In this paper, we provide a seminal view on recent advances in understanding the behavior of  $\alpha$ -klotho in female reproductive health, primarily focusing on the gaps in current research that underline insights into its function and mechanism, and suggest a new avenue for systematic future research.

**Keywords:** Klotho, female reproductive, perinatal health, hormone related cancer

## Introduction

Klotho (KL) was firstly identified by Kuro-o *et al.* in 1997 as an aging suppressor gene in mice [1] and then recognized as  $\alpha$ -klotho to be distinguished from the other two members of the klotho protein family:  $\beta$ -klotho and  $\gamma$ -klotho (klph or LctI) [2-4]. KL deficient mice developed multiple premature aging syndromes, including shortened life span, skin atrophy, osteoporosis, infertility, and emphysema [1]. On the other side of spectrum, overexpression of KL not only rescued all of the macroscopic phenotypes, histological and blood analyses observed in KL deficient mice, but also restored thymus and genital organs into nearly normal, both males and females became fertile [1].

The KL gene is located on chromosome 13q12 in human with the size of 50 kb [1]. There are five exons and four introns in the coding region of KL gene in human and the gene transcribes mRNAs of 3,036 nucleotides [5]. Up to now, three types of  $\alpha$ -KL protein have been identified: the full-length transmembrane  $\alpha$ -klotho, soluble  $\alpha$ -klotho, and secreted  $\alpha$ -klotho [5]. The full-length KL is a single pass transmembrane

protein composed of 1,012 amino acids [5] containing extracellular and intracellular domains. The extracellular domain consists of two separate domains: KL1 and KL2, which are associated with functions of  $\alpha$ -klotho [5, 6]. The membrane-bound full-length  $\alpha$ -klotho protein can be cleaved by the membrane-anchored proteases, including ADAM 10, ADAM 17, and BACE1 [5, 7]. The truncated protein cleaved out from the full-length one is known as soluble  $\alpha$ -klotho which contains KL1 only or both KL1 and KL2, without intracellular domain. The secreted  $\alpha$ -KL is a protein of short chain with 549 amino acids generated from alternative RNA splicing [7], which contains an N-terminal signal peptide followed by the KL1 domain only [5]. Full-length  $\alpha$ -KL acts as a co-receptor of fibroblast growth factor 23 (FGF23) to regulate phosphate homeostasis [8]. The soluble  $\alpha$ -KL and secreted  $\alpha$ -KL found in blood, urine, and cerebrospinal fluid are also called circulating  $\alpha$ -klotho [9]. Circulating  $\alpha$ -klotho acts as a humoral factor with an enzymatic activity [7], involved in multiple biological processes, such as angiogenesis, energy metabolism, nitric oxide production, and antioxidant enzymes synthesis [7].

In humans,  $\alpha$ -klotho is predominantly expressed in kidneys, parathyroid gland, adipose tissue and choroid plexus. It's also distributed in prostate, small intestine, placenta and umbilical cord blood [7, 9-12]. In female reproductive system,  $\alpha$ -klotho is expressed in breast tissues, ovary, salpinx and uterus. During pregnancy, it is predominantly expressed in the placenta [1, 3, 7, 9, 12, 13], and located in the syncytiotrophoblast brush border, while much less in the cytotrophoblast [13].

Pregnant women have a higher plasma  $\alpha$ -klotho level than non-pregnant women [7], and plasma  $\alpha$ -klotho level increases with the increasing gestational age [7, 13]. In contrast, abnormal  $\alpha$ -klotho expression was observed in certain pregnancy complications, such as preeclampsia [14, 15] and hormonal related cancers, such as breast cancer, cervical cancer and epithelial ovarian cancer (EOC) [16-18]. Therefore, our review will focus on the roles of  $\alpha$ -klotho in female reproductive organs, pregnancy outcomes and hormonal related cancer in females.

### **$\alpha$ -klotho in female reproductive system**

Deficiency of  $\alpha$ -KL gene led to aging-like symptoms in female reproductive system [1].  $\alpha$ -KL deficient mice were not apparently different from wild-type ones at 12-day-old, but abnormal phenotypes in female reproductive system became apparent at 8-week old [19]. Their vaginas didn't open, uteruses and ovaries were atrophic, ovaries contained only primary or secondary follicles but no Graafian follicles or corpus lutea [1, 20, 21], and they were out of estrus cycles and became infertile [22]. Over expression of  $\alpha$ -KL in  $\alpha$ -KL deficient mice restored these aging-like impairments. In Kuro-o's study, exogenous expression of  $\alpha$ -KL made  $\alpha$ -KL deficient mice fertile. Meanwhile, their genital organs and cells were restored to almost normal status [1]. Shiraki-Iida *et al.* also observed restoration of ovaries and uteruses, as well as matured follicles after overexpression of  $\alpha$ -KL in  $\alpha$ -KL deficient mice by recombinant adenoviral vector [19]. Moreover, Masuda *et al.* fed  $\alpha$ -KL deficient mice with zinc to induce expression of  $\alpha$ -KL, these zinc-fed mice showed ovarian follicles at various maturation stages and corpora lutea, while impairments of untreated mice were not restored [21].

Toyama *et al.* revealed the mechanisms of female mice sterility brought by  $\alpha$ -KL deficiency,

and found LH receptor (LHR) and aromatase P450 were not expressed in the ovary, FSH and GnRH were both able to restore them in follicles of ovary. Reduced production of FSH and LH in pituitary gland and absence of estrus cycle were observed in  $\alpha$ -KL deficient mice, and FSH or GnRH administration promoted advanced maturation of ovaries and uterus. In addition, wild type mice transplanted with ovaries from  $\alpha$ -KL deficient ones were fertile [22]. These results suggest that female reproductive organs in  $\alpha$ -KL deficient mice are potentially functional; Infertility in  $\alpha$ -KL deficient females is mainly due to absence of proper stimulus by gonadotropins. However, the synergistic effect of  $\alpha$ -KL deficiency with gonadotropins on development of female reproductive system should not be ruled out.

From human observation, one prospective cohort study (n=633) showed that, during preimplantation, both oocytes maturation and fertilization rates after normal intracytoplasmic sperm injection were positively correlated with serum  $\alpha$ -KL concentrations [23]. Furthermore, multivariate logistic regression indicated that higher serum  $\alpha$ -klotho levels during preimplantation is closely associated with higher clinical pregnancy rates ( $P < 0.001$ ) after adjusting age, ethnicity, BMI, oocytes maturation, fertilization and serum 1,25(OH)<sub>2</sub>D level [23]. Taken together, the role of  $\alpha$ -KL in female reproductive function still need further investigation.

### **$\alpha$ -klotho and reproductive outcomes**

#### *$\alpha$ -klotho and maternal outcomes*

Maternal hypertensive disorder is a group of diseases including preeclampsia (PE), eclampsia, gestational hypertension and chronic hypertension [24], of which the most common type is PE [25]. PE occurs in about 2-8% of pregnancies and is one of the leading causes of maternal and perinatal morbidity and mortality worldwide [26].  $\alpha$ -KL is predominantly expressed in the placenta in both animal and human [1, 7, 13]. In pregnant women with PE,  $\alpha$ -KL expression in placenta is abnormal. Jing *et al.* found  $\alpha$ -KL protein expression in placenta detected by IHC was ranked as follows: Normal pregnancy (n=20) > gestational hypertension (n=10) > preeclampsia (n=10) > eclampsia (n=20). Giannubilo *et al.* observed that  $\alpha$ -KL mRNA and protein levels were both decreased in placentas of pregnant women with PE (n=12),

when compared with normal pregnancies [14]. Cecati M *et al.* noted lower expression of both full-length membrane  $\alpha$ -KL and secreted  $\alpha$ -KL in PE placentas (n=34) compared with normal counterparts [25]. Fan *et al.* also observed decreased expression of both mRNA and protein of  $\alpha$ -KL in PE placentas (n=19) [27]. However, Loichinger *et al.* did not find significant difference of  $\alpha$ -KL level between placentas of PE mothers and controls [13]. Cecati *et al.* observed that heterozygosity and homozygosity for -744delA mutation were significantly more common in the PE group (n=34) [25]. Nevertheless, small sample size makes it difficult to demonstrate a relationship between -744delA polymorphism and PE development.

Maternal plasma  $\alpha$ -KL level is also different in normal pregnancies from PE. However, existing results are conflicting. **Table 1** shows results of studies which explore  $\alpha$ -KL in PE. Miranda *et al.* found there was no significant difference in plasma levels of  $\alpha$ -KL between women with PE without small-for-gestational age (SGA) and normal pregnant women, and it stayed constant after controlling potential confounding variables such as gestational age at venipuncture, maternal age, nullparity, ethnicity, and tobacco use [7]. Loichinger *et al.* reported higher plasma levels of  $\alpha$ -KL in patients with PE compared to normal pregnancies [13]. On the contrary, Our lab observed significant lower  $\alpha$ -KL level in PE group compared with normal counterparts [27].

To be noted, the potential bias brought by the heterogeneity of sample set cannot be ignored. For example, plasma klotho level was reported to be associated with smoking [28]. Mothers who smoked were excluded in Loichinger's study [13], while in other studies, smoking was not included in the exclusion criteria [7, 27]. In addition, Miranda *et al.* compared PE patients not complicated by SGA with normal pregnant women [7]. SGA is a common complication of PE, the risk of SGA in PE patients is nearly four times higher than normal pregnancies [29]. Thus, the representativeness of PE patients not complicating SGA of PE population is questionable. It should be noted that, despite different distributions, plasma  $\alpha$ -klotho level in PE group shown by Miranda *et al.* is quite different from that shown by Loichinger *et al.* (median and range, 1177.3 (762.4-2013.4) pg/mL) vs

(mean and SD, 2019 $\pm$ 1320 pg/mL). Moreover, different samples of blood used by researchers (serum [27] vs plasma [7, 13]) make it difficult to determine whether or not the maternal blood level of  $\alpha$ -KL is higher in PE patients than that in normal pregnancies.

Nitric oxide (NO) production and oxidative stress regulated by  $\alpha$ -KL may be the key component in PE development. Small vessel spasm and vascular lesions due to ischemia are the main pathological changes in hypertensive disease of pregnancy. And NO plays an important role in this process [30].  $\alpha$ -KL may affect the production of NO. Animal studies revealed that angiogenesis, which is dependent on endothelium-derived NO, was damaged in  $\alpha$ -KL deficient mice [25]. Oxidative stress has relation to the pathology of placenta and it is possibly influenced by  $\alpha$ -KL. In mice, overexpression of soluble  $\alpha$ -KL protected cardiovascular and renal function against oxidative stress probably by NO production [31]. Epidemiological evidence showed that higher concentration of malondialdehyde (MDA) could be used to quantify microvillous membrane lipid peroxide concentration. Our lab found that higher level of MDA in PE group, and inversely correlated with maternal serum  $\alpha$ -KL [27]. Furthermore, Loichinger *et al.* observed that among patients with PE, those with the highest level of  $\alpha$ -KL had significantly less accelerated villous maturation (AVM) [13], which is a marker of oxidative damage in the placenta [13, 32], supporting the ability of  $\alpha$ -KL in resistance to oxidative stress.

Besides hypertensive disorders during pregnancy,  $\alpha$ -KL is also related to infection during preterm gestations. Jennifer *et al.* noted that among patients with preterm labor or preterm prelabor rupture of membranes (pPROM), those with microbial invasion of the intra-amniotic cavity (MIAC) had lower plasma levels of  $\alpha$ -KL, even after adjusting for maternal age, tobacco use, gestational age at venipuncture [28]. This may suggest the protective role of  $\alpha$ -KL against infection during pregnancy.

Of note, there is a case report that abnormally high level of serum  $\alpha$ -klotho was associated with poor clinical outcome during pregnancy [33]. Sagiri *et al.* reported a 41-year-old pregnant women, whose serum level of  $\alpha$ -klotho was 1,971.12 $\pm$ 201.11 pg/ml (mean  $\pm$  SD), which was pretty high compared to 951.2 $\pm$ 323.9 pg/ml (mean  $\pm$  SD) of uncomplicated pregnan-

## α-klotho in women's health

**Table 1.** Studies on α-klotho level in PE

Author	Year	Sample	Number of PE	Age (year)	Gestational age (weeks)	Measurement	Level of Klotho	Compared to Normal
Jing <i>et al.</i> [30] (mean ± SD)	2011	Placenta	20	26.1±3.7	37.2±1.6	IHC analysis	Absorbance value: 124.26±2.44	Decreased
Giannubilo <i>et al.</i> [14]	2012	Placenta	12	Not provided	Not provided	RT-PCR, Western blot	Not reported	Decreased
Miranda <i>et al.</i> [7] median (range)	2014	Maternal plasma	58 (PE without SGA)	24 (19-30)	35.1 (30.1-38.7)	ELISA	1177.3 (762.4-2013.4 pg/mL)	No difference
Fan <i>et al.</i> [27] (mean ± SD)	2016	Maternal serum	19	28.6±5.6	36.6±2.8	ELISA	579.0±228.4 pg/ml	Decreased
		Placenta	19			RT-PCR, Western blot, IHC	No exact value	Decreased
Cecati <i>et al.</i> [25] (mean ± SD)	2016	Placenta	34	30.2±1.3	31.2±1.1	RT-PCR, Western Blot	No exact value	Decreased
Loichinge <i>et al.</i> [13] (mean ± SD)	2016	Maternal plasma	31	28.0±6.9	36.2±2.0	ELISA	2019±1320 pg/mL	Elevated
		Placenta				IHC	No exact value	No difference

cy in our previous study [27]. Her fetus was diagnosed with typical Klinefelter syndrome (47, XXY karyotype), and finally had an abortion [33]. Although we cannot draw any conclusion from a single case report, it suggests a possible relationship between abnormally high  $\alpha$ -KL level and some disorders.

### *$\alpha$ -klotho and perinatal outcomes*

In human, Miranda *et al.* reported that median maternal plasma concentration of  $\alpha$ -KL was lower in women with SGA (with or without PE), compared to those who delivered normal babies [7]. Shao *et al.* observed elevated  $\alpha$ -KL mRNA and protein in placentas of women with macrosomia, compared with those who delivered babies of normal weight. And such relationship was not altered by gestational diabetes [34], which was a risk factor of macrosomia. In addition, Fan *et al.* found serum level of  $\alpha$ -KL in both maternal and cord blood was positively correlated with fetal birth weight [27]. Although in neonates, plasma  $\alpha$ -KL levels are not significantly different between SGA and appropriate for gestational age neonates. Thus,  $\alpha$ -klotho probably has a positive role in prenatal growth.

$\alpha$ -KL is predominantly expressed in normal placentas [7, 13], especially in syncytiotrophoblast microvillous membrane [13, 35], which might be a major source of  $\alpha$ -KL during pregnancy. Of note, although maternal plasma level of  $\alpha$ -KL increased with gestation age [7, 13], placental  $\alpha$ -KL significantly decreased [13]. One possible explanation is more rapid shedding of placental  $\alpha$ -KL into circulation. Elevated level of ADAM17 detected in syncytiotrophoblasts supports this hypothesis [13]. Another evidence is that serum  $\alpha$ -KL level of umbilical cord blood was higher than levels in neonates at 4-day-old, their mothers and healthy non-pregnant volunteers [35]. However, Sihanidou *et al.* reported short half-life of soluble  $\alpha$ -klotho in vivo [36], proposing a doubt for the hypothesis.

There are several possible explanations of how  $\alpha$ -klotho affects newborns' birth weight: 1)  $\alpha$ -klotho promotes adipocyte differentiation in fetus [34]. 2)  $\alpha$ -klotho stimulates the pituitary gland to produce more growth hormone in fetus [34]. 3) Deletion of  $\alpha$ -klotho may result in impaired metabolism of calcium and phosphate in the fetus [7]. But all of them remain to be elucidated.

$\alpha$ -klotho may also play a role in postnatal growth.  $\alpha$ -KL deficient mice showed osteoporosis and postnatal growth retardation, characterized as lack of weight gain and a paucity of adipose tissue [1]. In human,  $\alpha$ -KL increases in neonates as postnatal age advances. Plasma concentration of  $\alpha$ -KL in neonates increased from 14 to 28 day of age, regardless of preterm or term neonates [36]. Moreover, plasma concentration of  $\alpha$ -KL at 14 and 28 day of age (1099 $\pm$ 480 pg/ml, 1277 $\pm$ 444 pg/ml, respectively) [36] was higher than the serum level at 4 day (582 $\pm$ 90 pg/ml) [35]. At 14 and 28 days, not only term neonates have higher plasma  $\alpha$ -KL levels than preterm ones, but plasma  $\alpha$ -KL is positively associated with body weight and length [36].

The increase of  $\alpha$ -KL with the advance of postnatal age may be related to its role in regulating metabolism. Sihanidou *et al.* observed that neonatal plasma level of  $\alpha$ -KL was positively correlated with serum 1,25(OH)<sub>2</sub>D at both 14 and 28 days after birth [36]. Ohata *et al.* revealed that  $\alpha$ -KL was one of the determinants of phosphate at day 4 of neonates by multiple regression analysis [35]. This uptrend of  $\alpha$ -klotho along with increase of age may be also attributed to the maturation of kidney tissues, which is a predominant site of  $\alpha$ -KL production [1]. Renal cellular proliferation and enlargement along with postnatal growth lead to increase in nephron size and function, which consequently lead to more  $\alpha$ -KL release [36]. The majority of nephrons are normally formed during the third trimester of pregnancy and nephrogenesis is completed at the time of birth in full-term infants [37]. In rats, expression of  $\alpha$ -KL in the kidney was faintly detected at day 18 of prenatal life and 1 day after birth, while markedly increased after 4 days of age [38], which confirms this thesis.

### **$\alpha$ -klotho and hormone-related cancer**

#### *$\alpha$ -klotho and breast cancer*

Wolf *et al.* was the first to discover the reduced expression of  $\alpha$ -klotho in breast cancer by IHC, and found higher  $\alpha$ -klotho expression in all normal breast samples and 90% of normal breast samples adjacent to tumor tissues (invasive ductal carcinoma or ductal carcinoma in situ (DCIS)), while only in 17% of ductal carcinoma in situ tissues and in 22% of invasive ductal carcinoma tissues [16]. In the study of

Rubinek's, higher  $\alpha$ -KL expression was found in all normal breast tissues and mild breast hyperplasia, but was reduced in 33% of tissues which were moderate to florid hyperplasia [39]. Dallol *et al.* also demonstrated downregulated  $\alpha$ -KL mRNA in breast tumors compared to non-tumor tissues [40].

Promoter methylation and histone deacetylation are two main mechanisms of downregulated expression of  $\alpha$ -KL in breast cancer [39, 40]. Rubinek and Dallol both observed upregulation of  $\alpha$ -KL mRNA levels after administration of demethylating agent 5-AZA [39, 40]. Furthermore, treatment with SAHA, an inhibitor of histone deacetylase, significantly enriched acetylated histone 3 at lysine (AcH3K9) in KLOTHO promoter region and elevated  $\alpha$ -KL mRNA expression in MDA-MB-231 cell line [39].

Dallol *et al.* found KLOTHO promoter methylation existed in 55% of 179 breast cancer samples of Saudi Arabia women, and speculated that methylation of KLOTHO promoter may contribute to the relative young age of breast tumor onset in Saudi Arabia women (mostly below age 50 compared to over 50 in Western countries) [40]. In contrast, Rubinek *et al.* observed promoter methylation in only 33% (8/23) of breast cancer tissues [39]. We should note that characteristics of samples in the two studies were different. In Rubinek's study, tumor samples from patients were of different pathological types (mild or moderate hyperplasia, DCIS, etc), whereas in Dallol's, patients were all diagnosed with invasive ductal carcinoma. Moreover,  $\alpha$ -KL methylation was not detected in normal breast tissues in Rubinek's study, but 15.38% (10/65) were detected in Dallol's study [39, 40], such difference may be caused by different assays used to measure the promoter methylation. Considering this, it might not be proper to conclude that  $\alpha$ -klotho promoter methylation contribute to early onset of breast tumor in Saudi Arabia women.

Sinha *et al.* reported treatment with combinations of GTPs and SFN synergistically reactivated  $\alpha$ -KL expression in MDA-MB-231 and MDA-MB-453 cells through demethylation and histone modification at  $\alpha$ -KL promoters [41], suggesting a potential way to trigger  $\alpha$ -klotho expression in human breast cancer cells.

Studies also found exogenous overexpression of  $\alpha$ -KL inhibited tumor cell growth in breast cancer. MCF-7 and MDA-MB-231 cells transfected with an HA-tagged  $\alpha$ -KL expression vector (pcDNA3-HA-KL) showed reduced number and size of surviving colonies (reduced by 84% and 72%, respectively), compared with empty controls [16]. In Ligumsky's study, overexpression of both  $\alpha$ -KL and KL1 could inhibit colony formation of MCF-7 and MDA-MB-231 cells [6]. Meanwhile, tumors from soluble-KL1-injected nude mice were smaller, and showed reduced proliferation and expression of the epithelial mesenchymal transition (EMT) marker vimentin [6].

Structure-Function analysis revealed that different domains of  $\alpha$ -KL have different tumor suppressor activities in breast cancer. KL1 and full length membrane  $\alpha$ -KL inhibited colony formation of MCF-7 and MDA-MB-231 cells, as well as interacted with IGF-1R and inhibited the IGF-1 pathway [6]. However, KL2 did not have such tumor suppressor ability. Furthermore, in vivo, soluble KL1 slowed colony formation in nude mice [6]. It seems that KL1 only is sufficient to mediate growth inhibition. In addition, it was proved that tumor suppressor activities of  $\alpha$ -KL were not mediated by enzymatic activity:  $\alpha$ -KL constructs without putative catalytic site, which was generated by computerized structural modeling, retained their tumor suppressor activity but showed reduced ability to modulate FGF23 signaling [6].

KL-VS is a functional variant of KLOTHO gene. A study of 1115 Ashkenazi Jewish women with BRCA1 mutation suggested an association between functional variant (FV) status and increased risk of breast cancer (HR 1.35, 95% CI 0.99-1.83,  $P=0.060$ ), and ovarian cancer risk (HR 1.54, 95% CI 0.97-2.45,  $P=0.068$ ) after type of cancer was adjusted [42]. However, a subsequent study among a larger group ( $n=9,080$ ) of ethnically diverse BRCA1 and BRCA2 mutation carriers found FV of  $\alpha$ -KL had no effect on either breast or ovarian cancer risk in BRCA1 mutation carriers ( $n=5,741$ ) (HR=1.02, 95% CI 0.93-1.12,  $P=0.66$ ; HR=1.01, 95% CI 0.84-1.20,  $P=0.95$ ) [43]. Considering that subjects in the former study were of a single race and sample size was small, the latter study is considered more persuasive.

Recent studies revealed potential mechanisms of  $\alpha$ -KL in tumorigenesis during breast cancer development: 1)  $\alpha$ -KL inhibits IGF signaling pathway.  $\alpha$ -KL overexpression in MCF-7 cells and MDA-MB-231 cells (transfected with pcDNA3-HA-KL vectors) reduced IGF-1 induced phosphorylation of not only IGF-1R, but its downstream targets: IRS-1, AKT1, GSK3b, and extracellular signal-regulated kinases (ERK)-1 and ERK-2. Moreover, treatment with soluble form of  $\alpha$ -KL also inhibited phosphorylation of IGF-1R [16]. Over expression of KL1 reduced phosphorylation of IGF-1R, AKT, and ERK1/2 in MCF-7 cells. Co-immunoprecipitation (Co-IP) assays indicated that  $\alpha$ -KL and KL1 interacted with endogenous IGF-1R [6, 16]. 2)  $\alpha$ -klotho enhances activation of the FGF pathway induced by bFGF [16]. The FGF pathway inhibits proliferation of breast cancer cells. Over expression of  $\alpha$ -KL in MCF-7 and MDA-MB-231 cell lines enhanced the phosphorylation of ERK1/2, which is an indicator of the activation of the pathway [16]. 3)  $\alpha$ -KL affects breast cancer tumorigenesis through interaction with FGFRs. FGF19 and its receptor FGFR4 were overexpressed in breast cancer, and such overexpression was negatively associated with methylation of KLOTHO gene, indicating that  $\alpha$ -KL expression may be required to maintain FGFR4 expression [40]. 4)  $\alpha$ -KL regulates metabolic activity in breast cancer. Reprogramming of energy metabolism is one of the signs of cancer. Overexpression of  $\alpha$ -KL activated AMP-activated kinase (AMPK) as well as its downstream effector acetyl CoA carboxylase (ACC) in MCF-7, T47D and MDA-MB-231 cell lines. Soluble  $\alpha$ -KL reduced expression of critical components of glucose metabolism, such as glucose transporter GLUT1 and the key glycolytic enzymes hexokinase 2 (HK2), phosphofructokinase1 (PFK1), pyruvate kinase M2 (PKM2) and pyruvate dehydrogenase kinase 1(PDK1) [44].

### *$\alpha$ -klotho and epithelial ovarian cancer*

Expression of  $\alpha$ -KL is also reduced in epithelial ovarian cancer (EOC). In Lojkin's study, high levels of  $\alpha$ -KL mRNA and protein were noted in only 16% (3/19) EOC cell lines (CSOC882, CaOV3, UWBI-289VECTOR). No detectable or very low levels of  $\alpha$ -KL were noted in 58% (11/19) EOC cell lines (TOV 112D, OV90, OV2008, CSOC 1031, ES2, PA1, A2780, OVCA-

432, OVCA3, OVCA5, OVCA8). Moreover, reduced expression of  $\alpha$ -KL was found in 39% (68/176) of EOC tissue, while in none of 24 normal ovary tissues [45, 46].

OVCA-432, SKOV-3, and ES2 cells transfected with full length membrane  $\alpha$ -KL or KL1 showed reduced number and size of surviving colonies [46]. EOC cells treated with soluble  $\alpha$ -KL (soluble human klotho, encoding the amino acids 34-981) showed reduced viability (Viability of OVCA-432, SKOV-3, and ES2 cells was reduced by 40%, 22% and 20% respectively) [46].  $\alpha$ -KL may suppress EOC cells by inhibiting EMT through suppressing mesenchymal marker Snail1 and Snail2, and upregulating E-cadherin expression [46].

Interestingly, Lu's prospective study showed that, in patients with EOC,  $\alpha$ -KL mRNA (secreted, membrane and total klotho, n=100) level was positively correlated with IGF-1 expression [18]. Kaplan-Meier survival analysis indicated that patients with detectable  $\alpha$ -KL had worse progressive-free and overall survival during follow-up when compared to those without  $\alpha$ -KL detected. Multivariate Cox proportional regression revealed that high expression of secreted  $\alpha$ -KL, rather than full length membrane  $\alpha$ -KL or total  $\alpha$ -KL, was significantly associated with increased risk of disease progression and death after controlling patient age at surgery, disease stage, tumor grade, histological type and residual tumor size, as well as IGF-I and IGFBP-3 expression [18].

It should be noted that in Lu's study, 26.5% of the samples had no detectable  $\alpha$ -KL [18], while Lojkin *et al.* observed  $\alpha$ -KL in all of the EOC samples [46]. The difference may be attributed to different characteristics of patients they chose, or different method they applied to detect  $\alpha$ -KL expression (RT-PCR vs IHC) [18, 46]. Given the above evidence, more population-based studies are needed to warrant the relationship between  $\alpha$ -klotho and EOC.

Tumor suppressor capability of  $\alpha$ -KL in EOC might be possibly associated with following mechanisms: 1) Inhibited activation of IGF-1 signaling pathway.  $\alpha$ -klotho inhibited IGF-1-mediated AKT activation, as well as ERK1/2 phosphorylation in SKOV3 and OvCa432 cells [45, 46]. 2) Downregulation of the estrogen receptor (ER). ER plays an important role in the

development of ovarian cancer [51]. Treatment with E2 increased transcriptional activity of the ER in SKOV-3 and OVCA432 cells, while treatment with  $\alpha$ -KL inhibited E2-mediated transcriptional activity [45, 46]. 3) Interaction of the two activities above. ER is a downstream effector of IGF-1 signaling in EOC. It is possible that  $\alpha$ -klotho inhibits the IGF-1 pathway, which in turn downregulates transcriptional activity of the ER [46].

### *$\alpha$ -klotho and cervical cancer*

Reduced  $\alpha$ -KL expression was also found in both cervical cancer cell lines and cancer tissues. Lower expression was detected in the CaSki and SNU-1299 cell lines, and extremely low levels of  $\alpha$ -KL mRNA were noted in SiHa and SNU-17 cell lines [17]. Lee *et al.* noted 40% (4/10) of invasive cervical carcinoma tissues had no detectable  $\alpha$ -KL [17]. Aviel-Ronen *et al.* observed no  $\alpha$ -KL expression in 18.4% (7/38) of adenocarcinoma (ADC) tissues and in 4.5% (2/44) of squamous cell carcinoma (SQCC) tissues, while high expression in all normal cervical tissues [47]. It is notable that downregulation of  $\alpha$ -KL seems to occur at the late phase of cervical tumorigenesis. In Lee's study, all of low-grade squamous intraepithelial lesion (LSIL) tissues (n=4), and high-grade squamous intraepithelial lesion (HSIL) tissues (n=6) had detectable  $\alpha$ -KL, while 40% (4/10) of invasive cervical carcinoma tissues had no  $\alpha$ -KL detected [17]. Furthermore, Chang *et al.* reported that in tissues of cervical intraepithelial neoplasia III (CIN III),  $\alpha$ -KL expression was nearly half of the normal tissues. Meanwhile, no  $\alpha$ -KL was detected in invasive cervical cancer tissues [48].

The transcriptional suppression in cervical cancer is correlated with DNA methylation and histone deacetylation. Methylation-specific PCR (MSP) and bisulfate genomic sequencing (BGS) analysis revealed that klotho-expressing cell lines (SNU-703, SNU-1160) had unmethylated promoter CpGs, while cell lines with low  $\alpha$ -KL expression (CaSki, SNU-17, and SNU-1299) exhibited prominently methylated promoter CpGs [17]. Moreover, treatment with DAC significantly restored  $\alpha$ -KL mRNA expression in the SNU-1299 cell line. In SiHa cell line, KLOTHO promoter was enriched in deacetylated histone H3, and  $\alpha$ -KL expression was restored by TSA [17].

$\alpha$ -KL acts as a tumor suppressor in cervical cancer. Ectopic expression of secreted KL in CaSki cells reduced the number of colonies by 57% [17]. SiHa cells transfected with  $\alpha$ -KL migrated slower into the wounded area and had decreased invasive capacity, compared with cells transfected with empty vectors [48].

$\alpha$ -KL mediates invasiveness of cervical cancer cells through affecting EMT and Wnt signaling pathway. EMT plays a major role in metastasis and progression of cervical cancer [49], and expression of  $\alpha$ -KL caused a reversal of EMT in cervical cancer cells. In SiHa cells transfected with  $\alpha$ -KL, increased expression of E-cadherin and decreased expression of N-cadherin, Twist and Slug were observed. Meanwhile, ectopic overexpression of  $\alpha$ -KL downregulated MMP7 and MM9 in SiHa cells, which play an important role in cell-matrix interaction and tumor invasion [49]. Furthermore,  $\alpha$ -KL inhibits Wnt/ $\beta$ -catenin pathway in cervical cancer [17, 48], and Wnt/ $\beta$ -catenin signaling has been reported to cause upregulation of Slug and Twist [50]. Thus,  $\alpha$ -KL affects EMT by mediating the Wnt pathway.

Furthermore, differential expression of  $\alpha$ -KL was noted in ADC and SCC. Aviel-Ronen *et al.* noted reduced protein and mRNA level of  $\alpha$ -KL in ADC compared with SCC [47], suggesting expression of  $\alpha$ -KL is probably tumor-type dependent.

### **Summaries and perspectives**

Deficiency of  $\alpha$ -KL leads to aging-like symptoms in genital organs and cells, sterility of mice, while overexpression of  $\alpha$ -KL restores these disorders. This role of  $\alpha$ -KL in mice fertility maybe relate to gonadotrophins.

Due to conflicting results of maternal  $\alpha$ -KL levels, the role of  $\alpha$ -KL in pregnancy disorders remains to be elucidated; however,  $\alpha$ -KL level is associated with infant birth weight, although whether such association attributes to elevated  $\alpha$ -KL expression along with maturation of relative organs, or paracrine effects of  $\alpha$ -KL on adipocyte or metabolism is still not clear.

$\alpha$ -klotho is a potential tumor suppressor and exhibited substantial decrease in gynecological cancers, including breast cancer, cervical cancer, and ovarian cancer. There are evidences



showing that  $\alpha$ -KL expression is associated with suboptimal debulking results and survival rates.

Further studies are required to clarify the role of  $\alpha$ -KL in the development of female genital system, gynecological cancers, hypertensive disorders of pregnancy and prenatal & postnatal growth of babies. There remains much to do to apply  $\alpha$ -KL to the promotion of maternal and child health.

### Acknowledgements

This work was supported by National Natural Science Foundation of China (30972463, 81172664) and Open Fund of Hubei Provincial Key Laboratory for Applied Toxicology, Hubei Provincial Academy for Preventive Medicine.

### Disclosure of conflict of interest

None.

**Address correspondence to:** Suqing Wang, Department of Nutrition and Food Hygiene, School of Health Sciences, Wuhan University, China; Hubei Provincial Key Laboratory for Applied Toxicology, Hubei Provincial Academy for Preventive Medicine, Hubei, China. Tel: 86-27-68759972; E-mail: swang-2099@whu.edu.cn

### References

- [1] Kuro-o M, Matsumura Y, Aizawa H, Kawaguchi H, Suga T, Utsugi T, Ohyama Y, Kurabayashi M, Kaname T, Kume E, Iwasaki H, Iida A, Shirakida T, Nishikawa S, Nagai R and Nabeshima YI. Mutation of the mouse klotho gene leads to a syndrome resembling ageing. *Nature* 1997; 390: 45-51.
- [2] Ito S, Kinoshita S, Shiraishi N, Nakagawa S, Sekine S, Fujimori T, Nabeshima YI. Molecular cloning and expression analyses of mouse  $\beta$  klotho, which encodes a novel Klotho family protein. *Mech Dev* 2000; 98: 115-9.
- [3] Kuro-o M. Klotho in health and disease. *Curr Opin Nephrol Hypertens* 2012; 21: 362-368.
- [4] Fon Tacer K, Bookout AL, Ding X, Kurosu H, John GB, Wang L, Goetz R, Mohammadi M, Kuro-o M, Mangelsdorf DJ and Kliewer SA. Research resource: comprehensive expression atlas of the fibroblast growth factor system in adult mouse. *Mol Endocrinol* 2010; 24: 2050-2064.
- [5] Xu Y and Sun Z. Molecular basis of Klotho: from gene to function in aging. *Endocr Rev* 2015; 36: 174-193.
- [6] Ligumsky H, Rubinek T, Merenbakh-Lamin K, Yeheskel A, Sertchook R, Shahmoon S, Aviel-Ronen S and Wolf I. Tumor suppressor activity of klotho in breast cancer is revealed by structure-function analysis. *Mol Cancer Res* 2015; 13: 1398-407.
- [7] Miranda J, Romero R, Korzeniewski SJ, Schwartz AG, Chaemsaitong P, Stampalija T, Yeo L, Dong Z, Hassan SS, Chrousos GP, Gold P and Chaiworapongsa T. The anti-aging factor alpha-klotho during human pregnancy and its expression in pregnancies complicated by small-for-gestational-age neonates and/or pre-eclampsia. *J Matern Fetal Neonatal Med* 2014; 27: 449-457.
- [8] Kuro-o M. Overview of the FGF23-Klotho axis. *Pediatr Nephrol* 2010; 25: 583-590.
- [9] Kuro-o M. Klotho. *Pflugers Arch* 2010; 459: 333-343.
- [10] Wang Y and Sun Z. Current understanding of klotho. *Ageing Res Rev* 2009; 8: 43-51.
- [11] Lim K, Groen A, Molostvov G, Lu T, Lilley KS, Snead D, James S, Wilkinson IB, Ting S, Hsiao LL, Hiemstra TF and Zehnder D. alpha-Klotho expression in human tissues. *J Clin Endocrinol Metab* 2015; 100: E1308-1318.
- [12] Li SA, Watanabe M, Yamada H, Nagai A, Kinuta M and Takei K. Immunohistochemical localization of Klotho protein in brain, kidney, and reproductive organs of mice. *Cell Struct Funct* 2004; 29: 91-99.
- [13] Loichinger MH, Townner D, Thompson KS, Ahn HJ and Bryant-Greenwood GD. Systemic and placental alpha-klotho: effects of preeclampsia in the last trimester of gestation. *Placenta* 2016; 41: 53-61.
- [14] Giannubilo SR, Cecati M, Saccucci F, Corradetti A, Emanuelli M and Tranquilli AL. PP035. Placental klotho protein in preeclampsia: a possible link to long term outcomes. *Pregnancy Hypertens* 2012; 2: 260-261.
- [15] Giannubilo SR, Cecati M, Saccucci F, Emanuelli M and Tranquilli AL. PP008. Placental klotho gene in preeclampsia. *Pregnancy Hypertens* 2013; 3: 70.
- [16] Wolf I, Levanon-Cohen S, Bose S, Ligumsky H, Sredni B, Kanety H, Kuro-o M, Karlan B, Kaufman B, Koeffler HP and Rubinek T. Klotho: a tumor suppressor and a modulator of the IGF-1 and FGF pathways in human breast cancer. *Oncogene* 2008; 27: 7094-7105.
- [17] Lee J, Jeong DJ, Kim J, Lee S, Park JH, Chang B, Jung SI, Yi L, Han Y, Yang Y, Kim KI, Lim JS, Yang I, Jeon S, Bae DH, Kim CJ and Lee MS. The anti-aging gene KLOTHO is a novel target for epigenetic silencing in human cervical carcinoma. *Mol Cancer* 2010; 9: 109.
- [18] Lu L, Katsaros D, Wiley A, de la Longrais IA, Puopolo M and Yu H. Klotho expression in epi-

- thelial ovarian cancer and its association with insulin-like growth factors and disease progression. *Cancer Invest* 2008; 26: 185-192.
- [19] Shiraki-Iida T, Iida A, Nabeshima Y, Anazawa H, Nishikawa S, Noda M, Kuro-o M and Nabeshima Y. Improvement of multiple pathophysiological phenotypes of klotho (kl/kl) mice by adenovirus-mediated expression of the klotho gene. *J Gene Med* 2000; 2: 233-242.
- [20] Kuro-o M. Klotho and aging. *Biochim Biophys Acta* 2009; 1790: 1049-1058.
- [21] Masuda H, Chikuda H, Suga T, Kawaguchi H and Kuro-o M. Regulation of multiple ageing-like phenotypes by inducible klotho gene expression in klotho mutant mice. *Mech Ageing Dev* 2005; 126: 1274-1283.
- [22] Toyama R, Fujimori T, Nabeshima Y, Itoh Y, Tsuji Y, Osamura RY and Nabeshima Y. Impaired regulation of gonadotropins leads to the atrophy of the female reproductive system in klotho-deficient mice. *Endocrinology* 2006; 147: 120-129.
- [23] Takemura T and Okabe M. Serum alpha-klotho concentrations during preimplantation can predict aging or quality of human oocytes and clinical pregnancy rates. *Springerplus* 2016; 5: 53.
- [24] Lo JO, Mission JF and Caughey AB. Hypertensive disease of pregnancy and maternal mortality. *Curr Opin Obstet Gynecol* 2013; 25: 124-132.
- [25] Cecati M, Giannubilo SR, Saccucci F, Sartini D, Ciavattini A, Emanuelli M and Tranquilli AL. Potential role of placental klotho in the pathogenesis of preeclampsia. *Cell Biochem Biophys* 2016; 74: 49-57.
- [26] Eiland E, Nzerue C and Faulkner M. Preeclampsia 2012. *J Pregnancy* 2012; 2012: 586578.
- [27] Fan C, Wang Y, Wang J, Lei D, Sun Y, Lei S, Hu M, Tian Y, Li R and Wang S. Clinic significance of markedly decreased alpha-klotho in women with preeclampsia. *Am J Transl Res* 2016; 8: 1998-2010.
- [28] Lam-Rachlin J, Romero R, Korzeniewski SJ, Schwartz AG, Chaemsaitong P, Hernandez-Andrade E, Dong Z, Yeo L, Hassan SS and Chaiworapongsa T. Infection and smoking are associated with decreased plasma concentration of the anti-aging protein, alpha-klotho. *J Perinat Med* 2013; 41: 581-594.
- [29] Odegård RA, Vatten LJ, Nilsen ST, Salvesen KA, Austgulen R. Preeclampsia and fetal growth. *Obstetrics Gynecology* 2000; 96: 950-5.
- [30] Jing W. Expression and clinical significance of Klotho protein in placentas from women with HDCP (in Chinese). Jilin University 2011.
- [31] Mitobe M, Yoshida T, Sugiyama H, Shiota S, Tsuchiya K and Nihei H. Oxidative stress decreases klotho expression in a mouse kidney cell line. *Nephron Exp Nephrol* 2005; 101: e67-74.
- [32] Salafia CM, Pezzullo JC, Lopez-Zeno JA, Simmons S, Minior VK and Vintzileos AM. Placental pathologic features of preterm preeclampsia. *Am J Obstet Gynecol* 1995; 173: 1097-1105.
- [33] Taguchi S, Funabiki M and Nakamura Y. Abnormally high levels of serum α-klotho may result in a poor clinical outcome for a 41-year-old woman. *Journal of Medical Cases* 2015; 6: 472-473.
- [34] Shao WJ, Wang DX, Wan QY, Zhang MM, Chen MM and Song WW. [Expression of mRNA and protein of Klotho gene in placental tissue of macrosomia and its relationship with birth weight of neonates]. *Zhonghua Fu Chan Ke Za Zhi* 2016; 51: 420-423.
- [35] Ohata Y, Arahori H, Namba N, Kitaoka T, Hirai H, Wada K, Nakayama M, Michigami T, Imura A, Nabeshima Y, Yamazaki Y and Ozono K. Circulating levels of soluble alpha-Klotho are markedly elevated in human umbilical cord blood. *J Clin Endocrinol Metab* 2011; 96: E943-947.
- [36] Sihanidou T, Garatzioti M, Lazaropoulou C, Kourlaba G, Papassotiriou I, Kino T, Imura A, Nabeshima Y and Chrousos G. Plasma soluble alpha-klotho protein levels in premature and term neonates: correlations with growth and metabolic parameters. *Eur J Endocrinol* 2012; 167: 433-440.
- [37] Gubhaju L, Sutherland MR and Black MJ. Preterm birth and the kidney: implications for long-term renal health. *Reprod Sci* 2011; 18: 322-333.
- [38] Ohyama Y, Kurabayashi M, Masuda H, Nakamura T, Aihara Y, Kaname T, Suga T, Arai M, Aizawa H, Matsumura Y, Kuro-o M, Nabeshima Y and Nagail R. Molecular cloning of rat klotho cDNA: markedly decreased expression of klotho by acute inflammatory stress. *Biochem Biophys Res Commun* 1998; 251: 920-925.
- [39] Rubinek T, Shulman M, Israeli S, Bose S, Avraham A, Zundeleovich A, Evron E, Gal-Yam EN, Kaufman B and Wolf I. Epigenetic silencing of the tumor suppressor klotho in human breast cancer. *Breast Cancer Res Treat* 2012; 133: 649-657.
- [40] Dallol A, Buhmeida A, Merdad A, Al-Maghrabi J, Gari MA, Abu-Elmagd MM, Elaimi A, Assidi M, Chaudhary AG, Abuzenadah AM, Nedjadi T, Ermiah E, Alkhayat SS and Al-Qahtani MH. Frequent methylation of the KLOTHO gene and overexpression of the FGFR4 receptor in invasive ductal carcinoma of the breast. *Tumour Biol* 2015; 36: 9677-9683.
- [41] Sinha S, Shukla S, Khan S, Tollefsbol TO and Meeran SM. Epigenetic reactivation of

- p21CIP1/WAF1 and KLOTHO by a combination of bioactive dietary supplements is partially ER $\alpha$ -dependent in ER $\alpha$ -negative human breast cancer cells. *Mol Cell Endocrinol* 2015; 406: 102-114.
- [42] Wolf I, Laitman Y, Rubinek T, Abramovitz L, Novikov I, Beeri R, Kuro OM, Koeffler HP, Catane R, Freedman LS, Levy-Lahad E, Karlan BY, Friedman E and Kaufman B. Functional variant of KLOTHO: a breast cancer risk modifier among BRCA1 mutation carriers of Ashkenazi origin. *Oncogene* 2010; 29: 26-33.
- [43] Laitman Y, Kuchenbaecker KB, Rantala J, Hogervorst F, Peock S, Godwin AK, Arason A, Kirchhoff T, Offit K, Isaacs C, Schmutzler RK, Wappenschmidt B, Nevanlinna H, Chen X, Chenevix-Trench G, Healey S, Couch F, Peterlongo P, Radice P, Nathanson KL, Caligo MA, Neuhausen SL, Ganz P, Sinilnikova OM, McGuffog L, Easton DF, Antoniou AC, Wolf I and Friedman E. The KL-VS sequence variant of Klotho and cancer risk in BRCA1 and BRCA2 mutation carriers. *Breast Cancer Res Treat* 2012; 132: 1119-1126.
- [44] Shmulevich R, Rubinstein T, Scherf T, Wolf I and Rubinek T. The tumor suppressor klotho: a master regulator of metabolism in breast cancer. *Cancer Res* 2015; 75.
- [45] Irina Lojkin, Omer Schwartzman, Tami Rubinek and Wolf I. The aging suppressor hormone Klotho inhibits ovarian cancer cell proliferation by down-regulation of the estrogen receptor. [abstract]. *Proceedings of the 104th Annual Meeting of the American Association for Cancer Research* 2013; 3557-3557.
- [46] Lojkin I, Rubinek T, Orsulic S, Schwarzmann O, Karlan BY, Bose S and Wolf I. Reduced expression and growth inhibitory activity of the aging suppressor klotho in epithelial ovarian cancer. *Cancer Lett* 2015; 362: 149-157.
- [47] Aviel-Ronen S, Rubinek T, Zadok O, Vituri A, Avivi C, Wolf I and Barshack I. Klotho expression in cervical cancer: differential expression in adenocarcinoma and squamous cell carcinoma. *J Clin Pathol* 2016; 69: 53-57.
- [48] Chang B, Kim J, Jeong D, Jeong Y, Jeon S, Jung SI, Yang Y, Kim KI, Lim JS, Kim C and Lee MS. Klotho inhibits the capacity of cell migration and invasion in cervical cancer. *Oncol Rep* 2012; 28: 1022-1028.
- [49] Qureshi R, Arora H and Rizvi MA. EMT in cervical cancer: its role in tumour progression and response to therapy. *Cancer Lett* 2015; 356: 321-331.
- [50] Howe LR, Watanabe O, Leonard J and Brown AM. Twist is up-regulated in response to Wnt1 and inhibits mouse mammary cell differentiation. *Cancer Res* 2003; 63: 1906-1913.
- [51] Schlumbrecht MP, Xie SS, Shipley GL, Urbauer DL and Broaddus RR. Molecular clustering based on ER $\alpha$  and EIG121 predicts survival in high-grade serous carcinoma of the ovary/peritoneum. *Mod Pathol* 2011; 24: 453-462.