Depressed expression of Klotho and FGF receptor 1 in hyperplastic parathyroid glands from uremic patients

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Fibroblast growth factor 23 (FGF23) exerts its effect by binding to its cognate FGF receptor 1 (FGFR1) in the presence of its co-receptor Klotho. Parathyroid glands express both FGFR1 and Klotho, and FGF23 decreases parathyroid hormone gene expression and hormone secretion directly. In uremic patients with secondary hyperparathyroidism (SHPT), however, parathyroid hormone secretion remains elevated despite extremely high FGF23 levels. To determine the mechanism of this resistance, we measured the expression of Klotho, FGFR1, and the proliferative marker Ki67 in 7 normal and 80 hyperplastic parathyroid glands from uremic patients by immunohistochemistry. All uremic patients had severe SHPT along with markedly high FGF23 levels. Quantitative real-time reverse transcription PCR showed that the mRNA levels for Klotho and FGFR1correlated significantly with their semi-quantitative immunohistochemical intensity. Compared with normal tissue, the immunohistochemical expression of Klotho and FGFR1 decreased, but Ki67 expression increased significantly in hyperplastic parathyroid glands, particularly in glands with nodular hyperplasia. These results suggest that the depressed expression of the Klotho–FGFR1 complex in hyperplastic glands underlies the pathogenesis of SHPT and its resistance to extremely high FGF23 levels in uremic patients.

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Secondary hyperparathyroidism (SHPT) is a common complication of chronic kidney disease. Phosphate retention, hypocalcemia, and calcitriol deficiency have long been considered to contribute to the pathogenesis of SHPT.[1–3] In addition, recent data suggest that fibroblast growth factor 23 (FGF23), a novel phosphaturic hormone, has a central role in the progressive decline of calcitriol levels and in the concomitant parathyroid hormone (PTH) hypersecretion in chronic kidney disease.[4–6]

FGF23 is a 32 kD protein that is mainly produced by osteocytes,[7] and it exerts its hormonal effects by binding to its cognate fibroblast growth factor receptor 1 (FGFR1) in the presence of its obligatory co-receptor, Klotho.[8,9] Klotho is a transmembrane protein that determines the tissue specificity of FGF23. In the kidney, FGF23 interacts with the Klotho–FGFR1 complex present in the distal tubule,[10] and thereby presumably inhibits sodium-dependent transporter and 1α-hydroxylase (CYP27B1) activities in the proximal tubule, leading to phosphaturia and reduced synthesis of calcitriol.[11,12] In patients with chronic kidney disease, FGF23 levels increase as kidney function declines to help maintain normal serum phosphate levels, but this results in the aggravation of SHPT because of decreased feedback inhibition by calcitriol.[4–6] Once on dialysis, serum FGF23 levels markedly increase in response to hyperphosphatemia and calcitriol therapy.[13–15]

Importantly, the parathyroid gland also expresses both FGFR1 and Klotho and is a target organ for FGF23.[16,17] Recent studies have shown that FGF23 directly decreases PTH gene expression and secretion. These data suggest that the direct action of FGF23 on PTH secretion is in contrast to its indirect action by inhibition of renal calcitriol production. It is, however, noteworthy that in uremic patients undergoing dialysis, such an indirect effect of FGF23 may be less evident, as renal production of calcitriol is substantially impaired and active vitamin D sterols are frequently used to control SHPT.

Thus, extremely elevated FGF23 levels would be expected to decrease serum PTH levels in uremic patients. However, in these patients, PTH secretion remains elevated despite extremely high FGF23 levels.[14,15] A similar paradox has been observed in refractory SHPT, in which parathyroid glands do not respond to calcium supplementation and calcitriol therapy, which should decrease PTH secretion. In the past, such a resistance to medical treatment has been explained by a decrease in the expression of calcium-sensing receptor and vitamin D receptor, particularly in glands with nodular hyperplasia, which is a more severe form of parathyroid hyperplasia.[18–22]

In the present study, we examined the expression of Klotho and FGFR1 in surgically excised parathyroid glands of uremic patients and compared it with the expression in normal human parathyroid tissue. The recognition of abnormal Klotho–FGFR1 complex expression would provide a new insight into the mechanisms involved in dysregulated PTH secretion and parathyroid cell proliferation in uremic patients with extremely high FGF23 levels.

RESULTS

Patient characteristics

Parathyroid tissue specimens were obtained from 5 patients with normal kidney function and 23 dialysis patients with SHPT. Clinical characteristics of the patients included in the study are shown in Table 1. Control patients had normal levels of serum whole PTH and FGF23, whereas all uremic patients had severe SHPT requiring parathyroidectomy and showed extremely high levels of serum FGF23, as reported previously.[23] Serum calcium, phosphate, and alkaline phosphatase levels were also significantly higher in dialysis patients than in control patients. Nearly all patients with SHPT (21 of 23) were treated with vitamin D sterols at the time of parathyroidectomy, and 43 percent (10 of 23) had a history of treatment with cinacalcet hydrochloride.

Histology and weight of removed parathyroid glands

A total of 7 normal parathyroid glands and 80 hyperplastic parathyroid glands were obtained from the participating patients. All normal parathyroid glands showed clusters of parenchymal cells mixed with a considerable amount of adipose tissue. Hyperplastic parathyroid glands were divided into two types: diffuse hyperplasia ($n = 19$) and nodular hyperplasia ($n = 61$). Diffuse hyperplasia was defined as an increased number of parenchymal cells with normal lobular structures, and nodular hyperplasia was defined as at least one well-circumscribed, encapsulated, and virtually fat cell-free accumulation of parenchymal cells.[24] All uremic patients with SHPT had at least one hyperplastic gland with nodular hyperplasia. The weight of glands with nodular hyperplasia (672 ± 80 mg) was significantly higher than those with diffuse hyperplasia (172 ± 41 mg, $P < 0.001$).

Table 1 | Baseline characteristics of the study population

Variable	Normal (*N*=5)	SHPT (*N*=23)	*P*-value
Age (years)	67 ± 2	54 ± 3	0.002
Sex (male/female)	3/2	9/14	0.36
Duration of dialysis (months)	NA	135 ± 12	—
BUN (mg/dl)	15.3 ± 2.2	60.0 ± 3.7	<0.001
Creatinine (mg/dl)	0.86 ± 0.08	11.19 ± 0.56	<0.001
Calcium (mg/dl)	9.45 ± 0.15	9.98 ± 0.10	0.019
Phosphate (mg/dl)	3.90 ± 0.32	6.08 ± 0.30	<0.001
Alkaline phosphatase (U/l)	196 ± 19	704 ± 189	0.014
PTH(1–84) (pg/ml)	19 ± 3	583 ± 155	0.001
FGF23 (pg/ml)	37 ± 13	12,629 ± 2697	<0.001

BUN, blood urea nitrogen; FGF23, fibroblast growth factor 23; PTH, parathyroid hormone; SHPT, secondary hyperparathyroidism.

Immunohistochemical expression of Klotho, FGFR1, and Ki67

Representative immunohistochemical staining of Klotho, FGFR1, and Ki67 is shown in Figure 1. Ki67 is a proliferation marker expressed in all phases of the cell cycle. In normal glands, expression of Klotho and FGFR1 revealed distinct staining along the parathyroid cell surface (Figure 1a and f), suggesting that the parathyroid is a target organ for FGF23 in humans also. In contrast, the expression of these proteins was substantially reduced in hyperplastic parathyroid glands from uremic patients, particularly in glands with nodular hyperplasia (Figure 1b–d, g–i). Ki67 expression showed mainly nuclear localization, and its positive cells were evidently increased in hyperplastic parathyroid glands compared with normal glands (Figure 1k–n). Interestingly, careful evaluation of serial sections revealed that expression of Klotho and FGFR1 were virtually negative in parathyroid cells of nodular lesions, whereas cells outside such lesions showed weak but definitive positive staining (Figure 2a, b, d–g). A serial section of the gland showed significantly increased Ki67-positive cells in the nodular lesion compared with that in the outside area (Figure 2c, h and i).

We scored the immunohistochemical signals of Klotho, FGFR1, and Ki67 as described previously to perform a semi-quantitative analysis.[21] To confirm the validity of the semi-quantification, we measured Klotho and FGFR1 mRNA levels by quantitative real-time reverse transcription PCR in aliquots from 41 different parathyroid glands. In these patients, half of the surgically removed parathyroid glands were used for immunohistochemistry and the other half were used for real-time reverse transcription PCR. Linear regression analysis showed that immunohistochemical expression of Klotho and FGFR1 correlated significantly with the respective mRNA levels ($r = 0.34$, $P = 0.028$; and $r = 0.36$, $P = 0.021$, respectively).

Next, we compared the semi-quantitative immunohistochemical expression of Klotho, FGFR1, and Ki67 in hyperplastic parathyroid glands obtained from uremic patients with that in normal parathyroid tissue. We found that both Klotho and FGFR1 scores decreased significantly in uremic hyperplastic glands compared with normal tissue, and that these trends were more pronounced in glands with nodular hyperplasia (Figure 3a and b). Ki67 scores increased significantly in glands with nodular hyperplasia compared with either normal tissue or glands with diffuse hyperplasia

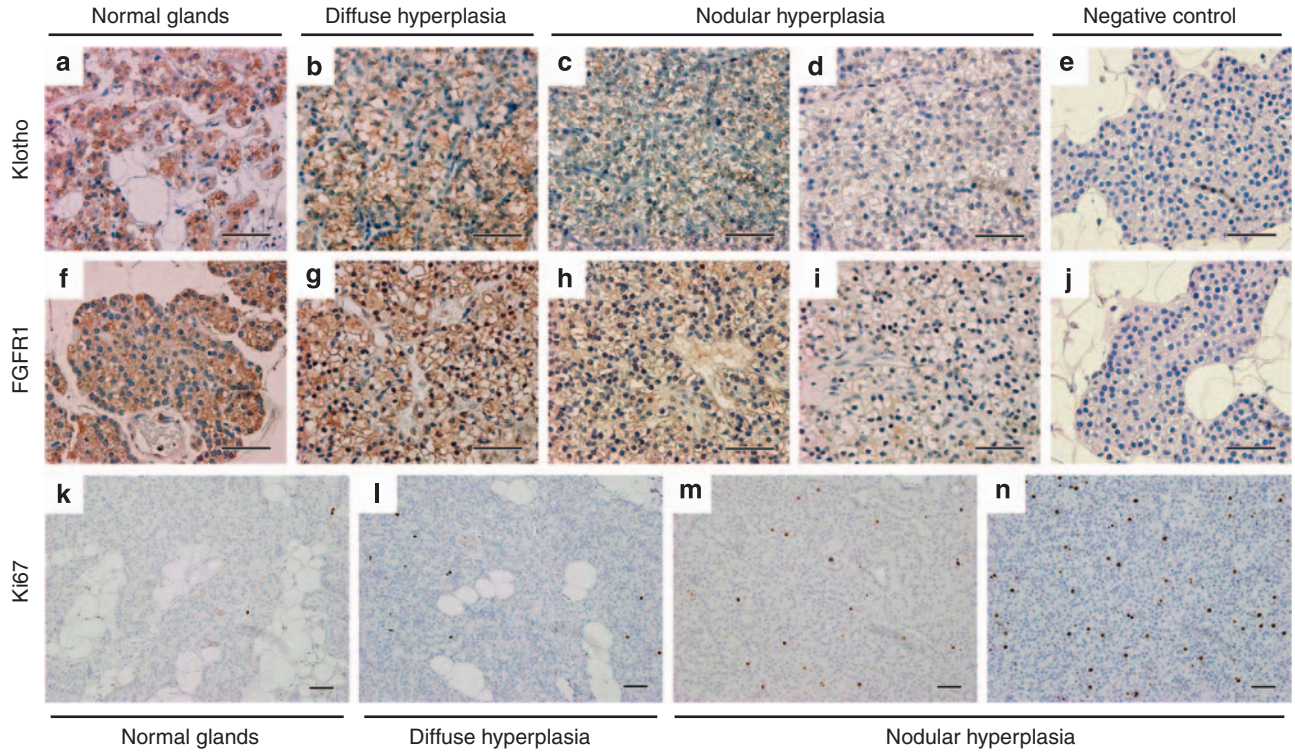

Figure 1 | Representative immunohistochemical staining of Klotho, fibroblast growth factor receptor 1 (FGFR1), and Ki67. (**a**–**e**) Klotho staining (**e**; negative control); (**f**–**j**) FGFR1 staining (**j**; negative control); (**k**–**n**) Ki67 staining. (**a**, **e**, **f**, **j**, **k**) Normal parathyroid glands; (**b**, **g**, **l**) diffuse parathyroid hyperplasia; (**c**, **d**, **h**, **i**, **m**, **n**) nodular parathyroid hyperplasia. Note the distinct staining of Klotho and FGFR1 along the cell surface in normal parathyroid glands. These expressions were substantially reduced in hyperplastic glands from uremic patients, particularly in glands with nodular hyperplasia. Ki67-positive cells were evidently increased in uremic parathyroid hyperplasia. Such an increase in Ki67-positive cells was particularly remarkable in nodular hyperplasia. Each area was scored as follows: grade 3, (**a** and **f**); grade 2 (**b** and **g**); grade 1 (**c** and **h**); and grade 0 (**d** and **i**). Original magnifications: (**a**–**j**), $\times 400$; (**k**–**n**), $\times 200$. Bars = 100 μm.

(Figure 3c). Ki67 score positively correlated with parathyroid gland weight ($r = 0.33$, $P = 0.003$). Notably, there was a significant correlation between Klotho and FGFR1 scores ($r = 0.71$, $P < 0.001$). This agrees with the fact that Klotho is co-expressed with FGFR1.[16] Klotho score was negatively correlated with parathyroid gland weight ($r = -0.24$, $P = 0.033$), and there was a nearly significant negative correlation between FGFR1 score and parathyroid gland weight ($r = -0.21$, $P = 0.057$). However, no significant correlation was found between either Klotho or FGFR1 score with Ki67 score.

DISCUSSION

In the present study, we evaluated Klotho and FGFR1 expression in parathyroid glands from uremic patients with severe SHPT and from control patients with normal kidney function, and showed that these expressions were significantly downregulated in hyperplastic parathyroid glands compared with normal parathyroid tissue. Furthermore, the reduction in Klotho and FGFR1 expression was more remarkable in glands with nodular hyperplasia than those with diffuse hyperplasia.

FGF23 is a hormone that has a broader role in the pathogenesis of alterations in mineral and bone metabolism[25] by interacting with FGFR in the presence of Klotho.[8,9] Klotho directly binds with FGFR1c, 3c, and 4, and increases their affinity to FGF23,[8] whereas Klotho-dependent FGF23 signaling defined by upregulation of the gene, early growth responsive 1 (*Egr-1*), is restricted to interaction with FGFR1c.[9] Indeed, a recent study showed that neither FGFR3 nor FGFR4 is the principal mediator of FGF23 effects in the kidney, suggesting that the Klotho–FGFR1 complex is the main target for FGF23.[10]

Importantly, Klotho and FGFR1 are also co-expressed in parathyroid glands.[16] A previous study showed that FGF23 administration increases *Egr-1* expression in the parathyroid glands, implying that the parathyroid is a physiological target for FGF23.[8] A subsequent study using rats and *in vitro* parathyroid cultures showed that FGF23 suppresses PTH secretion.[16] Other researchers have also reported similar findings using primary bovine parathyroid cell cultures.[17] Thus, it is clear that FGF23 is a negative regulator of PTH secretion at least in normal physiology. Nevertheless, in uremic patients undergoing dialysis therapy, PTH hypersecretion and parathyroid cell proliferation is observed despite elevated FGF23 levels.[14,15] In this context, our finding that the Klotho–FGFR1 complex is severely depressed in hyperplastic glands may shed light on the inability of extremely

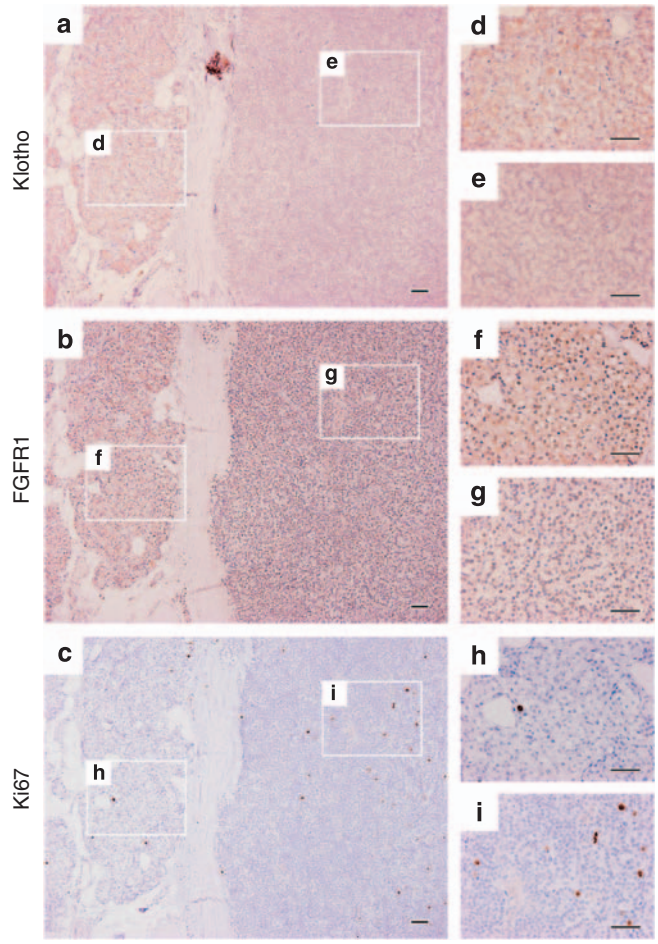

Figure 2 | Immunohistochemical staining of nodular lesions surrounded by diffuse hyperplastic parathyroid tissue. (**a**, **d**, **e**) Klotho staining; (**b**, **f**, **g**) fibroblast growth factor receptor 1 (FGFR1) staining; (**c**, **h**, **i**) Ki67 staining. Boxed areas are shown at higher magnification to right of each frame, as indicated. Both Klotho and FGFR1 expressions were markedly decreased in nodular areas, whereas diffuse hyperplastic cells outside the nodular lesion showed weak but definitive positive staining. Ki67-positive cells were observed predominantly in nodular areas. Original magnification: (**a**–**c**), $\times 100$; (**d**–**i**), $\times 400$. Bars = 100 μm.

high FGF23 levels in sufficiently suppressing PTH secretion in uremic patients. Recent studies suggest that FGF23 levels increase as kidney function declines and are associated with early and progressive calcitriol deficiency, and thus, may contribute to the development of SHPT.[4–6] In addition, progressive depression of the Klotho–FGFR1 complex in hyperplastic parathyroid glands may also have a role in SHPT progression by inducing resistance to the inhibitory effect of FGF23 on PTH secretion. In this study, however, we were unable to directly confirm that the depressed expression of Klotho and FGFR1 cause functional impairment of FGF23 on the parathyroid glands. Future experimental studies are needed to investigate fully the mechanisms underlying the resistance of PTH hypersecretion to extremely high FGF23 levels in uremia. Specifically, whether or not systemic or local upregulation of Klotho could mediate abnormal PTH secretion and parathyroid cell proliferation is of interest and worthy of further research.

The mechanism by which Klotho and FGFR1 expression is reduced in uremic parathyroid hyperplasia is unclear. A similar reduction in Klotho expression also occurs in parathyroid adenoma from patients with primary hyperparathyroidism.[26] In that study, parathyroid Klotho mRNA levels were inversely correlated to serum calcium level, which is in agreement with a previous study showing that low extracellular calcium is a stimulus for Klotho-mediated PTH secretion.[27] Relevantly, Klotho expression is also decreased in the kidney obtained from chronic kidney disease patients.[28] Studies in animal models have shown that systemic administration of calcitriol or phosphorus-restricted diet enhanced the renal expression of Klotho.[29,30] Whether these factors modulate the expression of Klotho and/or FGFR1 in the parathyroid glands needs to be determined in future research.

Our study also showed that even in the same patient, both Klotho and FGFR1 expressions were more severely reduced in nodular hyperplasia compared with diffuse hyperplasia. Thus, it is clear that the severity of parathyroid hyperplasia itself is associated with a reduction in the Klotho–FGFR1 complex in uremic patients. In the past, progressive reduction in calcium-sensing receptor and vitamin D receptor expression has been observed during the course of parathyroid hyperplasia.[18–22] A reduction in vitamin D receptor expression may reportedly precede the onset of parathyroid cell proliferation,[31] and more recent studies suggest that activation of the epidermal growth factor receptor by tumor growth factor-α is the cause for both hyperplastic growth and vitamin D receptor reduction.[32] On the other hand, reduced calcium-sensing receptor in the parathyroid glands may follow the development of hyperplasia and then contribute to further parathyroid growth.[33] Future studies should elucidate whether depressed expression of the Klotho–FGFR1 complex has a role in the pathogenesis of SHPT or it is only a secondary change caused by the progression of parathyroid hyperplasia.

We previously reported that serum FGF23 levels predict future refractory SHPT in dialysis patients.[14,15] Although the mechanism of this finding is unclear, it is possible that chronic phosphate retention, as reflected by elevated FGF23 levels, may contribute to further progression of parathyroid hyperplasia, because high phosphate level directly stimulates PTH secretion and parathyroid cell proliferation.[34,35] Another possibility is that high levels of FGF23 at baseline may be a consequence of prolonged active vitamin D administration for severe hyperparathyroidism,[13] which may be related to future resistance to vitamin D therapy. Furthermore, the results of this study propose that increased levels of FGF23 may reflect depression of the Klotho–FGFR1 complex, which is associated with a more severe form of parathyroid hyperplasia. These possibilities should be examined in future studies.

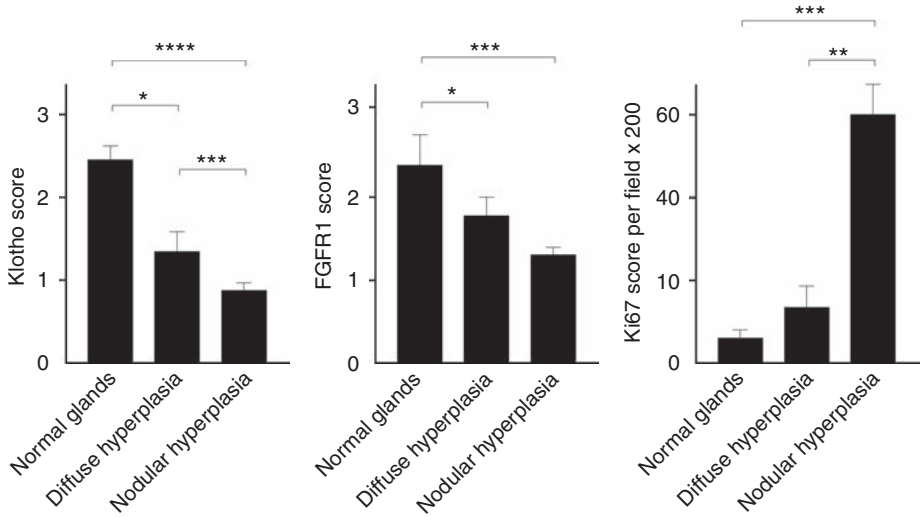

Figure 3 | Semi-quantification expression of Klotho, fibroblast growth factor receptor 1 (FGFR1), and Ki67 in parathyroid tissue of each group. The values shown are means ± s.e.m. *$P<0.1$; **$P<0.05$; ***$P<0.01$; ****$P<0.001$.

Finally, we did not find a significant association of either Klotho or FGFR1 expression with parathyroid cell proliferation as evaluated by Ki67 score, although the expression of the Klotho–FGFR1 complex was markedly downregulated in parallel with the severity of parathyroid hyperplasia and an increase in parathyroid gland weight. The precise reason for these findings is unclear; however, it is possible that the inclusion of patients who have a history of cinacalcet treatment may have influenced the results, as calcimimetics prevent excessive parathyroid cell proliferation and gland hyperplasia in uremic rats.[36,37] Further studies are needed to examine whether FGF23 mediates parathyroid cell proliferation in the presence of the Klotho–FGFR1 complex and whether such an effect is altered in uremia.

In conclusion, Klotho and FGFR1 expression decreased significantly in uremic parathyroid hyperplasia, particularly in glands with nodular hyperplasia. The results of this study suggest that the depressed expression of the Klotho–FGFR1 complex in hyperplastic glands may explain, at least in part, the resistance to extremely high FGF23 levels that would be expected to decrease the serum PTH levels. Additional studies are needed to determine whether downregulation of the Klotho–FGFR1 complex has a role in abnormal PTH secretion and parathyroid growth in uremia. Further insights into the FGF23–Klotho–FGFR1 system is important in understanding the role of FGF23 in the pathogenesis of SHPT, and in developing therapeutic approaches to treat SHPT in uremic patients who have extremely elevated FGF23 levels.

MATERIALS AND METHODS

Parathyroid gland tissues

Normal parathyroid glands were obtained in conjunction with thyroid surgery from patients with normal kidney function, and hyperplastic parathyroid glands were obtained from dialysis patients with SHPT who underwent total parathyroidectomy with autotransplantation. According to the guidelines released by the Japanese Society for Dialysis Therapy,[38] the indications for parathyroidectomy were the presence of severe hyperparathyroidism (persistent high serum intact PTH levels >500 pg/ml) associated with hyperphosphatemia (serum phosphate >6.0 mg/dl) and/or hypercalcemia (serum calcium >10.0 mg/dl) that was refractory to medical therapy. This study adhered to the principles of the Declaration of Helsinki and was approved by the ethical committee of the Kobe University School of Medicine. All patients provided an informed consent.

Antibodies

A polyclonal anti-human Klotho antibody was kindly provided by Kyowa Hakko Kirin (Tokyo, Japan). This antibody was generated by immunizing rabbits with a synthesized peptide (PLQPATGDVASDSYNNVFRDT) corresponding to a sequence in the human Klotho protein (amino acids 116–138). Affinity-purified antibody was obtained by extraction from antiserum using the peptide immobilized on agarose gel (SulfoLink kit; Pierce, Rockford, IL, USA). A rabbit anti-FGFR1 polyclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and a mouse monoclonal anti-human Ki67 antibody was purchased from DAKO (Glostrup, Denmark).

Immunohistochemistry

Parathyroid glands were fixed in 10% formalin and embedded in paraffin. The sections (3 μm) were deparaffinized in xylene and rehydrated through an ethanol series. Sections were heated in a microwave for 20 min in Target Retrieval Solution at pH 6.0 (DAKO) for Klotho staining and in 0.01 mmol/l citrate buffer at pH 6.0 for Ki67 staining. Endogenous peroxidase was inactivated with hydrogen peroxide for 5 min. After blocking with 10% goat serum for 15 min, the sections were incubated with anti-Klotho antibody (1:2500 dilution, 60 min), anti-FGFR1 antibody (1:150 dilution, 30 min), and anti-Ki67 antibody (1:75 dilution, 30 min). Universal Negative Control Rabbit (DAKO) was used as a negative control. Sections for Klotho staining were incubated for 30 min with

Table 2 | Primers used for RT-PCR

Gene	Forward primer	Reverse primer
FGFR1	CCATCGACCATGGATGGTTTC	TGGGATTACAGGCGTGAGCA
Klotho	TGAGGTCCTGTCTAAACCCTGTGTC	ATGTGCAAGGCCCTCAACAAG
GAPDH	GCACCGTCAAGGCTGAGAAC	TGGTGAAGACGCCAGTGGA

FGFR1, fibroblast growth factor receptor 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RT, reverse transcription.

peroxidase-labeled polymer conjugated to goat anti-rabbit immunoglobulins (EnVision System/HRP; DAKO), whereas sections for FGFR1 and Ki67 staining were incubated with anti-rabbit/mouse secondary antibody for 15 min and with streptavidin–horseradish peroxidase for 15 min (LSAB2 System-HRP; DAKO). Finally, all the sections were stained with 3,3′-diaminobenzidine tetrahydrochloride for 5 min and counterstained with hematoxylin for 1 min. Every step was followed by three washes with phosphate-buffered saline for 5 min, and all the procedures were performed at room temperature.

Semi-quantification

Semi-quantitative immunohisochemical analysis was performed as previously described.[21] In brief, the immunoreactivities for Klotho, FGFR1, and Ki67 antigen were evaluated in six areas that were randomly selected by one observer. The Klotho and FGFR1 signals were scored according to the percentage of positive staining along the cell surface using the following grading criteria: grade 3, more than 75%; grade 2, 50–75%; grade 1, 25–50%; and grade 0, below 25% (Figure 1). The number of Ki67-positive cells in each area, counted at a magnification of $\times 200$, was designated as the Ki67 score. This analysis was performed by three independent observers (the intra- and inter-observer coefficients of variation were all $<10\%$). After taking an average of the scores, Klotho, FGFR1, and Ki67 scores were assigned to each gland.

RNA isolation and quantitative real-time reverse transcription PCR

Parathyroid tissues were immediately freeze-dried in liquid nitrogen and stored at $-80\,^{\circ}C$ until RNA isolation. Total RNA was isolated using Isogen (Nippon Gene, Tokyo, Japan). First-strand cDNA was synthesized using a TaKaRa RNA PCR kit (AMV) (Takara Biochemicals, Osaka, Japan). Quantitative real-time PCR analysis was performed by using the Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) and the FastStart Universal SYBR Green Master mix (Roche, Indianapolis, IN, USA). The relative gene expression was normalized to the gene expression of glyceraldehyde 3-phosphate dehydrogenase in the same sample. Sequences of primers used for quantitative real-time reverse transcription PCR are listed in Table 2. Specificity of the PCR products was verified by melting curve analysis and agarose gel electrophoresis.

Laboratory methods

Blood samples were obtained 1 or 2 days before parathyroidectomy. Samples were stored for <2 h at $5\,^{\circ}C$ until centrifugation. On arrival at the laboratory, the blood samples were centrifuged at 3000 r.p.m. for 10 min, aliquoted, and stored at $-80\,^{\circ}C$ until analysis. Serum PTH(1–84) levels were measured using a third-generation PTH assay (Whole PTH; Scantibodies Laboratories, Santee, CA, USA). Serum FGF23 levels were determined using a sandwich ELISA kit (Kainos Laboratories, Tokyo, Japan) that exclusively detects the full-length FGF23 peptide. Serum calcium, phosphorus, albumin, alkaline phosphatase, blood urea nitrogen, and creatinine were measured using standard methods. The measured serum calcium levels were adjusted to albumin levels using the following equation: corrected calcium = serum measured calcium + (4−albumin).

Statistical analysis

All values are expressed as means ± s.e.m. Statistical significance was determined by Student's *t*-test and Fisher's exact test for two-group comparisons and by one-way ANOVA (analysis of variance) for multiple-group comparison followed by Bonferroni's *post-hoc* test. Pearson's correlation coefficient analyses were used to examine the relationships between each parameter. $P<0.05$ was considered statistically significant. All computations were performed using Dr SPSS II for Windows version 11.01 J (SPSS Japan, Tokyo, Japan).

DISCLOSURE

All the authors declared no competing interests.

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