of CD patients and 18% of UC patients and between −2.5 and −1.0 SD in 50% and 55% of subjects with CD and UC, respectively (Table).

Multiple regression analyses for variables associated with BMD *Z* scores at various sites

Multiple regression analyses were done for BMD including BMI, plasma concentrations of PK, MK-7, and 25OH-D as independent variables. Serum PTH level was excluded since coinclusion of 25OH-D and PTH caused multicollinearity to skew the results. As shown in Table , BMI was a significant predictor of BMD at weight-bearing sites such as the lumbar spine, femoral neck, and total hip. Plasma MK-7 and 25OH-D concentrations were significant predictors of femoral neck BMD. Plasma PK concentration was a significant predictor of BMD at the distal one-third of radius and lumbar spine.

Analysis of food intake in CD and UC patients

Food intake could be evaluated in 25 patients (15 with CD and 10 with UC). Fat intake was significantly lower and protein intake was significantly higher in patients with CD than those with UC. The results were similar when expressed as the percentage of total energy intake. The adequate intakes (AI) for calcium in Japan are 600–650 mg for men and 550–600 mg for women. AI for vitamin K is 75 μg for men and 65 μg for women, respectively, and that for vitamin D is 5 μg [ ]. As a whole, although the average calcium intake was below AI, vitamin K and D intakes apparently exceeded AI (Table).

Ten patients with CD were on enteral nutrition (EN) with almost fat-free formula; Elental® (Ajinomoto Pharma, Tokyo, Japan) with 18.8%, 1.4%, and 79.8% of total energy contributed by protein, fat, and carbohydrate, respectively. One patient with UC was on total parenteral nutrition. When nutrient intake was compared between CD patients with EN and those without EN, the former had higher protein and carbohydrates intakes and lower fat intake than the latter. Regarding other nutrients intake, there was no significant difference between the two groups except calcium. There were no significant differences in plasma vitamin K and 25OH-D concentrations between these groups (data not shown).

Table 2 BMD in patients with CD and UC

	CD (n=18)	UC (n=22)	p value
BMD (g/cm^2)			
Lumbar spine (L1–4)	0.880±0.072	0.931±0.138	0.152
Femoral neck	0.697±0.105	0.768±0.126	0.064
Total hip	0.801±0.120	0.910±0.136	0.012
Distal one-third of radius	0.634±0.066	0.664±0.084	0.222
Z scores			
Lumbar spine (L1–4)	−0.96±0.57**	−0.14±1.13	0.005
Femoral neck	−1.00±0.78**	−0.09±1.16	0.005
Total hip	−0.85±0.91**	0.27±1.11	0.001
Distal one-third of radius	−2.19±0.94**	−1.29±1.79**	0.064
T scores			
Lumbar spine (L1–4)	−1.18±0.59	−0.79±1.06	0.155
Femoral neck	−1.14±0.85	−0.56±1.05	0.067
Total hip	−0.95±0.97	−0.11±1.08	0.014
Distal one-third of radius	−2.31±1.00	−1.83±1.81	0.055

Values represent the mean±SD, and comparison between CD and UC groups was made with unpaired t test
**p<0.01, statistically significant difference from zero with one-sample t test in the Z score

Table 3 Multiple regression analyses for the determination of independent factors for BMD

Sites	R^2	Variable	β coefficient	p value
Lumbar spine	0.529	BMI	0.663	0.005
		Plasma PK	0.612	0.035
Femoral neck	0.748	BMI	0.363	0.028
		Plasma MK-7	0.295	0.036
		Plasma 25OH-D	0.484	0.037
Total hip	0.731	BMI	0.438	0.012
Distal one-third of radius	0.388	Plasma PK	0.813	0.016

Only significant predictors are shown. Determinants of independent predictors for BMD at each site were analyzed by multivariate analysis with forced entry. Variables included were BMI, plasma 25OH-D, PK, and MK-7

Multiple regression analyses for plasma vitamin K and 25OH-D concentrations

Multiple regression analyses revealed that fat intake was a significant determinant of plasma PK and 25OH-D levels. Vitamin K intake was a significant predictor for plasma MK-7 level (Table).

Discussion

In this study, we have studied the IBD-induced osteoporosis in relation to vitamin K and D status of the patients. Decreased BMD and high-turnover bone was far more pronounced in patients with CD than those with UC.

Although glucocorticoid treatment is one of the postulated pathogenic factors for osteoporosis in IBD [, , ,], current use of glucocorticoid was not associated with decreased BMD in the present study. Unfortunately, the possible involvement of glucocorticoid could not be

Table 4 Food intake in CD and UC patients

	IBD (n=25)	CD (n=15)		UC (n=10)	p value	EN p value
		EN therapy (n=10)	Non-EN therapy (n=5)			
Energy (kcal)	1,707±479 (1,580)	1,961±465 (1,796)	1,412±320 (1,501)	1,602±466 (1,524)	0.338[a]	0.055[a]
Energy intake from EN (kcal)	–	810±318 (750) (min 300–max 1200)	–	–	–	–
Proportion of total energy intake from EN (%)	–	42.0±16.8 (39.1) (min 20–max 77)	–	–	–	–
Protein (g)	68.2±19.3 (62.8)	81.9±21.1 (79.8)	60.3±12.3 (61.9)	58.5±11.8 (57.0)	0.022[b]	0.028[b]
Fat (g)	29.9±13.9 (28.3)	22.1±10.0 (24.0)	29.1±7.5 (30.7)	38.1±15.8 (38.1)	0.030[b]	0.164[b]
Carbohydrates (g)	287.8±98.4 (274.3)	359.0±85.3 (339.3)	223.5±60.3 (242.1)	248.9±85.5 (258.9)	0.098[b]	0.005[b]
Calcium (mg)	483±250 (431.0)	662±230 (675)	380±144 (351)	356±214 (354.5)	0.032[b]	0.014[b]
Vitamin K (μg)	131.1±124.6 (73.0)	96.8±68.8 (66.0)	207.0±220.9 (73.0)	127.5±102.2 (97.0)	0.846[a]	0.337[b]
Vitamin D (μg)	9.6±10.4 (6.9)	9.3±7.4 (7.4)	10.2±13.3 (1.5)	9.6±12.5 (6.6)	0.782[a]	0.893[b]
Macronutrient (% energy)						
Protein	16.2±2.9 (15.6)	16.7±2.0 (16.0)	17.4±2.4 (17.4)	15.2±3.7 (14.4)	0.008[a]	0.617[b]
Fat	16.4±7.7 (14.9)	10.1±4.0 (10.6)	18.8±3.9 (19.0)	21.5±7.6 (21.1)	0.009[b]	0.004[b]
Carbohydrates	66.5±8.7 (66.1)	73.4±6.4 (72.7)	62.7±5.1 (61.3)	61.5±7.8 (61.9)	0.017[b]	0.005[b]

Values represent the mean±SD with values in parentheses being the median. "p value" and "EN p value" represent the comparison between CD and UC patients and the comparison between CD subjects with EN and those without EN, respectively

[a] Comparisons between CD and UC patients and that between CD with EN and without EN were done with unpaired t test depending on normality

[b] Comparisons between CD and UC patients and that between CD with EN and without EN were done with Mann–Whitney test depending on normality

evaluated in more detail, since most of them were referred to the university hospital from another hospital and cumulative dose of glucocorticoid could not be precisely calculated. We believe, however, that glucocorticoid use is unlikely to be mainly responsible for the decreased BMD in the current subjects based on the following consideration. Trabecular bone is mainly affected in glucocorticoid-induced osteoporosis (GIO) [ ]. In GIO, decreased BMD is most prominent at the lumbar spine with trabecular predominance [ ]. In contrast is the present finding that decreased BMD was most marked at the distal one-third of radius, a site of cortical predominance.

Table 5 Multiple regression analyses for the predictor(s) of plasma 25OH-D, PK, and MK-7 levels

	R^2	Variable	β coefficient	p value
Plasma PK	0.586	Fat intake	0.620	0.030
Plasma MK-7	0.464	Vitamin K intake	0.708	0.036
Plasma 25OH-D	0.452	Fat intake	0.584	0.046

Only significant predictors are shown. Independent predictor for plasma PK, MK-7, or 25OH-D concentrations was analyzed by multivariate analysis with forced entry. Serum CRP level and intakes of protein, fat, and carbohydrates were included in all analyses. Vitamin D intake was additionally included in the analysis for plasma 25OH-D concentration. For plasma PK and MK-7, vitamin K intake was additionally included

Another possible factor includes disease severity. IBD is associated with increased production of inflammatory cytokines, e.g., IL-1, IL-6, and TNF-α which are potent stimulators of osteoclastic bone resorption [–]. Although circulating concentration of these cytokines could not be measured, serum level of CRP was evaluated as an inflammation marker. Although serum CRP level was higher in CD patients, it was not associated with BMD (data not shown).

Low BMI is another factor to be associated with IBD-related osteoporosis [,], but the current results that the average BMI was in the normal range and BMD at nonweight-bearing site was also decreased, which make it unlikely that the reduced BMD in these subjects is related to their BMI.

Then, we focused our attention to the possible involvement of vitamin K and D deficiency. Unfortunately, no single measure can represent the vitamin K status with PK and MK-7 being the two major circulating forms. PK is rich in green vegetables, whereas MK-7 content is extraordinarily high in fermented soy "natto," which is a common food in Japan, but not elsewhere [,]. Large standard deviation in plasma MK-7 concentration probably reflects that some Japanese favors, but some dislike "natto." Indeed, a large geographic difference in plasma MK-7 concentration in Japan was reported to be due to the frequency of natto intake [ ]. Since most vitamin K intake

comes from green vegetables in America and Europe [,], previous reports on the plasma concentration of vitamin K from outside Japan focused on PK [,]. Although circulating vitamin K levels have been measured with various methods, the present data were obtained with our newly developed LC-APCI-MS/MS procedure with stable isotope-labeled internal standard yielding high sensitivity and specificity [ ]. In our recent report from the Nagano study using the same assay procedure, mean plasma PK level was 1.52 ng/mL, 1.74 ng/mL, and 1.29 ng/mL in healthy women aged 30–49, 50–69, and over 70 years, respectively [ ]. Thus, blood level of vitamin K was much lower in IBD patients than that in the healthy Japanese measured by the same assay. The data in the Nagano study may be higher than those in the average Japanese, since many participants in the Nagano study were farmers with much vegetable consumption, for which further discussion will be made in the next paragraph.

Then, we considered the physiological relevance of the above data. We measured serum levels of PIVKA-II and ucOC as the sensitive markers of vitamin K deficiency in the liver and bone, respectively, with the cut-off values being 28 mAU/mL for PIVKA-II and 4.5 ng/mL for ucOC. Both levels were significantly higher in CD patients than those with UC. These results, together with the decreased plasma levels of PK and MK-7 in CD patients, strongly suggest that circulating vitamin K levels are decreased at least in patients with CD. Decreased plasma levels of 25OH-D, PK, and MK-7 are likely to have physiological significance considering that they were determinants of BMD at some measurement sites as shown in Table , as well as the above-mentioned elevated concentrations of PIVKA-II and ucOC.

The average and median concentration for ucOC, but not for PIVKA-II, was above the cut-off value in these subjects, especially CD patients. Serum PIVKA-II level exceeded the cut-off level in only 25% and 4% of patients with CD and UC, respectively. In contrast, serum ucOC concentration was above the cut-off value in 92% and 36% of patients with CD and UC, respectively. These differences could be explained by a pharmacokinetic feature called "first-pass effect." Vitamin K absorbed from the gastrointestinal tract is transported to the liver via the portal vein where it is used for the γ-carboxylation of clotting factors [,]. Only the vitamin K unutilized in the liver will be available to the bone. Therefore, the bone is likely to be much more susceptible to vitamin K deficiency than the liver. Thus, serum ucOC level well reflects the skeletal vitamin K deficiency, but needs to be interpreted with caution that it is also affected by bone turnover as exemplified with its association with BAP.

The average serum concentration of 25OH-D was 11.5 and 20.2 ng/mL in CD and UC patients, respectively. Serum PTH concentration was reciprocally higher in CD than in UC. Thus, most IBD patients, especially those with CD, were considered to be vitamin D deficient.

The next consideration relates to the factor(s) responsible for the deficiency of these vitamins. As shown in Table , there was no significant difference in vitamin K and D intakes between CD and UC, which suggests that the difference in blood levels of these vitamins could not be ascribed to the difference in their intake. Malabsorption of these vitamins would be the most likely explanation for the apparent discrepancy, which is compatible with the previous report that the absorption of exogenously administered vitamin D_2 was severely disturbed in CD, but not in UC [ ].

As the basis for the malabsorption of vitamin K and D, compromised ability of the intestine to absorb these vitamins would be the most fundamental because of intestinal inflammation or intestinal resection in some cases. In the current study, multiple regression analyses revealed that fat intake was a significant determinant of plasma concentrations of both PK and 25OH-D. Many patients in the current study were under nutritional therapy with restricted fat intake, since excessive fat intake is considered to worsen the intestinal inflammation in IBD patients. These results suggest that restricted fat intake could be another factor responsible for the impaired absorption of vitamin K and D, which, however, is not supported by some previous studies. For example, Tangpricha et al. [ ] reported that vitamin D dissolved in fat-free orange juice was effectively absorbed from the intestine and indicated that fat content of the diet little influenced vitamin D absorption. Thus, further studies, favorably the intervention ones, are required on the role of fat restriction on the absorption of fat-soluble vitamins.

Unlike PK, vitamin K intake was the significant predictor for plasma MK-7 level. The difference between two vitamin K analogs may reflect their pharmacokinetic difference such as the far longer half-life of MK-7 than PK [ ], although further detailed studies are needed. Actually, this study is a baseline valuation. Follow-up study is now under way to evaluate the patients' vitamin status and BMD with milder food restriction with more use of immunosuppressants and biomodulators.

In the present study, vitamin K and D status of IBD patients was both studied, which was not adopted before. The intake of vitamins and their plasma concentration were simultaneously evaluated, which was not usually the case in the previous studies. These would be the strength of the current study. We have to mention two limitations of this study. First, the number of subjects studied was not so large. Thus, it could not be determined whether vitamin K and D deficiency observed in the current study was associated with increased fracture risk as reported in the

previous report [ ]. Next, the patients were under nutritional therapy with restricted fat intake. Thus, further studies with larger number of subjects with wider variety of background profiles are necessary to generalize the present findings.

In summary, BMD was decreased and plasma concentrations of PK, MK-7, and 25OH-D were quite low in patients with IBD, especially CD, despite apparently sufficient intake of these vitamins. Impaired intestinal absorption of these fat-soluble vitamins is likely to be associated with vitamin K and D deficiency and bone loss in IBD.

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Conflicts of interest None.

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Original Article

Hypovitaminosis D and K are highly prevalent and independent of overall malnutrition in the institutionalized elderly

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There have been methodological problems for studying hypovitaminosis D and K in the elderly. First, studies were done either by evaluating food intake or measuring their circulating levels, but rarely by both in Japan. In this paper, vitamin D and K intakes and their circulating levels were simultaneously determined. Second issue is whether hypovitaminosis D and K are independent of general malnutrition, prevalent in the elderly. We tried to statistically discriminate them by principal component analysis (PCA). Fifty institutionalized elderly were evaluated for their circulating 25 hydroxy-vitamin D (25OH-D), intact parathyroid hormone (PTH), phylloquinone (PK), menaquinone-7 (MK-7) levels, and their food intake. Although average vitamin D intake (7.0 μg/day) exceeded the Japanese Adequate Intake (AI) of 5.0 μg/day, average serum 25OH-D concentration was in the hypovitaminosis D range (11.1 ng/mL). Median vitamin K intake was 168 μg/day, approximately 2.5 times as high as AI for vitamin K. Nevertheless, plasma PK and MK-7 concentrations were far lower than those of healthy Japanese elderly over 70 years old. PCA yielded four components; each representing overall nutritional, vitamin K_2, vitamin D, and vitamin K_1 status, respectively. Since these components are independent of each other, vitamin D- and K-deficiency in these subjects could not be explained by overall malnutrition alone. In summary, institutionalized elderly had a high prevalence of hypovitaminosis D and K, and the simultaneous determination of their circulating level and dietary intake is mandatory in such studies. PCA would yield fruitful results for eliminating the interference by confounders in a cross-sectional study.

Key Words: hypovitaminosis D, hypovitaminosis K, principal component analysis, adequate intake, institutionalized elderly

INTRODUCTION

Vitamin D is of utmost importance in enhancing the intestinal absorption of calcium and phosphorus,[1,2] with its deficiency causing skeletal mineralization defect; rickets and osteomalacia. Recently, it has come to the general attention that inadequate supply of vitamin D, even in its milder form (vitamin D insufficiency), is associated with increased risk of fracture through negative calcium balance, hence secondary hyperparathyroidism.[1,2] Vitamin D insufficiency is also reported to be associated with muscle weakness. Recent clinical studies have indicated that intervention with vitamin D supplementation reduced the incidence of falling in elderly subjects.[3] Clinically important non-vertebral fractures, such as hip and wrist fractures are triggered by falling. Thus, vitamin D insufficiency would render the elderly subjects more prone to fracture through its effects both on the skeleton and muscle. Recently, lower serum level of 25 hydroxy-vitamin D (25OH-D) was reported to be a significant risk factor even for mortality.[4]

Vitamin D insufficiency is quite common in the elderly population,[5,6] and institutionalized elderly are at even higher risk for vitamin D insufficiency.[7-10] Factors hitherto postulated to be responsible include low dietary vitamin D intake,[7,9] reduced dermal capacity to produce vitamin D with aging and minimal sun exposure.[11,12]

In contrast to vitamin D, the skeletal action of vitamin K has called our attention only quite recently. The only biological action of vitamin K has been considered to be its role as the coenzyme of γ-glutamyl carboxylase (GGCX) in the liver, by which additional carboxyl group is introduced into the glutamic acid residue in four of the

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Table 1. Background profiles and results from blood tests of the study subjects.

	Total	Male	Female	*p* value
n	50	15	35	-
Age (y)	87.6±8.0 (88.5)	84.9±7.9 (83.0)	88.7±7.8 (90.0)	0.133
Level of care needed	3.6±1.1 (4.0)	3.3±1.0 (3.0)	3.7±1.2 (4.0)	0.228
Body height (cm)	144.0±11.6 (142.0)	157.0±7.8 (159.0)	138.4±7.8 (139.0)	< 0.01
Body weight (kg)	43.6±9.3 (43.2)	50.3±7.9 (49.9)	40.7±8.3 (38.1)	0.001
Body mass index (kg/m2)	21.0±3.8 (20.1)	20.5±3.4 (19.6)	21.3±4.0 (20.2)	0.476
Serum albumin (g/dL)	3.7±0.4 (3.7)	3.8±0.4 (3.9)	3.6±0.4 (3.6)	0.136
Serum total cholesterol (mg/dL)	184±37 (184)	186±26 (195)	183±41 (183)	0.828
Serum triglyceride (mg/dL)	98±41 (92)	96±47 (75)	98±39 (93)	0.403
Serum aminotransferase (U/L)	22±11 (19)	20±7 (17)	22±12 (19)	0.603
Serum alanine aminotransferase (U/L)	16±10 (13)	16±7 (13)	16±12 (12)	0.235
eGFR (mL/min./1.73m2)	61±20 (60)	67±19 (67)	59±21 (57)	0.208
Serum 25-hydroxyvitamin D (ng/mL)	11.1±3.1 (11.2)	10.3±3.5 (9.3)	11.5±3.0 (11.6)	0.274
Serum parathyroid hormone (pg/mL)	30.8±11.8 (30.0)	29.9±11.1 (31.0)	31.3±12.2 (30.0)	0.736
Plasma phylloquinone (ng/mL)	0.73±0.70 (0.58)	0.62±0.29 (0.60)	0.77±0.82 (0.53)	0.992
Plasma menaquinone-7 (ng/mL)	0.53±0.37 (0.45)	0.59±0.47 (0.47)	0.51±0.32 (0.44)	0.849

Data are expressed as mean±SD with the values in parentheses showing the median.
Comparison of indices between males and females were done by unpaired t test or Mann-Whitney test depending on normality.
eGFR; estimated Glomerular Filtration Rate.

Table 2. Daily dietary intakes of the study subjects.

	Total	Male	Female	*p* value
Energy (kcal)	1322±159 (1387)	1374±96 (1416)	1300±175 (1386)	0.160
Protein (g)	51.0±5.8 (53.3)	53.1±3.6 (54.6)	50.2±6.3 (53.5)	0.091
Fat (g)	32.8±3.9 (34.6)	34.2±2.4 (35.3)	32.2±4.3 (34.5)	0.095
Carbohydrates (g)	178±20 (186)	185±12 (189.7)	175±21 (186)	0.093
Calcium (mg)	494±53 (504)	503±50 (506)	490±54 (502)	0.157
Vitamin D (μg)	7.0±1.4 (7.7)	7.4±0.9 (7.8)	6.9±1.5 (7.6)	0.107
Vitamin K (μg)	155±30 (168)	164±19 (172)	151±33 (168)	0.107

Data are expressed as mean±SD with the values in parentheses showing the median. Comparison of indices between male and women were done by unpaired t test or Mann-Whitney test depending on normality.

blood coagulation factors (II, VII, IX, X) to yield γ-glutamic carboxyl (Gla) residue.[13] Other extrahepatic proteins are also γ-carboxylated by GGCX, such as osteocalcin (bone Gla protein; BGP) and matrix gla protein (MGP).[14] Recent evidences suggest that vitamin K deficiency is associated with increased risk of fracture. When subjects were categorized into quartiles according to their vitamin K intake, fracture risk in the lowest quartile was twice as high as that in the highest quartile.[15] The age-adjusted incidence of vertebral fracture was significantly higher in subjects with low plasma phylloquinone levels than those with high plasma levels in Japanese women.[16] In addition, the association of circulating vitamin K level and bone mineral density (BMD) has also been reported. For example, low plasma phylloquinone concentration was associated with low BMD at the femoral neck in men, and lower spine BMD in postmenopausal women without estrogen replacements.[17] High serum concentration of undercarboxyled osteocalcin (ucOC), which is a sensitive indicator of skeletal vitamin K insufficiency, was a significant risk factor of hip fracture independent of BMD.[18,19]

Plasma phylloquinone level is subject to alteration by aging,[20,21] and elderly subjects have been reported to have low plasma phylloquinone concentrations.[22] Of note is the report that elderly nursing home residents generally had a poor dietary vitamin K intake compared to the ambulatory elderly.[23]

Studies on the role of hypovitaminosis D and K in the elderly, especially the institutionalized ones are greatly hampered by the fact that they are also generally malnourished. Arguments against the significance of these vitamins have been made that decreased serum concentrations of these vitamins is merely a reflection of overall malnutrition. In this paper, we have tried to statistically discriminate hypovitaminosis D and K from general malnutrition by using principal component analysis (PCA), which has been employed in clinical nutrition for the analyses of dietary pattern.[24,25]

MATERIALS AND METHODS

Subjects

The study subjects were 50 institutionalized elderly (male 15, female 35) in a nursing home, Kayu-Shirakawa. Exclusion criteria were routine medication that has potential interference with vitamin D or vitamin K status. Detailed information about this study was given and written consent was obtained from the subject or the proxy. The study protocol was approved by the ethical committee in Kyoto Women's University.

Laboratory Data

Blood was obtained after overnight fasting. After centrifugation, serum was kept frozen at –30°C until analysis. Serum concentration of 25OH-D was measured by radioimmunoassay (RIA) (DiaSorin, Stillwater, MN, USA). Circulating level of intact parathyroid hormone (PTH) was measured by electro chemiluminescent immunoassay (ECLIA) (Roche Diagnostics, Mannheim, Germany). Plasma vitamin K_1 (phylloquinone; PK), and menaquinone-7 (MK-7) levels were determined by high-performance liquid chromatography-tandem mass-mass spectrometry with atmospheric pressure chemical ionization (LC-APCI-MS/MS) using a HPLC system (Shimadzu, Kyoto, Japan) and API3000 LC-MS/MS System (Applied Biosystems, Foster City, CA) with ^{18}O-labeled vitamin K as the internal standard.[26]

Nutrition Intake Study

Since the subjects were institutionalized and their diet was supplied from the institution, their nutrients and energy intake were calculated by multiplying the supplied nutrients on the basis of the Standard Tables of Food Composition in Japan, 5th ed. with the average percentage intake in a preceding month by the staff.[27] Percentage intake was assessed for each subject at every meal, and the monthly average percentage intake was calculated. Based on these records, their intake of energy and nutrients was calculated using software (Healthy Maker Pro 501, Mushroom Software Corp, Okayama, Japan).

Statistical Analyses

Statistical analyses were performed with SPSS 15.0J (SPSS Japan Inc., Tokyo, Japan). Comparison of two independent groups was made with Student's t-test or Mann-Whitney test depending on normality. Multiple regression analyses by stepwise method were performed to determine independent factors for circulating levels of vitamin D and K levels. The relationship between various nutritional indices and circulating vitamin D- and K- levels was analyzed with principal component analysis (PCA), which is a statistical method to summarize the various parameters into a small number of summary factors (components). These components are obtained in such a way that the first component is extracted from the initial raw data with the maximal amount of information (eigenvalue), and the second one is extracted from the remaining information. Therefore, each component is mutually independent. Components with the eigenvalue greater than 1 were adopted, as in usual practice.

RESULTS

Biochemical markers and Circulating Concentrations of Vitamin D and K

Baseline characteristics and data from blood examination are shown in Table 1. There was no gender difference in the age and level of care needed, which is a 5-grade score in the long-term care insurance in Japan with a higher number indicating the need for more intensive care. The level of care needed was higher than grade 3 in 78% of subjects. Most of the present subjects required wheelchair for transportation. Body height and body weight were significantly higher in males than in females. Body mass index (BMI), or serum albumin, total cholesterol and triglyceride concentrations did not significantly differ between the two groups. Generally, serum albumin level less than 3.5 g/dL is considered to indicate malnutrition. Serum albumin level was below this value in 26% of subjects. Inasmuch as the advanced age and high level of care needed, nutritional parameters remained within the reference range in most of the subjects. None of the study subjects had severe hepatic or renal dysfunction. There is a general consensus that a serum 25OH-D concentration less than 20 ng/mL indicates hypovitaminosis D.[2] Serum 25OH-D concentration was <10 ng/mL in 40% of subjects, 10-20 ng/mL in 58%, and ≥20 ng/mL in only one subject. None of the subjects had a serum PTH level above the cut-off value (65 pg/mL). Plasma PK and MK-7 concentrations in all of the subjects were 0.73±0.70 ng/mL and 0.53±0.37 ng/mL, respectively. In the present study, serum PK was less than 1 ng/ml and serum MK-7 was less than 1 ng/ml, in 85% and 90% of the subjects, respectively. The interpretation for these values will be given in the "Discussion" section. There were no gender differences in plasma vitamin K levels, serum 25OH-D or PTH.

Nutritional intake in the study subjects

The nutrients intake in the males and females were not statistically different as shown in Table 2. During the preparation of this paper, Dietary Reference Intake (DRI) for Japanese 2010 (DRI 2010) was released on May 29, 2009.[28] Since this work was done in 2006, however, consideration is made basically according to DRI 2005.[29] The intake of macronutrients such as protein, fat and carbohydrates appeared appropriate for their age and sex. The adequate intakes (AI) for calcium in Japan are 750 mg for men and 650 mg for women over 70 years. The AI for vitamin D is 5 μg/day, and that for vitamin K is 75 μg/day for men and 65 μg/day for women respectively. Although average calcium intakes in both groups were lower than the AI in DRI 2005, the average daily vitamin D intake was 7.0 μg, which is 140% of the AI in DRI 2005. The average daily intake of vitamin K in whole subjects was 155 μg, which is more than twice the AI for each gender. Thus, apparently these subjects had sufficient intakes of vitamin D and K based on AI in DRI 2005.

Multiple regression analyses for the determination of independent factor for circulating vitamin D, K concentrations.

In multiple regression analyses, vitamin D intake was a significant determinant of serum 25OH-D level, although the R^2 was low. Serum triglyceride level was the only significant predictor for plasma MK-7 concentration, and vitamin K intake and serum triglyceride concentrations significantly contributed to plasma PK level (Table 3).

Principal Component Analysis (PCA)

Since institutionalized elderly are generally malnourished, it is quite important to determine whether the low vitamin D - and K -status is independent of overall malnutrition or not. Then PCA was performed with the parameters included for analysis being serum albumin, triglyceride, cholesterol, 25OH-D, PTH levels and plasma PK, MK-7

Table 3. Multiple regression analyses for the determination of independent factors for circulating vitamin D, K concentrations.

	R^2	*p* value	Variable	β	*p* value
Serum 25OH-D	0.095	0.033	Vitamin D intake	0.309	0.033
Plasma PK	0.181	0.011	Vitamin K intake	0.290	0.042
			Triglyceride	0.380	0.009
Plasma MK-7	0.255	<0.001	Triglyceride	0.505	<0.001

Only significant predictors are shown. The abbreviations are β for β coefficient, and *p* for *p* value. Independent predictor for serum 25OH-D or plasma PK, MK-7 concentrations was analyzed by multivariate analysis with stepwise regression. Age, level of care needed and serum triglyceride and total cholesterol concentrations were included in all analyses. Vitamin D intake was additionally included in the analysis for plasma 25OH-D concentration. For plasma PK and MK-7, vitamin K intake was additionally included.

Table 4. Principal component analysis of nutrition indices

	Component 1	Component 2	Component 3	Component 4
Serum Albumin	0.880	0.004	0.047	0.059
Serum triglyceride	0.229	0.734	0.119	0.380
Serum total cholesterol	0.800	0.320	-0.046	-0.060
Serum 25OH-D	0.434	-0.457	-0.658	-0.033
Serum PTH	0.156	-0.273	0.877	-0.090
Plasma PK	-0.014	0.030	-0.071	0.986
Plasma MK-7	0.117	0.832	-0.238	-0.152

Factor loadings to four components after varimax rotation are shown. Loadings greater than 0.35 are shown in bold
Four components thus obtained were considered to represent the following nutritional status; component 1: overall nutritional status, component 2: vitamin K_2 status, component 3: vitamin D status, and component 4: vitamin K_1 status.

concentrations. Four components were obtained and explained 82% of the variance. The first component was composite of high albumin, total cholesterol and 25OH-D, and second component consisted of high triglyceride, low 25OH-D, and high MK-7. The third component was composite of low 25OH-D and high PTH, and the fourth component was composed of high triglyceride and high PK. The interpretation of each component was made as follows; the first component representing overall nutritional status, the second component, vitamin K_2 status, the third component, vitamin D status, and the fourth component representing vitamin K_1 status (Table 4).

DISCUSSION

Nutritional status would be adequately assessed by both evaluating the subjects' food intake and measuring their circulating or urinary markers. This principle would hold true especially in the elderly, since they are at high risk for malabsorption or utilization defects of nutrients. Unfortunately in Japan, vitamin D and K status in the elderly has been studied either by evaluating their food intake, as in the annual National Nutrition Survey Japan (NNS-J) or by measuring circulating level of these vitamins,[21,30-33] but rarely by both.[12,34]

Institutionalized elderly have been our special concern, since they are much more susceptible to hypovitaminosis D and K deficiency than the healthy elderly. The NNS-J in 2006 showed that subjects over 70 years of age, including both genders, had the following daily nutrients intakes: energy 1761 kcal, calcium 551 mg, vitamin D 9.0 μg, vitamin K 273 μg,[35] which were higher than those of the subjects in the present study. Gastrointestinal absorption of nutrients in the present study subjects would be impaired also. These considerations led us to simultaneously evaluate both vitamin D and K intakes and its circulating levels in the present study.

Before the interpretation of our data, determination procedure for vitamin K deserves some discussion. There have been discrepancies on the plasma concentration of vitamin K in the previous literature, which is at least partly due to the different determination procedure employed. Recently we have developed a novel procedure for the determination of vitamin K analogs with high sensitivity and specificity, based on high-performance liquid chromatography-tandem mass-mass spectrometry with atmospheric pressure chemical ionization (LC-APCI-MS/MS).[26] With this procedure, plasma concentrations of PK and MK-7 were 0.73±0.70 ng/mL (median 0.58 ng/mL) and 0.53±0.37 ng/mL (median 0.45 ng/mL), respectively in the current study. In our recent study, plasma concentrations for PK and MK-7 were 1.29±1.09 ng/mL (median 0.94 ng/mL) and 4.21±6.81 ng/mL (median 2.14ng/mL), respectively in the healthy Japanese elderly over 70 years old using the same assay procedure.[21] In the same study, lowest concentration of plasma vitamin K level to avoid the elevation of serum ucOC concentration was 2.5 ng/ml for PK and 6.4 ng/ml for MK-7.[21] Since serum ucOC level is a sensitive indicator of skeletal vitamin K insufficiency, these figures can yield a rough estimate of circulating vitamin K levels needed by the skeleton.

The median intake of vitamin K in the current subjects was 168 μg, which was more than twice the AI in DRI 2005. The AI for vitamin K was not altered in DRI 2010. Dietary vitamin K intake has been identified as an important determinant of plasma phylloquinone concentration

in previous studies.[36,37] In the present study, vitamin K intake was also significantly associated with plasma PK, but not with plasma MK-7. Since they were not supplied with fermented soybean; natto, which contains exraordinary amount of MK-7,[38] phylloquinone from green vegetables is likely to be the major contributors to the total vitamin K intake in our subjects. Thus plasma PK alone correlated with total vitamin K intake, adjusted by serum triglyceride. These data strongly suggest that these subjects are vitamin K-deficient in spite of the fact that their dietary intake is far above the AI in according to DRI 2005, and increased vitamin K intake would be effective in improving plasma PK levels in institutionalized elderly in present study.

As in the case of vitamin K, average dietary intake of vitamin D was around 7 μg/day, which is approximately 140% of the AI in subjects in the present study. Nevertheless, the average serum 25OH-D concentration was only 11.1 ng/mL. Thus, most subjects in the present study had hypovitaminosis D in spite of apparently sufficient vitamin D intake.

Although the multiple regression analysis has identified vitamin D intake as the significant contributor to serum 25OH-D concentration, the R^2 value was low, which indicates that the current model could explain only a small portion of variation. Several factors could be responsible for the above results. First, because of walking disability and other physical dysfunction, the chance of sun exposure was minimal in most of the current study subjects, but it was not null. Thus, sun exposure may also partly explain the above results. Unfortunately, however, detailed information about sun exposure was unavailable. Furthermore, ADL itself has been reported to be related to serum 25OH-D levels,[39] on which detailed information is not available in the current study. Secondly, the intestinal absorption of vitamin D is likely to decrease due to factors such as compromised intestinal ability for nutrients absorption and limited fat intake.[40] Nevertheless, oral vitamin D intake seems to be of value in the institutionalized elderly for improving their vitamin D status. Cashman *et al.* reported dose-dependent increase in serum 25OH-D concentration after incremental supplementation with vitamin D_3 in free-living adults over 64 years of age.[41] Although AI for vitamin D slightly increased to 5.5 μg/day in recently issued DRI 2010, the elderly subjects are likely to require much more vitamin D intake to avoid hypovitaminosis D considering the various problems to interfere with absorption and utilization as discussed above. A second issue with regard to the above discussion; disturbed intestinal absorption and limited fat intake, will also apply to the discrepant intake and circulating level of vitamin K.

Although serum 25OH-D level was extremely low, average serum PTH level was within the reference range. Circulating 25OH-D concentrations showed significant negative correlation with serum PTH levels ($r = -0.293$, $p = 0.041$; data not shown), which suggests that the negative feedback regulation of PTH secretion by vitamin D is not impaired in the current population. Kuchuk *et al.* reported that the elevation of serum PTH concentration by vitamin D deficiency is moderate in its magnitude, and usually fell into the reference range.[42] Thus they stressed the importance of serum 25OH-D level, and argued that for bone health maintenance and physical performance in the elderly, serum 25OH-D concentration above 50-60 nmol/L (20-24 ng/mL) was required.

Although the institutionalized elderly are considered to be generally malnourished,[43-45] nutritional status appeared rather satisfactory in the present study subjects in face of hypovitaminosis D and K. Then we analyzed the relationship between the overall nutrition and circulating levels of vitamin D and K by PCA. The PCA have yielded four components representing: overall nutritional status, vitamin D status, vitamin K_2 status, and vitamin K_1 status respectively. Serum 25OH-D also exhibited some association with the first component, representing the overall nutritional status. One of the reasons for the above results would be that 25OH-D is bound to vitamin D-binding protein (DBP) and albumin during its transport in circulation.[46] Since these components are independent of each other by their definition, these results suggest that hypovitaminosis D and K in the institutionalized elderly do not merely reflect general malnutrition, and have their own role. Confounders are serious challenge in the clinical studies. In the intervention studies, randomization would eliminate the interference by the confounders. It would be less problematic in the case of cohort studies. Adjustment for confounders is quite difficult in the cross-sectional studies like the current one. Multivariate analyses such as PCA would be of help in eliminating the interference by confounders in this type of studies.

In conclusion, institutionalized elderly had high prevalence of hypovitaminosis D and K in spite of their dietary intake exceeding the AI in DRI 2005 in Japan, which suggests that the requirement for these vitamins would be higher in these subjects. Additionally, hypovitaminosis D and K were shown to be independent of general malnutrition by PCA, which would be a useful analytical procedure for eliminating the interference by confounders in cross sectional studies.

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AUTHOR DISCLOSURES

None of the authors have any conflicts of interest.

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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 FGFR1 correlated 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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KEYWORDS: chronic kidney disease; FGF23; FGFR1; Klotho; parathyroid; secondary hyperparathyroidism

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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

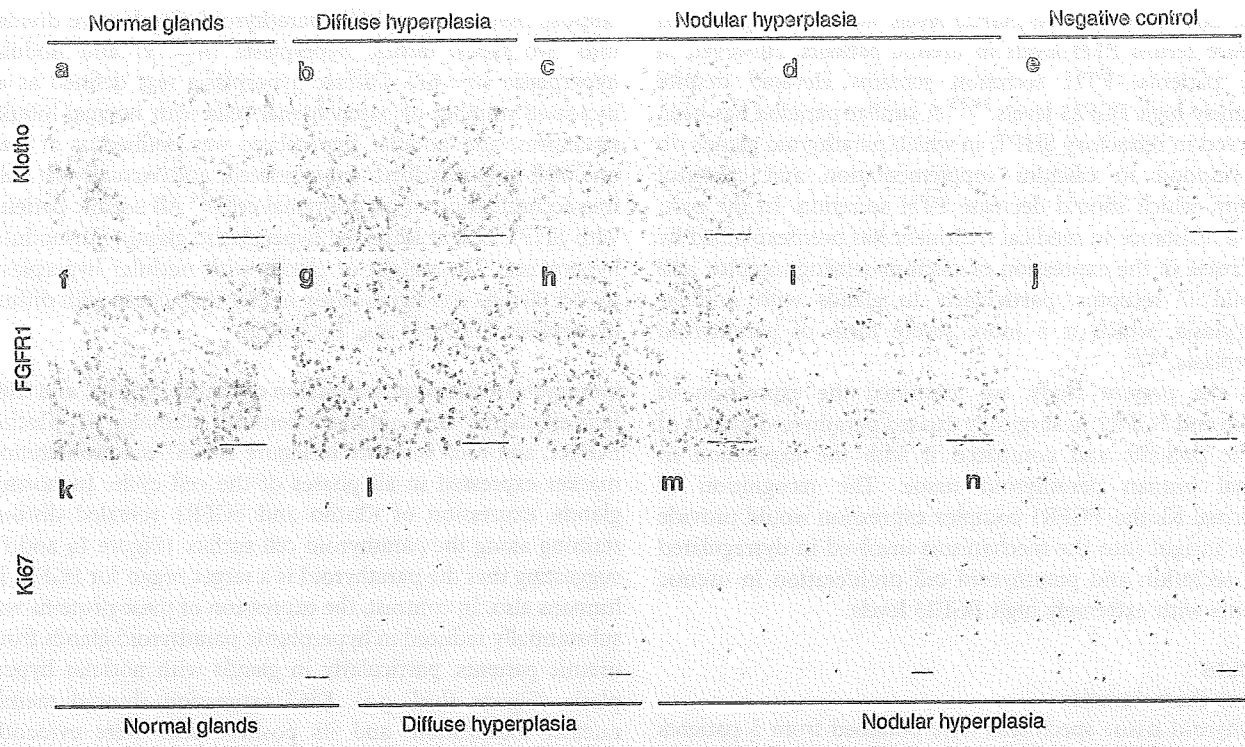

Figure 1 | Representative immunohistochemical staining of Klotho, fibroblast growth factor receptor 1 (FGFR1), and Ki67. (a–e) Klotho staining (e; negative control); (f–j) FGFR1 staining (j; negative control); (k–n) Ki67 staining. (a, e, f, j, k) Normal parathyroid glands; (b, g, l) diffuse parathyroid 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), × 400; (k–n), × 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

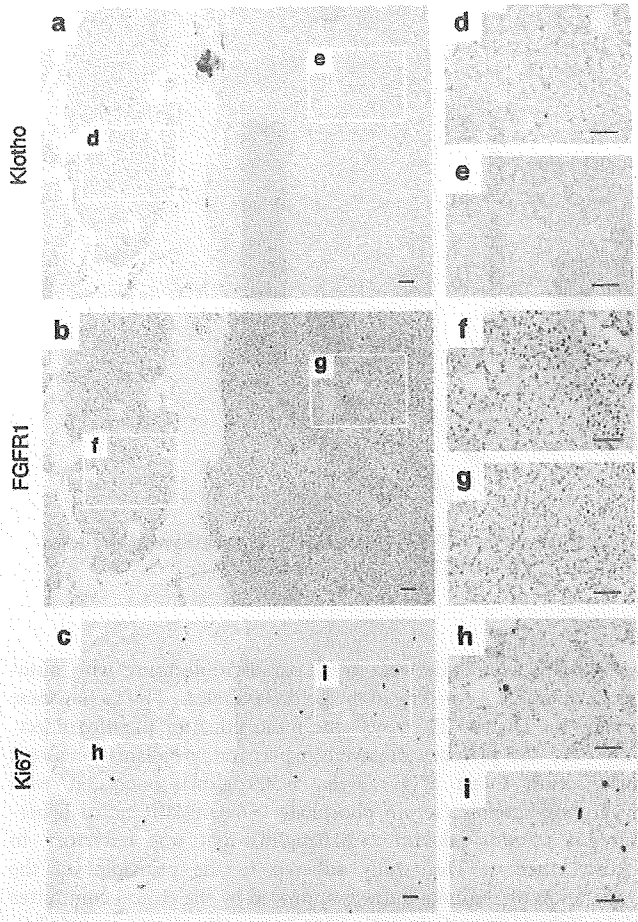

Figure 2 | Immunohistochemical staining of nodular lesions surrounded by diffuse hyperplastic parathyroid tissue. (**a**, **d**, **e**) Klotho staining; (**b**, **f**, **g**) fibroblast growth factor receptor 1 (FGFR1) staining; (**c**, **h**, **i**) Ki67 staining. Boxed areas are shown at higher magnification 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**), × 100; (**d**–**i**), × 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.

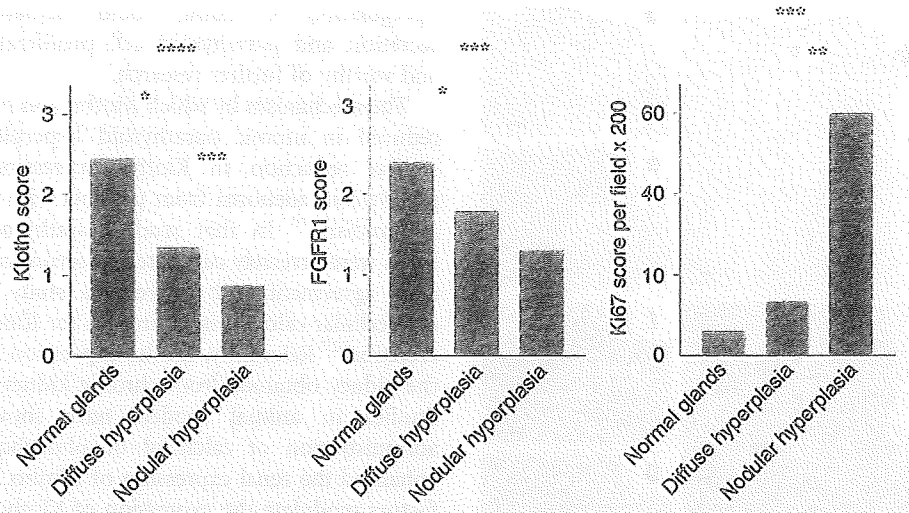

Figure 3 | Semi-quantification expression of Klotho, fibroblast growth factor receptor 1 (FGFR1), and Ki67 in parathyroid tissue of each group. The values shown are means ± s.e.m. *$P<0.1$; **$P<0.05$; ***$P<0.01$; ****$P<0.001$.

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 °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 °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 °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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ORIGINAL ARTICLE

The impact of lifestyle factors on serum 25-hydroxyvitamin D levels: a cross-sectional study in Japanese women aged 19–25 years

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Abstract Insufficient levels of serum 25-hydroxyvitamin D [25(OH)D] lead to low bone mineral density (BMD) by increasing serum levels of intact parathyroid hormone (PTH), and are associated with a high mortality rate. Therefore, the 25(OH)D level is used as an indicator of frailty in older persons. To obtain higher serum 25(OH)D levels, management of lifestyle habits and nutrient intake is important beginning in a person's younger years. This study evaluated the degree of association between serum 25(OH)D concentrations and lifestyle factors in young Japanese women. A cohort study was conducted from December 2003, and the survey was finished by February 2004. The subjects were 274 Japanese women aged 19–25 years old. The parameters evaluated in these subjects included: (1) serum concentrations of 25(OH)D, intact PTH, calcium, and phosphorus; (2) BMD in the lumbar spine and hip; and (3) lifestyle factors (nutrient intake, physical activity, and duration of sunlight exposure). The serum 25(OH)D level was negatively associated with the intact PTH level (Spearman; $r = -0.17$, $P = 0.006$). The BMD was significantly higher in the high 25(OH)D and low intact PTH group than the other group ($P < 0.05$). The serum 25(OH)D level was significantly correlated with daily intake of dietary vitamin D ($r = 0.20$, $P = 0.001$), the mean number of steps taken per day ($r = 0.16$, $P = 0.010$) and the mean time spent in sedentary activity ($r = -0.14$, $P = 0.018$) among the lifestyle factors evaluated. Multiple regression analysis showed the degree of association between lifestyle factors and serum 25(OH)D to be small ($R^2 = 0.084$). Daily intake of dietary vitamin D and daily walking may be useful for increasing the serum 25(OH)D level in young Japanese women.

Keywords 25-Hydroxyvitamin D · Nutrient intake · Physical activity · Japanese women

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Introduction

Vitamin D in the body is primarily produced in basal epidermis by ultraviolet radiation, and it is also supplied from intake of foods [,]. Vitamin D is then transported to the liver where it is metabolized to 25(OH)D. 25(OH)D is converted in the kidneys to active 1,25 dihydroxyvitamin D [1,25$(OH)_2$D], and exerts its effects as a bone metabolic hormone with intact parathyroid hormone (PTH) [ ].

It is well established that serum 25(OH)D concentration is the best clinical indicator of vitamin D status. A lower level of serum 25(OH)D leads to bone fractures [,] and serum 25(OH)D concentration is reported to fall [ ] in the elderly, with the vitamin D deficiency being common in the