Tumor Suppressor Activity of Klotho in Breast Cancer Is Revealed by Structure–Function Analysis

Hagai Ligumsky1,2, Tami Rubinek1, Keren Merenbak-Lamin1,2, Adva Yeheskel3, Rotem Sertchook4, Shiri Shahmoon1,2, Sarit Aviel-Ronen5, and Ido Wolf1,2

Abstract

Klotho is a transmembrane protein containing two internal repeats, KL1 and KL2, both displaying significant homology to members of the β-glycosidase family. Klotho is expressed in the kidney, brain, and various endocrine tissues, but can also be cleaved and act as a circulating hormone. Klotho is an essential cofactor for binding of fibroblast growth factor 23 (FGF23) to the FGF receptor and can also inhibit the insulin-like growth factor-1 (IGF-1) pathway. Data from a wide array of malignancies indicate klotho as a tumor suppressor; however, the structure–function relationships governing its tumor suppressor activities have not been deciphered. Here, the tumor suppressor activities of the KL1 and KL2 domains were examined. Over-expression of either klotho or KL1, but not of KL2, inhibited colony formation by MCF-7 and MDA-MB-231 cells. Moreover, in vivo administration of KL1 was not only well tolerated but significantly slowed tumor formation in nude mice. Further studies indicated that KL1, but not KL2, interacted with the IGF-1R and inhibited the IGF-1 pathway. Based on computerized structural modeling, klotho constructs were generated in which critical amino acids have been mutated. Interestingly, the mutated proteins retained their tumor suppressor activity but showed reduced ability to modulate FGF23 signaling. These data indicate differential activity of the klotho domains, KL1 and KL2, in breast cancer and reveal that the tumor suppressor activities of klotho can be dissected from its physiologic activities.

Implications: These findings pave the way for a rational design of safe klotho-based molecules for the treatment of breast cancer.

Mol Cancer Res; 13(10); 1398–407. ©2015 AACR.

Introduction

Klotho (α-klotho) is a transmembrane protein expressed in the distal convoluted tubules of the kidneys and the choroid plexus, as well as in various endocrine and exocrine tissues (1), but it can also be cleaved, shed, and act as a circulating hormone (2, 3). Klotho-deficient mice manifest a syndrome resembling human aging, whereas overexpression of klotho extends lifespan (1, 4). Major physiologic activities of klotho include regulation of phosphate and calcium homeostasis as well as of metabolic activity (5–7). To date, several mechanisms of action of klotho have been described. Klotho is an essential cofactor for binding of FGF23 to the FGF receptor (FGFR; refs. 5, 8), it modulates bFGF signaling (9, 10), inhibits the insulin and the insulin-like growth factor (IGF)-1 pathways (4, 9), and regulates the activity of the transient receptor potential vanilloid type 5 (TRPV5) calcium channel (11, 12). The common link between these diverse activities has not been elucidated yet.

In the past years klotho has emerged as a potent tumor suppressor in a wide array of malignancies, including breast, pancreas, colon, gastric, and lung cancers, as well as in melanoma (9, 10, 13–16). We have previously shown that klotho is abundantly expressed in normal breast and pancreatic tissues and that upon malignant transformation its expression is reduced (9, 10, 17). Restoring klotho expression, or treatment with the soluble protein, inhibited proliferation of breast and pancreatic cancer cells (9, 10), and further studies indicated it as an inhibitor of the IGF-1 pathway (4, 9).

Mouse klotho is a 1,014-amino-acid–long protein (1). Its intracellular domain is composed of 10 amino acids and has no known functional role (1). The extracellular domain is composed of a signal sequence (SS), responsible for directing the protein to the cell surface, and two internal repeats, KL1 and KL2, each about 450-amino acids long (1), which exhibit only weak similarity to each other (21% amino-acid identity; ref. 1). The discrete activities of each domain have not been well-characterized yet. Cleavage of the extracellular domain of klotho can yield either the entire extracellular domain (130 kDa) or the discrete domains KL1 and KL2 (2, 18, 19). Although only the full-length protein can serve as a cofactor for the activity of FGF23 (5, 8), either the full-length protein or KL1 can inhibit proliferation of pancreatic cancer cells (10).

Note: Supplementary data for this article are available at Molecular Cancer Research Online (http://mcr.aacrjournals.org/).

H. Ligumsky performed this work in partial fulfillment of the requirements for a Ph.D. degree.

Corresponding Author: Ido Wolf, Tel Aviv Medical Center, 6 Weizmann Tel Aviv, Israel; 64229; Phone: 972-52-736-0558; Fax: 972-3-697-3030; E-mail: idow@tlvmc.gov.il

doi: 10.1158/1541-7786.MCR-15-0141
©2015 American Association for Cancer Research.
Each klotho domain displays significant homology to members of the glycoside hydrolase family 1 enzymes (1). Two glutamic acid residues are required for the enzymatic activity of family 1-β-glycosidases, one acts as a nucleophile and the other as an acid/base catalyst (20). Although these critical amino acids are not conserved in klotho, klotho was shown to hydrolyze β-glycoside and β-d-glucuronide (20). In support of these activities, mutations of critical amino acids, considered to be essential for its enzymatic activity, abolished the ability of klotho to increase TRPV5 currents (11). Yet, these mutations did not affect its ability to inhibit Wnt signaling. To our knowledge, the role of these residues in mediating klotho tumor suppressor activities has not been studied yet.

We aimed to decipher klotho structure-function relationships that are involved in mediating its tumor suppressor activities in breast cancer. Our findings demonstrate that KL1, but not KL2, can inhibit growth of breast cancer cells in vitro and in vivo. Structural modeling and site-directed mutagenesis indicate that amino acids, essential for modulation of FGF23 signaling, are not required for klotho tumor suppressor activity.

Materials and Methods

Chemicals and antibodies

The chemicals used were FGF23, IGF-1 and soluble human KL1 (hKL1) (PeproTech Inc.), and G418 (Invitrogen). Antibodies used were total-IGF-1BR (Santa Cruz Biototechnology), phospho-AKT1 (S473), phospho-IGF-IR (Y1131), total pan-AKT (Cell Signaling Technology), diprophosphorylated-Thr183/Tyr185 and total-ERK1/2 (Sigma), and HA (Covance). Anti-klotho antibodies directed against KL1 domain (KM2076) and KL2 domain (KM2119) were a kind gift from Kyowa Hakko Kogyo Co., Ltd.

Cells and transfections

MCF-7, MDA-MB-231, and HEK-293 cells were obtained from the ATCC. Cells were received from Dr. Hadassa Degani’s lab at Weizmann Institute of Science, which obtained them directly from the ATCC and were not passaged more than 6 months after resuscitation. All transfections used Lipofectamine 2000 (Invitrogen).

Structural model construction of KL1 and KL2 domains

Template structures for molecular modeling of human klotho were selected by BLAST search against the Protein Data Base (PDB). The BLAST search suggested the structures of the human Cytosolic Neutral beta-Glycosylceramidase (klotho-related Protein, KL2P) as best template (42% sequence identity to KL1 and 33% to KL2 domain). A crystal structure of KL2P with highest resolution (1.60 Å) and good stereochemistry was selected (PDB entry 2E9L). Structural model of KL1 was built using PDB 2E9L (21) as a template. The template was selected using HHpred (22). The model was built using Modeller (23). The model structure was refined using the Rosetta Relax protocol (v. 3.2) (24). KL1 model was assessed using ModFold (25) and ConSurf (26), using a multiple sequence alignment of 150 KL1 homologues collected from Uniprot (27) using Blast (28) and aligned with Mafft (29). Structural model of KL2 was based on a sequence alignment between KL2 domain, the template structure, and 14 representative sequences of family 1 Glycoside hydrolase, which was carried out using several multiple alignment algorithms (Expresso, Promals3D, and FFAS03). A set of 20 all-atoms model of KL2 was built using the restrained-based modeling approach as implemented in the program MODELLER 9V6 based on the different alignments. The model was evaluated using MODELLER objective function, DOPE, ProSA, and Anolea. The model showing the best score as judged by these evaluation methods was saved for further studies and presented here.

Generation of klotho expression constructs

Mouse HA-tagged full-length klotho (FL-KL) in pcDNA3.1 was a generous gift from Y. Nabeshima (Kyoto University, Japan). Mouse KL1 and KL2 constructs were prepared in two steps, in order to contain the original FL-KL SS. For the first step, the SS was PCR amplified and restriction sites were introduced using specific primers: 5′-ATCGAATTCTAGAAGGCTAGCAGACGAGC-3′ (EcoRI) and 5′-ATCAAGCTTGGCGCCCGAGGGAGGACGAGCAG-3′ (HindIII). KL1 and KL2 were PCR amplified and restriction sites were introduced using specific primers. For KL1, 5′-ATCAAGCTTAGTGCTTTGAGCTCTGCTGAC-3′ (HindIII) and 5′-ATCTCCAGCTCTCTCTTCTGGCTACATACCCCC-3′ (XhoI). KL2, 5′-ATCAAGCTTAAACCTGCTAGTGCTCTCTCTCTGAGCC-3′ (HindIII) and 5′-ATCTCCAGCTCTCTCTTCTGGCTACATACCCCC-3′ (XhoI). Plasmids were digested with the suitable enzymes, and the SS was ligated to KL1 and KL2. Subsequently, the constructs were sequenced for verification and subcloned into pcDNA3.1 backbone.

Mouse KL1 with intra-cyttoplasmic/transmembrane (IC/TM) was prepared as followed: at first, an IC/TM fragment was PCR amplified and restriction sites were introduced using specific primers: 5′-ATCTCCAGCTCTTCTCTGCTCTCATGC-3′ (XhoI) and 5′-GATCTCCGACCTATAACCTCTCTCTGAGCC-3′ (XhoI). Then the fragment and KL1 plasmid were digested with the suitable enzymes, and the IC/TM was ligated to KL1.

Human FL-KL (hFL-KL) in pEF and human full-length without IC/TM (hFL-KL ΔIC/TM) was a generous gift from M. Kuro-O (UT Southwestern, Dallas, TX).

Directed mutagenesis of klotho putative enzymatic active site

Prior to mutagenesis, constructs were transferred to a Topo plasmid to preserve the HA tag. The selected amino acids for substitution were chosen in order to preserve the three-dimensional conformation of the molecule. First, a single mutation was made at amino acid 416 by converting Glu to Gln. FL-KL and KL1 constructs were PCR amplified using specific primers for mutation insertion: 5′-GGCTCCCTGGTGGCGCATG-3′ (muta-

Colony assays

Two days following transfection with the indicated plasmids, G418 (750 µg/mL for all cell lines) was added to the culture media. At day 14, cells were stained using crystal violet (Sigma) and quantitated using ImageJ software.

Western blot analysis

Cells were harvested, lysed, and total protein was extracted with RIPA buffer (50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1%
NP-40, 0.25% Na-deoxycholate, 1 mmol/L EDTA, 1 mmol/L NaF) together with a protease inhibitor cocktail (Sigma). Lysates were resolved on 10% SDS-PAGE and immunoblotted with the indicated antibodies. Band intensities were quantified using ImageJ software.

**Immunoprecipitation**

MCF-7 cells were harvested as described above, and 400 µg protein lysates were incubated with protein A/G (Pierce) beads at 4°C for 1 hour to eliminate nonspecifically bound proteins. These beads were subsequently used as a negative control. The supernatants were then incubated with 2 µl anti total IGF-1R for 4 hours and then protein A/G was added. Following an overnight incubation, the beads were washed six times in RIPA buffer, and the immunoprecipitated materials were separated by SDS–polyacrylamide gel electrophoresis and detected by Western blotting.

**Mice tumor xenograft studies**

Mice maintenance and experiments were carried out under institutional guidelines of the Sheba Medical Center. Experiments were conducted as described (30). Six-week-old female athymic nude mice were injected with 0.75 × 10⁵ MDA-MB-231 cells subcutaneously into both flanks. After 5 days, mice were treated with daily i.p. injections of soluble hKL1: 10 µg/kg, or saline (n = 6 per group). Tumor size was measured weekly with a digital caliper, and the volume was estimated using the equation V = (a × b²) × 0.5236, where a is the larger dimension and b is the perpendicular diameter.

**Immunohistochemistry analysis**

After mice were euthanized, tumors were removed and fixed in 4% paraformaldehyde. Paraffin-embedded tumors were serially sectioned and stained with hematoxylin & eosin (H&E), and immunohistochemically stained for Ki-67 and vimentin. Stained sections were examined microscopically by an experienced pathologist. For plasma analyses, immediately after mice were euthanized, blood was drawn from the heart and plasma was analyzed at the automated blood lab, Sheba Medical Center.

**Results**

**Structural modeling of the KL1 and KL2 domains**

We generated a computational structure model of KL1 and KL2 domains based on similarity of klotho to KLrP (Fig. 1A). Crystallographic studies indicated KLrP as a classic family 1 glycoside hydrolases that display the Koshland-retaining mechanism. Two glutamic residues serve as its catalytic residues: Glu-373 is the nucleophile and Glu-165 is the acid/base catalyst. The model indicates that both KL1 domain (residues 57–506) and KL2 domain (residues 515–953) share significant homology to glycoside hydrolase family 1 and are predicted to have similar TIM-Barrel fold (ref. 31; Fig. 1A). According to the model, Glu-414 in KL1 domain can serve as the nucleophile residue (Fig. 1B). Although the corresponding residue to the acid/base catalyst Glu-165 is Asn-239, which cannot serve as an acid/base catalyst, Asp-238 may function as an alternative acid/base catalyst and it is in the required distance from the nucleophile (2Å). Thus, KL1 may serve as an active enzyme. In KL2, on the other hand, the critical nucleophilic residue (Glu-373) is replaced by Ser-872, which cannot serve as the nucleophilic residue (Fig. 1C). Thus, the model suggests that KL2 is not an active glycoside hydrolase.

KL1 inhibits colony formation and suppresses in vivo growth of breast cancer cells

In order to evaluate the differential activities of KL1 and KL2, we constructed expression vectors of KL1 and KL2 domains, both with an SS, directing them to the membrane (Fig. 2A and B) and enabling them to be secreted (Supplementary Fig. S1). The effect of overexpression of these domains on growth of breast cancer cells was assessed using colony formation assays. MCF-7 and MDA-MB-231 breast cancer cells were transfected with either klotho, KL1, or KL2 expression vectors or an empty vector. Transfected cells were cultured in media containing G418 for 2 weeks and stained to determine the number of surviving colonies. Klotho and KL1 expression significantly reduced the number and size of surviving colonies, whereas KL2 showed only a modest effect (Fig. 2C and D). The tumor suppressor activity of KL1 was also verified in vivo. For this study, MDA-MB-231 cells were injected to both flanks of athymic mice, and mice were treated with daily i.p. injections of either control vehicle (n = 6) or soluble hKL1 (10 µg/kg, n = 6) for 4 weeks. Already after 12 days of treatment, a significant inhibition of tumor growth was noted among KL1-treated mice (P < 0.001, for comparison of tumor volume between the control and hKL1-treated group), lasting to the end of the experiment (Fig. 3A). Tumor weight at the end of the experiment was significantly lower (P < 0.001) in hKL1-treated group (Fig. 3B and C). Immunohistochemistry analysis revealed a significant reduction in the number of cells expressing the proliferation marker Ki-67 and the mesenchymal marker vimentin. Stained sections were examined microscopically by an experienced pathologist. For plasma analyses, immediately after mice were euthanized, blood was drawn from the heart and plasma was analyzed at the automated blood lab, Sheba Medical Center.

**Statistical analysis**

Results are presented as mean ± SD. Continuous variables were compared using the t test. All significance tests were two-tailed, and a P value of <0.05 was considered as statistically significant.

**Differential modulation of IGF-1 signaling by KL1 and KL2**

We have previously shown that klotho efficiently suppresses activation of the IGF-1 pathway and interacts with the IGF-1R in breast cancer cells (9). In order to analyze the differential effects of KL1 and KL2 on IGF-1 signaling, MCF-7 cells were transfected with either klotho, KL1, or KL2 expression vectors or an empty vector, starved for 48 hours, treated with IGF-1 (100 ng/ml, 15 minutes) and analyzed using Western blotting for the level of expression and phosphorylation of IGF-1R, AKT, and ERK1/2. KL1 as well as klotho reduced IGF-1 induced phosphorylation of these proteins, whereas KL2 had only minor effect (Fig. 4A). Co-immunoprecipitation (Co-IP) assays using MCF-7 cells expressing either klotho, KL1, or KL2 indicated that only klotho and KL1 can interact with the endogenous IGF-1R (Fig. 4B).

**Only full-length, membrane-bound klotho can transduce FGF23 signaling**

Membrane-bound klotho is an essential cofactor for activation of the FGFRs by FGF23 (8). To our knowledge, the ability of KL1 and KL2 to modulate FGF23 signaling has not been determined.
yet. In order to test this, MCF-7 and HEK293 cells were transfected with either klotho, KL1, or KL2 expression vectors or an empty vector, starved for 48 hours, treated with FGF23 (100 ng/mL, 15 minutes), and analyzed using Western blotting for the level of expression and phosphorylation of ERK1/2. Neither KL1 nor KL2 were able to mediate FGF23-induced phosphorylation of ERK1/2 (Fig. 4C). Removing the IC/TM domain (FL-KL-IC/TM), which anchors klotho to the cell membrane, also impaired its ability to transduce FGF23 signal (Fig. 4D).

In order to test if membrane-bound KL1 can mediate FGF23 signaling, we generated a KL1 domain fused with the IC/TM region of klotho (KL1-IC/TM, Fig. 2A). HEK 293 cells were transfected with these constructs, serum starved for 48 hours, stimulated with either a vehicle or 100 ng/mL FGF23 for 15 minutes, and analyzed using Western blotting for the phosphorylation of ERK1/2. KL1-IC/TM failed to transduce the FGF23 signal (Fig. 4D).

Glu-416 and Asp-240 are not required for klotho tumor suppressor activity
Mutations of klotho putative active sites abolished its effect on TRPV5 and on the renal phosphate transporter NaPi-2a (11, 32, 33), but did not affect its ability to inhibit Wnt signaling (34). To our knowledge, the role of these residues in mediating klotho tumor suppressor activities has not been studied yet. In order to decipher the role of these amino acids in mediating the tumor suppressor activity of klotho, a series of mouse klotho and KL1 constructs were designed, in which amino acids have been mutated. Glu-416 (putative nucleophile, corresponds to human Glu-414) was converted to glutamine and Asp-240 (putative acid/base catalyst, corresponds to human Asp-238) was converted to asparagine (Fig. 1B). These mutations are not expected to alter the tertiary structure of the protein. The ability of the various mutated proteins to inhibit colony formation of MCF-7 and MDA-MB-231 cells was determined as described above. In both breast cancer cell lines, the mutated klotho constructs, FL-KL or KL1, harboring single (416) or double (240-416) mutations, retained the antiproliferative effect exhibited by klotho. Surprisingly, the mutated klotho and KL1 proteins displayed even a more potent antiproliferative activity compared with their wild-type counterparts. For example, in MCF-7 cells, FL-KL, FL-KL-416, and FL-KL-240-416 reduced number of colonies to 61%, 37%, and 45% of control, respectively (Fig. 5A). In MDA-MB 231 cells, FL-KL, FL-KL-416,
and FL-KL-240-416 reduced number of colonies to 32%, 18%, and 16% of control, respectively (Fig. 5B).

Dissecting klotho tumor suppressor activities from its physiologic activities may enable the development of safe klotho-based therapies. We, therefore, aimed to study whether klotho mutant, which potently slows proliferation of cancer cells, can modulate FGF23 signaling. As only FL-KL can function as a cofactor for FGF23 (Fig. 4C and D), we analyzed only FL-KL mutants. To this aim, HEK293 cells were transfected with either FL-KL, FL-KL-416, and FL-KL-240-416 expression vectors or an empty vector, starved for 48 hours, treated with FGF23 (100 ng/mL, 15 minutes), and analyzed using Western blotting for the level of expression and phosphorylation of ERK1/2. Although the WT-FL and KL-416 were able to mediate FGF23-induced phosphorylation of ERK1/2 (Fig. 5C), transfection with FL-KL-240-416 only partially increased ERK phosphorylation (Fig. 5C).

Discussion

This structure–function analysis of klotho in breast cancer indicates KL1 as sufficient to mediate growth inhibition, whereas KL2 does not have a significant tumor suppressor activity. Furthermore, our data suggest that the activity of KL1, as well as of the full-length protein, is independent of the amino acids of its putative catalytic site.

Distinct activities of klotho, KL1, and KL2 have been demonstrated in several systems. The FGF23 coreceptor function is mediated only by full-length, membrane-anchored klotho (refs. 5, 8; Fig. 3C and D). On the other hand, either KL1 or KL2 were sufficient to activate TRPV5 (11). In contrast, several klotho activities were shown to be KL1 dependent. Thus, it was demonstrated that KL1 is essential and sufficient to mediate the anti-inflammatory activity of klotho in senescent cells, whereas KL2 did not show any such activity (35). Similarly, KL1 is mandatory and sufficient to inhibit Wnt signaling by klotho, whereas KL2 is devoid of this activity (34). Our data suggest that klotho tumor suppressor activities are KL1 dependent and that KL2 by itself cannot significantly inhibit proliferation of breast cancer cells.

Administration of KL1 slowed tumor formation by breast cancer cells in nude mice. Tumors from KL1-treated mice were smaller, showed reduced proliferation, and reduced expression of the EMT marker vimentin. Furthermore, their blood albumin...
levels were higher and LDH levels lower compared with control-treated mice. Treatment did not adversely affect either weight or kidney functions. This favorable toxicity profile can be attributed to the inability of KL1 to serve as a coreceptor for FGF23. These data support our previous observation using pancreatic cancer cells (10) and suggest that treatment with KL1 is safe and may

### Table 1. Comparison of blood chemistry parameters between the control and mice treated with soluble hKL1 (10 µg/kg)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (N = 6)</th>
<th>Soluble hKL1 (N = 6)</th>
<th>P value (con. vs. soluble hKL1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea (mg/dL)</td>
<td>56.6 ± 5.03</td>
<td>57.33 ± 7.06</td>
<td>0.85</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>0.24 ± 0.02</td>
<td>0.25 ± 0.02</td>
<td>0.46</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>205.2 ± 26.57</td>
<td>204.5 ± 24.92</td>
<td>0.96</td>
</tr>
<tr>
<td>Phosphorous (mg/dL)</td>
<td>7.8 ± 0.39</td>
<td>7.4 ± 0.89</td>
<td>0.48</td>
</tr>
<tr>
<td>Sodium (mg/dL)</td>
<td>142.6 ± 5.73</td>
<td>148 ± 2</td>
<td>0.057</td>
</tr>
<tr>
<td>Calcium (mg/dL)</td>
<td>9.04 ± 0.67</td>
<td>9.42 ± 0.58</td>
<td>0.15</td>
</tr>
<tr>
<td>Chloride (mg/dL)</td>
<td>107.8 ± 1.83</td>
<td>110.67 ± 2.16</td>
<td>0.34</td>
</tr>
<tr>
<td>Potassium (mg/dL)</td>
<td>7.14 ± 1.07</td>
<td>6.53 ± 1.57</td>
<td>0.37</td>
</tr>
<tr>
<td>Uric acid (mg/dL)</td>
<td>1.92 ± 0.28</td>
<td>1.65 ± 0.43</td>
<td>0.25</td>
</tr>
<tr>
<td>SGOT (AST), IU/L</td>
<td>574.86 ± 323.23</td>
<td>232.67 ± 122.01</td>
<td>0.04*</td>
</tr>
<tr>
<td>SGPT (ALT), IU/L</td>
<td>273.75 ± 199.85</td>
<td>75.83 ± 53.36</td>
<td>0.04*</td>
</tr>
<tr>
<td>LDH, IU/L</td>
<td>2534.33 ± 1548.54</td>
<td>698.75 ± 251.42</td>
<td>0.04*</td>
</tr>
<tr>
<td>Alkaline phosphatase (IU/L)</td>
<td>49.8 ± 14.34</td>
<td>81.17 ± 16.94</td>
<td>0.01*</td>
</tr>
<tr>
<td>Protein (g/dL)</td>
<td>5.02 ± 0.5</td>
<td>5.53 ± 0.37</td>
<td>0.08</td>
</tr>
<tr>
<td>Albumin (g/dL)</td>
<td>2.66 ± 0.21</td>
<td>3.02 ± 0.29</td>
<td>0.05*</td>
</tr>
<tr>
<td>Globulin (g/dL)</td>
<td>2.56 ± 0.32</td>
<td>2.5 ± 0.34</td>
<td>0.35</td>
</tr>
</tbody>
</table>

NOTE: Blood chemistry parameters from vehicle and soluble hKL1-treated nude mice harboring MDA-MB-231 human breast cancer cells tumors. Plasma was analyzed by automated blood lab. SGOT, Serum glutamic oxaloacetic transaminase; SGPT, Serum glutamic pyruvic transaminase.

* P < 0.05, soluble hKL1-treated mice versus control vehicle. Significant values are in bold.

Figure 3.
Soluble hKL1 inhibits growth of breast tumors in nude mice. A, MDA-MB-231 cells were injected into both flanks of 6-week-old female athymic nude mice. The mice (N = 6 per group) were treated with daily i.p. injections of soluble hKL1 (10 µg/kg) or vehicle control (saline) for 3 weeks. Tumors volumes were measured weekly (**, P < 0.001, for tumor volume between soluble hKL1-treated and control group). B, representative photographs of mice with tumors in soluble hKL1-treated group and control group before they were sacrificed. C, tumor weight was measured on day of sacrifice (**, P < 0.01 for comparison of tumor weight between the control group and the mice treated with soluble hKL1). D, H&E, Ki67, vimentin, and cytokeratin AE1/AE3 immunostaining magnification, ×400. E, mice weight of soluble hKL1-treated group and control group at the beginning and the end of the i.p. injections.
serve as an effective novel strategy for the treatment of breast cancer.

Although ample data indicate klotho as a potent tumor suppressor in a wide array of malignancies (9, 10, 15, 16, 36), its precise mechanism of action is still a matter of debate. Mechanisms implicated in its tumor suppressor activities include inhibition of insulin and IGF-1 signaling, modulation of FGF signaling, and inhibition of Wnt signaling (4, 5, 8, 9, 12, 15, 37). We noted a correlation between the ability to inhibit the IGF-1 pathway, interaction with the IGF-1R, and inhibition of proliferation. Thus, klotho and KL1 slowed proliferation, interacted with the IGF-1R, and inhibited the IGF-1 pathway. Conversely, KL2, which did not slow proliferation, did not inhibit the pathway or interacted with the receptor. These data provide an indirect support to the hypothesis that inhibition of the IGF-1 pathway is a major mediator of klotho activities in breast cancer. Interestingly, the same klotho region required for tumor suppression is the one required for the inhibition of the IGF-1 pathway and of Wnt signaling (10, 34). Thus, it is possible that a common mechanism that involves inhibition of the Wnt and IGF-1 signaling pathways is responsible to klotho tumor suppressor activities.

While klotho enables activation of the FGFR pathway by FGF23, it inhibits activation of the IGF-1 pathway. Thus, treatment with klotho can lead to either activation or inhibition of the ERK pathway, the downstream effector of both pathways. Although this may seem to be counterintuitive, it is important to note that the activity of the ERK pathway is tissue and cell type dependent (38). The ERK pathway regulates a wide array of physiologic and pathologic processes, including proliferation, development, and metabolism, and while in the kidneys, as a downstream effector of FGF23 signaling, it participates in regulating phosphorus homeostasis (39), in cancer cells it enhances proliferation as a downstream effector of multiple growth factors, including IGF-1 (40). Furthermore, the effect of klotho on various
tyrosine kinase receptors is also complex. Although klotho enhances activation of FGFRs by FGF23 (5, 8), either klotho or KL1 inhibit activation of the FGFRs by bFGF (10). As klotho regulates glycosylation pattern of various membrane proteins (33, 41), it is possible that some of its activities are mediated by regulating glycosylation pattern of the IGF-1R and FGFRs.

MCF-7 cells express somewhat higher klotho levels compared with MDA-MB-231 cells (9). Yet, MCF-7 cells are more sensitive to the tumorigenic effects of IGF-1 (42–45). These data suggest a resistance to endogenous klotho in MCF-7 cells. The mechanisms mediating this resistance to endogenous klotho remain to be elucidated. The IGF-1 signaling may play a less prominent role in MDA-MB-231 cells (44), and other signaling pathways, including Wnt signaling, are important mediators of tumorigenesis in these cells. As klotho is a potent inhibitor of Wnt signaling in other malignancies, it is possible that some of its effects in breast cancer are mediating by affecting Wnt signaling (37, 46).

Several studies demonstrated a critical role for residues within klotho’s putative active site in mediating some of the activities of klotho. Thus, mutations of these residues abolished its effect on TRPV5 and on the renal phosphate transporter NaPi-2a (11, 32, 33), but did not affect its ability to inhibit Wnt signaling (34). Our studies indicate that these amino acids are not required for klotho tumor suppressor activity. These data strengthen a possible relationship between klotho tumor suppressor activity and Wnt inhibition and suggest that enzymatic activity of klotho may not be required for its activity against cancer cells. On the other hand, the klotho double mutant showed reduced ability to mediate FGF23 signaling. This observation indicates that klotho’s diverse activities are mediated by different regions and may lead for the development of klotho-based molecules showing

Figure 5.
Mutating klotho putative enzymatic active site does not affect klotho anticancerous activity while compromising FGF23 signaling. A and B, MCF-7 and MDA-MB-231 cells were transfected as described above. Transfected cells were cultured in media containing G418 for 2 weeks. Colonies were fixed and stained with crystal violet and photographed. All experiments were conducted at least 3 times, and representative wells for each condition are shown. Quantitation of the colonies was performed using ImageJ (*, P < 0.05, FL-KL, FL-KL-416, FL-KL-240-416, KL1, KL1-416, and KL1-240-416 vs. matched control; #, P < 0.05, FL-KL-416, FL-KL-240-416 vs. WT FL-KL and KL1, KL1-416 and KL1-240-416 vs. WT KL1). C, HEK 293 cells were transfected with either FL-KL, FL-KL-416, FL-KL-240-416, KL1, KL1-416, and KL1-240-416 or pcDNA3 (vector control). Following 48-hour serum starvation, cells were stimulated with vehicle or 100 ng/mL FGF23 for 15 minutes and snap-frozen in liquid nitrogen. Cell lysates were prepared for Western blot using antibodies against phosphorylated ERK1/2 (pERK1/2) and total ERK1/2.
The acquisition of data, provided animals, acquired and managed patients, development of methodology, conception and design, acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H. Ligumsky, K. Menenbashk-Lamin, S. Aviel-Ronen, I. Wolf

Authors’ Contributions
Conception and design: H. Ligumsky, T. Rubinek, I. Wolf
Development of methodology: H. Ligumsky, T. Rubinek, I. Wolf
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H. Ligumsky, K. Menenbashk-Lamin, S. Aviel-Ronen, I. Wolf

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Ligumsky, T. Rubinek, A. Yeheksel, R. Sentchok, S. Aviel-Ronen, I. Wolf

Grant Support
This study was supported in part by the Israeli Science Foundation (grant number 1112/09), the Israel Cancer Association Research Grant by Peter & Nancy Brown in memory of Eric & Melvin Brown, and the Sackler Faculty of Medicine, Tel Aviv University, Tel-Aviv, Israel.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received March 23, 2015; revised May 21, 2015; accepted June 10, 2015; published OnlineFirst June 25, 2015.
43. de Blaquiere GE, May FE, Westley BR. Increased expression of both insulin receptor substrates 1 and 2 confers increased sensitivity to IGF-1 stimulated cell migration. Endocr Relat Cancer 2009;16:635–47.
Function Analysis

−

Tumor Suppressor Activity of Klotho in Breast Cancer Is Revealed by Structure–Function Analysis

Hagai Ligumsky, Tami Rubinek, Keren Merenbakh-Lamin, et al.


Updated version

Access the most recent version of this article at: doi:10.1158/1541-7786.MCR-15-0141

Supplementary Material

Access the most recent supplemental material at:
http://mcr.aacrjournals.org/content/suppl/2015/06/26/1541-7786.MCR-15-0141.DC1

Cited articles

This article cites 46 articles, 18 of which you can access for free at:
http://mcr.aacrjournals.org/content/13/10/1398.full#ref-list-1

E-mail alerts

Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions

To request permission to re-use all or part of this article, use this link http://mcr.aacrjournals.org/content/13/10/1398. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.