The physiological roles of ALP are not well understood, but strong evidence is provided by the rare genetic disease hypophosphatasia (HPP). Hypophosphatasia is an inherited disorder characterized by a defect in skeletal mineralization caused by TNSALP deficiency.[4] Various mutations in the *TNSALP gene* have been analyzed.[5-10] Elevated extracellular concentrations of inorganic pyrophosphate (PPi), phosphoethanolamine (PEA), and pyridoxal-5'-phosphate (PLP) have been observed in HPP.[4]

Osteoporosis is defined as a skeletal disorder characterized by compromised bone strength, predisposing elderly people to an increased risk of fracture.[11] Osteoporosis results from complex interactions between genetic and environmental factors. Several genes have been implicated as genetic determinants of osteoporosis.[12-13] Recently, we found a significantly higher association between single nucleotide polymorphisms (SNPs) in the *TNSALP gene* (787T>C) (rs3200254 and rs3200255) associated with the bone mineral density (BMD) among 501 postmenopausal women.[14] We genotyped two single nucleotide polymorphisms (787T>C [Tyr246His] and 876A >G [Pro275Pro]), which were shown to be in complete linkage disequilibrium. There was a significant difference in BMD and the BMD score adjusted for age (z-score) among haplotypes, which was the lowest among 787T/876A homozygotes, highest among 787T>C/876A>G homozygotes, and intermediate among heterozygotes. In subgroups divided by age, haplotypes were significantly associated with BMD in older postmenopausal women (>74 years; p=0.001), but not in younger postmenopausal women (≤74 years; p=0.964).[14] These results indicate that the effect of haplotypes on BMD depended on age. Furthermore, these results suggest that variation in TNSALP may be an important determinant of age-related bone loss, and that the phosphate metabolism pathway may provide a novel target for the prevention of osteoporosis.

In the present study, we aimed to clarify the association between serum bone-specific alkaline phosphatase activity and serum biochemical parameters among the TNSALP genotype (787T>C) groups to obtain information for the planning of desirable nutritional management for bone health.

METHODS

The institutional review board of the Japan Women's University approved the protocol, and the study was carried out according to the guidelines of the Declaration of Helsinki. Informed consent was obtained from all the subjects. Participants were excluded if they had metabolic disease. The study population consisted of 97 healthy Japanese males and 96 healthy Japanese females living in Tokyo.[15] All subjects were unrelated volunteers and aged 22.1±1.8 (mean±SD), with a height of 164.9±8.9 cm, weight of 57.2±9.2 kg, and BMI of 21.0±2.3 kg/m^2, respectively.

Fasting blood samples were obtained and sera were kept frozen at -80℃ until measurement. Calcium and phosphorus were measured employing the o-cresolphthalein complexion color development and enzymatic methods, respectively. Alkaline phosphatase activity was determined employing the method of Bessey *et al.*[16] A bone formation marker, bone-specific alkaline phosphatase (BAP), was determined by enzyme immunoassay (DS Pharma Biomedical Co, Ltd, Osaka, Japan). Serum-intact osteocalcin (OC) was measured using an immuno-radiometric assay (Mitsubishi Kagaku Bio Clinical Laboratories Inc, Tokyo, Japan).

All subjects were genotyped for TNSALP polymorphism (Tyr246His, 787T>C) (rs3200254 and rs3200255 were archived in dbSNP at http://www.ncbi.nlm.nih.gov/SNP). Deoxyribonucleic acid was extracted from whole blood (QIAamp DNA Blood kit, Qiagen), and a 219-bp segment of the TNSALP gene including polymorphism sites was amplified by the polymerase chain reaction (PCR).[14] TNSALP polymorphism was determined by direct sequencing using the thermo sequence Cy 5.5 dye terminator cycle sequencing kit (Amersham Biosciences Corp) with a Gene Rapid sequencer (Amersham Biosciences Corp). The amino acid sequence was numbered from the N-terminal of the mature protein, ie, Met at the translation initiation site was −17.[17]

Dietary nutrient intakes were measured based on 3-day food records, taken up to the day before blood examinations. Trained personnel reviewed the food records, and the nutrient content was determined with the use of Eiyo-Kun software (Kenpaku-sha, Japan).

Values are shown as means±SD, and Spearman rank correlation coefficients were calculated to analyze the relation between two parameters. Serum parameters were compared among genotypic categories using ANOVA. Significance was considered at p<0.05. Chi-square tests were conducted to examine whether the genotype frequencies were in Hardy-Weinberg equilibrium. Analysis was conducted using SPSS17.0J (SPSS Inc., USA)

RESULTS

In all subjects (n=193), the mean (±SD) levels of serum BAP and ALP activity were 26.9±7.8 and 192.9±48.0 U/L, respectively. The levels of serum calcium and phosphorus were 9.7±0.4 and 3.6±0.5 mg/dL, respectively. The level of serum osteocalcin was 7.9±3.1 ng/mL. The mean (±SD) dietary intakes of energy, calcium, phosphorus, and vitamin D were 2,078±555 kcal/day, 556±223 mg/day, 1,059±302 mg/day, 5.8±4.8 μg/day, respectively.

The distribution of TNSALP gene polymorphism (Y246H, 787T>C) did not deviate from the Hardy-Weinberg expectations (p>0.05). The allele frequencies were 0.453 for the T allele and 0.547 for the C allele in all subjects. Forty-three subjects showed the 787T (246Tyr, TT-type) homozygote, 89 subjects were heterozygous (TC-type), and 61 subjects showed the 787C (246Tyr, CC-type) homozygote. There was no significant differences among these genotype groups in terms of the height, weight, and serum parameters (ALP, BAP, OC, calcium and phosphorus), as shown in Table 1. There was also no significant differences among these genotype groups in terms of dietary intake: energy, calcium (mg/1,000 kcal/day), phosphorus (mg/1,000 kcal/day), and vitamin D (μg/1,000 kcal/day), as shown in Table 2.

The associations between serum BAP and serum biochemical parameters are shown in Table 3. There was a significant positive correlation between the level of BAP and ALP in all these genotypes. In 787C (246Tyr, CC-type) homozygotes, there was a significant positive corre-

Table 1. Body and serum parameters

	TT (n=43)	TC (n=89)	CC (n=61)	*p*-values
Height (cm)	165.0 ± 10.2	164.6 ± 8.0	165.4 ± 9.2	N.S.
Weight (kg)	57.7 ± 10.3	56.7 ± 8.9	57.7 ± 9.0	N.S.
ALP (U/L)	193.9 ± 56.9	191.2 ± 42.5	194.7 ± 49.3	N.S.
BAP (U/L)	26.6 ± 7.5	26.9 ± 7.0	27.1 ± 9.0	N.S.
OC (ng/mL)	7.7 ± 3.0	7.7 ± 3.1	8.5 ± 3.3	N.S.
Calcium (mg/dL)	9.8 ± 0.4	9.7 ± 0.4	9.8 ± 0.5	N.S.
Phosphorus (mg/dL)	3.6 ± 0.5	3.5 ± 0.5	3.7 ± 0.5	N.S.

Each value represents the mean ± SD.
ALP: alkaline phosphatase; BAP: bone-specific alkaline phosphatase; OC: osteocalcin
N.S.: not significant

Table 2. Dietary intake of energy, calcium, phosphorus, and vitamin D

	TT (n=43)	TC (n=89)	CC (n=61)	*p*-values
Energy (kcal/day)	2,064 ± 567	1,998 ± 506	2,205 ± 600	N.S.
Calcium (mg/1,000 kcal/day)	279 ± 103	265 ± 88	276 ± 95	N.S.
Phosphorus (mg/1,000 kcal/day)	532 ± 90	502 ± 82	518 ± 91	N.S.
Vitamin D (μg/1,000 kcal/day)	3.1 ± 2.8	2.6 ± 1.9	2.9 ± 1.9	N.S.

Each value represents the mean ± SD.
N.S. : not significant

lation between the level of BAP and OC (r=0.502, p<0.001), and there was a significant negative correlation between the levels of serum BAP and phosphorus (r= -0.299, p=0.019) (Table 3). A significant negative correlation between serum BAP and phosphorus was not present in heterozygotes (TC-type), nor in 787T homozygotes (TT-type) (Table 3).

DISCUSSION

This study investigated the contribution of SNPs at the human TNSALP associated with BMD on phosphate metabolism. As shown in Table 3, our study revealed that TNSALP genotypes have an effect on the relationship between serum BAP and phosphorus statuses. Interestingly, a significant negative correlation between serum BAP and phosphorus was observed in 787T>C homozygotes (CC-type), but not in heterozygotes (TC-type), nor 787T homozygotes (TT-type). These results suggest that serum bone-specific ALP activity may be reflected by the level of serum phosphorus differently depending on the TNSALP genotypes. In addition, a significant positive correlation between serum BAP and OC was observed in 787T>C homozygotes (CC-type) and in heterozygotes (TC-type), but not in 787T homozygotes (TT-type) (Table 3). OC is one of the secreted osteoblast-specifc proteins, and both BAP and OC are serum markers of bone-formation. Bone-specific ALP is thought be a more immature osteoblastic marker than OC. Although the reason why serum BAP was associated with serum OC levels in some TNSALP genotypes is unclear, we supposed that the genetic variance may affect the association between serum BAP and OC levels at the differential stage of osteoblasts.

We previously reported that BMD was lowest among 787T homozygotes (TT-type), highest among 787T>C homozygotes (CC-type), and intermediate among heterozygotes (TC-type) among 501 postmenopausal women.[14] The present study demonstrated that TNSALP 787T>C has an effect on, not only age-related bone loss, but also phosphate metabolism in young adult subjects.

The polymorphic 787T>C base change causes an amino acid substitution of histidine for tyrosine at position 246 in TNSALP. There was no significant difference in the levels of ALP-specific activity between 787T and 787T>C using the mouse stromal cell line ST2 cells, derived from mouse bone marrow transiently expressing SNPs of the human TNSALP (787T or 787T>C) gene.[18] However, the expression of the protein translated from 787T>C (His-246) had a lower Km value than 787T (Tyr-246).[18] The Km value indicates the concentration of the substrate at 1/2 Vmax (maximum velocity), and the kinetic affinity for the substrate. The kinetic affinity may affect the mediation of the specificity and modulation of activity, and may contribute to regulatory effects on phosphate

Table 3. Association between the level of serum BAP and serum biochemical parameters

BAP	TT (n=43)		TC (n=89)		CC (n=61)	
	r	*p*-values	*r*	*p*-values	*r*	*p*-values
ALP	0.831	0.000***	0.818	0.000***	0.750	0.000***
OC	0.276	0.073	0.298	0.005**	0.502	0.000***
Calcium	0.008	0.959	-0.008	0.940	-0.098	0.450
Phosphorus	-0.055	0.725	-0.114	0.286	-0.299	0.019*

ALP: alkaline phosphatase; BAP: bone-specific alkaline phosphatase; OC: osteocalcin
* : p<0.05, ** : p<0.01, *** : p<0.001

metabolism. Previously, we reported that Pi starvation increased TNSALP activity and regulated its expression in ST2 cells.[19,20] These results suggest that the possible role of the Pi sensing system in biological functions of ALP might have been evolutionarily conserved.

In mineralizing tissues, the ratio of Pi to PPi, which is an inhibitor of the formation of hydroxyapatite crystals, is important. TNSALP may play a crucial role in the mineralizing process, and may regulate extracellular PPi concentrations by hydrolyzing PPi, which is an inhibitor of the formation of hydroxyapatite crystals.[21] The production of PPi is controlled by the nucleoside triphosphate pyrophosphohydrolase (NTPPPH) isozymes, such as plasma cell membrane glycoprotein-1 (NPP1). Current knowledge of mice lacking TNSALP suggests that TNSALP has essential physiological functions in the metabolism of phospho-compounds.[22] Millán *et al* showed that bone mineralization in double-KO mice lacking both TNSALP and NPP1 is essentially normal, providing evidence that TNSALP and NPP1 are key regulators of the extracellular PPi concentrations required for bone mineralization. [22]

Most recently, a genome-wide association study of the serum phosphate concentration has been reported, and the group reported polymorphisms in seven loci associated with the serum phosphorus concentration.[23] Interestingly, the most robust association in the study was for SNP RS1697421, which is located adjacent to the TNSALP gene.[23] In the present study, TNSALP 787T>C had an effect on the level of serum phosphorus in young adult subjects. As shown in Table 3, we demonstrated the effect of the candidate osteoporosis-susceptibility gene TNSALP on phosphate metabolism, and suggested that the level of bone-specific ALP activity was significantly correlated with serum phophorus in 787T>C, but not in 787T homozygotes. As there are limitations of this association study due to the small sample size, further analysis of bone metabolism including the phosphate metabolism pathway is necessary for the prevention and treatment of osteoporosis, and will help elucidate the molecular and cellular functions of TNSALP.

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AUTHOR DISCLOSURES

None of the authors has any conflict of interest.

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Short Communication

Associations between serum bone-specific alkaline phosphatase activity, biochemical parameters, and functional polymorphisms of the tissue-nonspecific alkaline phosphatase gene in a Japanese population

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日本族群之血清中骨特異性鹼性磷酸酶活性、生化指標及組織非特異性鹼性磷酸酶基因的功能多型性之相關

前言：在過去的研究中，我們已證實了在組織非特異性鹼性磷酸酶(TNSALP)基因中的單一核苷酸多型性(787T>C)與骨礦物質密度(BMD)有關。在停經婦女中，TNSALP 787T 同型合子(TT 型)者其 BMD 最低；而 787T>C 同型合子(CC 型)者 BMD 則是最高。在本研究中，主要探討健康年輕的日本受試者，其 TNSALP 基因型對於血清中骨特異性鹼性磷酸酶(BAP)、血清鈣與磷相關性的影響。方法：健康青年受試者共 193 位，檢測該基因多型性及測量血清中 BAP、鈣與磷。由抽血檢查前 3 天的食物記錄來估算其膳食營養素攝取情形。結果：依據 TNSALP 的基因型加以分組，發現基因型為同型合子 CC 型者，其血清中 BAP 及磷呈顯著負相關；但在異型合子 TC 型及同型合子 TT 型者身上皆無此相關。結論：在本研究中，顯示了年輕成人受試者，其 TNSALP 基因中的單一核苷酸多型性(787T>C)會影響血清中 BAP 與磷之相關。此結果說明了 TNSALP 的變異可能是影響磷代謝的重要決定因子，而此資料對於預防骨質疏鬆症之防治策略可能是有用的。

關鍵字：骨特異性鹼性磷酸酶、磷、單一核苷酸多型性、組織非特異性鹼性磷酸酶、年輕成人受試者

Original Article

Effects of gamma-glutamyl carboxylase gene polymorphism (R325Q) on the association between dietary vitamin K intake and gamma-carboxylation of osteocalcin in young adults

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Introduction: It has been demonstrated that single nucleotide polymorphism (SNP) (R325Q, 974G>A) in the gamma-glutamyl carboxylase (GGCX) gene is associated with the bone mineral density (BMD). In the present study, we investigated the effect of GGCX polymorphism (974G>A) on the correlations among the vitamin K intake, level of serum vitamin K, and ratio of undercarboxylated osteocalcin (ucOC) to intact osteocalcin (OC) in healthy young Japanese subjects. Methods: Healthy young adult subjects (n=189) were genotyped for the polymorphism, and we measured the levels of serum vitamin K, intact OC, ucOC, and dietary nutrient intakes. Results: Dietary vitamin K intake from vegetables was significantly correlated with the level of serum phylloquinone (PK), and vitamin K intake from fermented beans, natto, was also significantly correlated with the level of serum menaquinone-7 (MK-7). Moreover, the total dietary vitamin K intake showed a significant negative correlation with the ratio of ucOC to intact OC. Interestingly, on grouping by the GGCX genotype, there was a significant interaction between the ratio of ucOC to intact OC with vitamin K intake in homozygotes (GG-type) and heterozygotes (GA-type) ($p<0.001$). These results suggest that an adequate nutritional strategy is necessary for people with high-risk genotypes (GG- or GA-type). Conclusions: We demonstrated the effects of SNP (974G>A) in the GGCX gene on the correlation between dietary vitamin K intake and gamma-carboxylation of serum OC. Our data may be useful for planning strategies to prevent osteoporosis.

Key Words: vitamin K intake, phylloquinone (PK), menaquinone-7 (MK-7), single nucleotide polymorphisms (SNP), gamma-glutamyl carboxylase (GGCX)

INTRODUCTION

Osteoporosis is defined as "a skeletal disorder characterized by compromised bone strength, predisposing to an increased risk of fracture".[1] Osteoporosis is an established and well-defined disease that affects more than 75 million people in Europe, the USA, and Japan.[2] How fracture restricts daily activities to reduce the quality of life (QOL) has been studied. The development of osteoporosis is caused by complex interactions between genetic factors and the lifestyle. Estrogen deficiency after menopause is important, and physical activity and calcium/vitamin D/vitamin K intakes are closely associated. The prevention of osteoporosis involves increasing the peak bone mass in adolescence and minimizing bone loss caused by aging and menopause. Dietary intake and nutritional education are essential from a young age.

Vitamin K is a nutrient closely involved in bone metabolism.[3] It acts as a cofactor for γ-glutamyl carboxylase (GGCX), and is well-known to participate in the activation of blood coagulation factors and bone mineralization.[4] Vitamin K facilitates post-translational carboxyla-

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tion of glutamyl residues in selected proteins.[5-9] Three vitamin K-dependent proteins (osteocalcin: OC, matrix Gla protein, and protein S) are found in bone; OC is the most abundant.[10-12] It is produced in osteoblasts, and fully carboxylated OC binds to the calcium ions of hydroxyapatite.[13] The amount of OC which is not carboxylated (undercarboxylated OC: ucOC) is considered a sensitive index of the vitamin K status of bone, and an elevated ratio of ucOC to intact OC is thought to be associated with low dietary intakes of vitamin K.[14-16] In previous studies, elevated ucOC was associated with an increased risk of hip fracture in elderly people.[17-20]

All forms of vitamin K have 1, 4-naphthoquinone as a common ring structure, and natural vitamin K exists in two molecular forms, vitamin K_1 (phylloquinone: PK) and vitamin K_2 (menaquinone: MK-n). Green leafy vegetables contain the highest content of PK and significantly contribute to the total vitamin K intake. Vitamin K_2 is classified into MK-1~14 due to the repeat structure of the side chain, with isoprene comprising the side chain. The MK-4 form shows marked physiological activities, and is included in many animal-derived foods such as meat. It is the major form of vitamin K in tissues, and dietary PK is converted into MK-4 by two possible routes *in vivo*.[21] Japanese fermented beans, bacillus natto (referred to as natto), contain large amounts of menaquinone-7 (MK-7) synthesized by bacteria.[22] The level of serum MK-7 is significantly higher in those who eat natto frequently. Kaneki *et al* identified a significant positive correlation between the level of MK-7 in serum and habit of eating natto in postmenopausal women.[23]

Previous studies demonstrated that a significant correlation between the single nucleotide polymorphisms of GGCX (R325Q, 974G>A) (rs699664 archived in the dbSNPs at http://www.ncbi.nlm.nih.gov/) was associated with bone mineral density among postmenopausal Japanese women.[24] The BMI-adjusted z score in a subpopulation older than 75 years was significantly higher in those with 325Q (AA-type) than in those with 325R (GG-type) or 325R/Q (GA-type). Recently, we reported the effects of the GGCX gene on the correlation between the level of serum MK-7 and gamma-carboxylation of serum OC in young male subjects (n=60).[25] In the present study, we aimed to clarify the effect of GGCX polymorphism (R325Q) on the correlations among the vitamin K intake and ratio of ucOC to intact OC in healthy young subjects to obtain basic information for the planning of nutritional management to promote bone health.

METHODS

The study protocol was approved by the Institutional Review Board of Japan Women's University, and written informed consent was obtained from all study subjects. Young subjects living in Tokyo, Japan, were recruited.[26] Participants were excluded if they had metabolic disease. The study population consisted of 97 healthy Japanese males and 92 healthy Japanese females. The subjects were aged 22.1±1.8 y (mean±SD), with a height of 165±8.9 cm, weight of 57.4±9.2 kg, and body mass index (BMI) of 21.0±2.3 kg/m^2.

Fasting blood samples were obtained and sera were kept frozen at -80℃ until measurement. The concentration of ucOC, as a sensitive marker of vitamin K deficiency, was measured with a new electro-chemiluminescence immunoassay (Sanko Jyunyaku Co, Ltd, Ibaraki, Japan), as described by Tsugawa *et al.*[27] The specific antibody to ucOC was purchased from Takara Bio Co, Ltd (Kyoto, Japan). Serum-intact OC was measured by immuno-radiometric assay (Mitsubishi Chemical Medience Inc, Tokyo, Japan). A bone formation marker, serum bone-specific alkaline phosphatase (BAP), was determined by enzyme immunoassay (Mitsubishi Chemical Medience Inc, Tokyo, Japan). The serum concentration of vitamin K (PK and MK-7) was measured using a new liquid chromatography-atmospheric pressure chemical ionization-tandem mass-mass spectrometry (LC-APCI-MS/MS) method[28] or high-performance liquid chromatography (HPLC) with a fluorescence detection method.[29]

Dietary nutrient intakes were measured based on 3-day food records taken up to the day before blood examinations. Trained personnel reviewed the food records, and nutrient content was determined with the use of Eiyo-Kun software (Kenpaku-sha, Japan).

All subjects were genotyped for GGCX polymorphism (R325Q, 974G>A) (dbSNP: rs699664). The DNA was extracted from whole blood (QIAamp DNA Blood Kit, Qiagen), and a 265-bp segment of the GGCX gene including polymorphism sites was amplified by the polymerase chain reaction (PCR) (forward primer: 5'-TGTTCTCCTACGTCATGCTGGCCAG-3'). The presence of GGCX polymorphism was determined by direct sequencing using the thermo sequence Cy 5.5 dye terminator cycle sequencing kit (Amersham Biosciences Corp, Piscataway, NJ, USA) with a Gene Rapid sequencer (Amersham Biosciences Corp).

Analysis was conducted using IBM SPSS Statistics version 20 (IBM Corp, Chicago, IL, USA). Values are shown as the mean±SD. Spearman rank correlation coefficients were calculated to analyze the relation between two parameters. The GGCX gene polymorphism was assessed using Bonferroni corrections for multiple comparisons. Significance was considered at $p<0.05$.

RESULTS

The levels of serum PK and MK-7 were 1.0±0.7 and 7.8±13.6 ng/ml, respectively. The levels of serum intact OC, ucOC, the ratio of ucOC to intact OC, and BAP were 8.0±3.1 ng/ml, 5.7±3.2 ng/ml, 0.7±0.3, and 26.9±7.8 U/ml, respectively.

Dietary nutrient intakes were measured based on 3-day food records taken up to the day before blood examinations. In all subjects (n=189), the mean (±SD) total dietary vitamin K intake was 207±117 μg/day. Vitamin K intake from vegetables, which are the main PK source, was 92±63 μg/day, and that from natto, which is the main MK-7 source, was 37±68 μg/day. The daily mean (±SD) energy, calcium, and vitamin D intakes of the subjects were 2,088±555 (kcal), 555±224 (mg), and 5.8±4.7 (μg), respectively.

As shown in Figure 1A, there was a significant positive correlation between the vitamin K intake from vegetables and concentration of serum PK (r^2=0.021, $p<0.05$). The vitamin K intake from natto also showed a significant positive correlation with the concentration of serum MK-

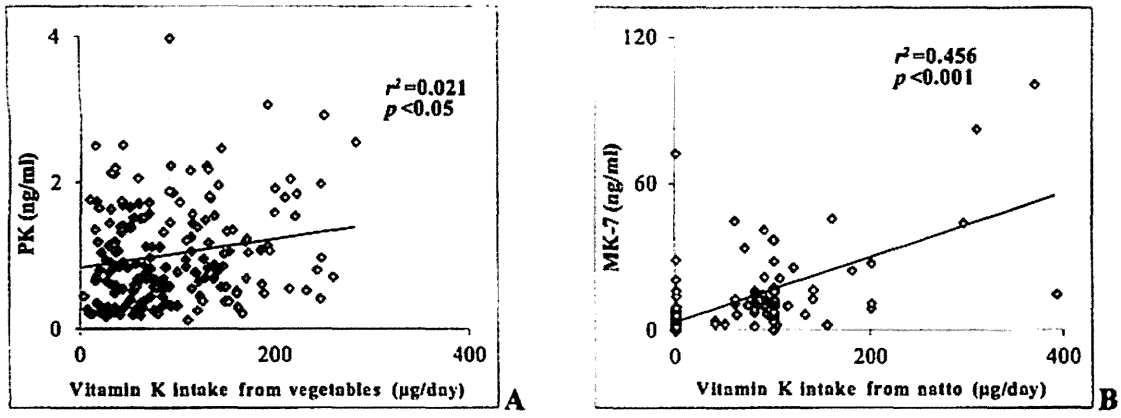

Figure 1. Association between vitamin K intake from vegetables and the concentration of serum PK (A), and association between vitamin K intake from natto and the concentration of serum MK-7 (B). There were significant negative correlations between the two parameters (A: y=0.002x + 0.8375, r^2=0.021, p<0.05, B: y=0.1328x + 2.8942, r^2=0.456, p<0.001).

7 (r^2=0.456, p<0.001) (Figure 1B).

The ratio of ucOC to intact OC is considered a sensitive marker of the vitamin K status in bone tissues. Figure 2A shows the correlation between the dietary vitamin K intake and ratio of ucOC to intact OC. The total dietary vitamin K intake showed a significant negative correlation with the ratio of ucOC to intact OC (r^2=0.213, p<0.001). In addition, there was a significant correlation between the concentration of PK or MK-7 and ratio of ucOC to intact OC (r^2=0.111, p<0.001, r^2=0.201, p<0.001, respectively) (Figures 2B and 2C).

In all subjects (n=189), eighty showed the 325R (GG-type) homozygote, 89 were heterozygous (GA-type), and 20 showed the 325Q (AA-type) homozygote. In male subjects (n=97), 42 showed the 325R (GG-type) homozygote, 46 were heterozygous (GA-type), and 9 showed the 325Q (AA-type) homozygote. In female subjects (n=92), 38 showed the 325R (GG-type) homozygote, 43 were heterozygous (GA-type), and 11 showed the 325Q (AA-type) homozygote.

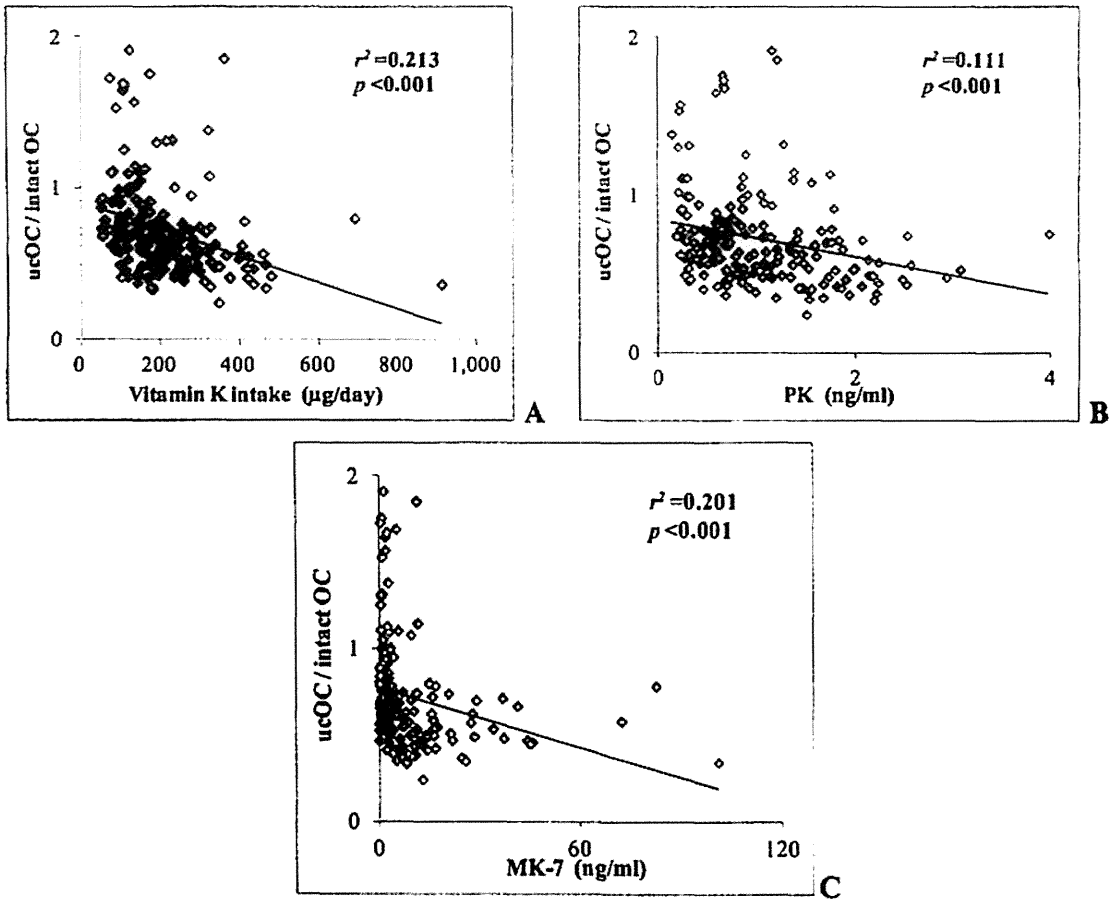

Figure 2. Association between the ratio of ucOC to intact OC and total vitamin K intake (A), and the concentration of PK (B) or MK-7 (C). There were significant negative correlations between the two parameters (A: y=-0.0009x + 0.909, r^2=0.213, p<0.001, B: y=-0.1169x + 0.8464, r^2=0.111, p<0.001, C: y=-0.0058x + 0.772, r^2=0.201, p<0.001).

Table 1. Body parameters and calcium/vitamin D intake

Genotype groups	n	age (year)	height (cm)	body weight (kg)	body mass index (m^2/kg)	calcium/vitamin D intake
GG	80	22.0 ± 1.7	164.4 ± 8.4	57.2 ± 10.7	21.1 ± 2.7	152 ± 131
GA	89	22.1 ± 1.8	165.7 ± 9.3	57.4 ± 8.2	20.8 ± 2.0	173 ± 156
AA	20	22.5 ± 2.1	165.0 ± 9.1	58.3 ± 7.0	21.5 ± 1.8	185 ± 165

Each value represents the mean ± SD.
There were no significant differences among these genotype groups.

Table 2. Serum vitamin K status and bone markers.

Genotype groups	n	PK (ng/ml)	MK-7 (ng/ml)	intact OC (ng/ml)	ucOC (ng/ml)	ucOC/ intact OC	BAP (U/ml)
GG	80	1.0 ± 0.7	6.3 ± 9.3	8.1 ± 2.5	5.5 ± 2.6	0.7 ± 0.3	26.9 ± 6.7
GA	89	1.0 ± 0.6	9.0 ±17.3	7.9 ± 3.7	6.0 ± 3.8	0.8 ± 0.4	27.2 ± 9.1
AA	20	1.1 ± 0.7	8.2 ± 8.4	7.8 ± 2.8	5.0 ± 2.3	0.6 ± 0.2	25.8 ± 6.5

Each value represents the mean ± SD.
PK: phylloquinone, MK-7: menaquinone-7, OC: osteocalcin, ucOC: undercarboxylated osteocalcin, BAP: bone-specific alkaline phosphatase.
There were no significant differences among these genotype groups.

As shown in Table 1, there was no significant difference among these genotype groups with regard to age, height, body weight, BMI, and calcium/vitamin D intake. Moreover, there was no significant difference among these genotype groups in terms of the level of serum PK, MK-7, intact OC, ucOC, the ratio of ucOC to intact OC, BAP activity, the total vitamin K intake, vitamin K intake from vegetables, and vitamin K intake from natto (Tables 2 and 3).

We investigated the relation between total vitamin K intake and ratio of ucOC to intact OC by GGCX genotype. A significant correlation between the ratio of ucOC to intact OC and total vitamin K intake was observed in the GG-type (r^2=0.294, p<0.001) and GA-type (r^2=0.160, p<0.001), but not in the AA-type (Figure 3). In addition, we investigated the relation between serum concentration of PK or MK-7 among the GGCX genotypes. There was a significant correlation between the ratio of ucOC to intact OC and concentration of PK in the GG-type (r^2=0.153, p<0.001) and GA-type (r^2=0.052, p<0.05), but not in the AA-type (Figure 4). There was also a significant correlation between the ratio of ucOC to intact OC and concentration of MK-7 in the GG-type (r^2=0.255, p<0.001) and GA-type (r^2=0.179, p<0.001), but not in the AA-type (Figure 5).

DISCUSSION

The present findings indicate the correlation between the dietary vitamin K intake and vitamin K status in healthy young Japanese adults. We determined the concentrations of serum PK and MK-7 using the LC-APCI-MS/MS technique or HPLC with fluorescence detection.[28-29] Kamao *et al* reported that there was a favorable correlation between the values obtained by LC-APCI/MS and those obtained by HPLC with fluorescence detection using internal standards for PK (y=0.841x + 0.035, r^2= 0.988) and MK-7 (y=0.908x - 0.386, r^2= 0.986).[29] The results of the LC-APCI-MS/MS method mostly agree with HPLC with the fluorescence detection method. By employing these effective methods, we clarified a significant positive correlation between vitamin K intake from vegetables, estimated from the 3-day food records, and the concentration of serum PK (r^2=0.021, p<0.05) (Figure 1A). This also indicated that PK is reliable for estimating dietary intake of PK related to serum PK. As shown in Figure 1B, we also clarified that dietary MK-7 intake from natto was significantly correlated with the concentration of serum MK-7 (r^2=0.456, p<0.001). Previously, we demonstrated that the concentration of serum MK-4 was very low (0.07 ± 0.05 ng/ml) compared with that of PK (0.56 ± 0.34 ng/ml) or MK-7(6.97 ± 13.30 ng/ml) in 60 healthy young Japanese males.[25] In addition, very similar data were obtained in healthy Japanese females by Tsugawa *et al.*[27] Therefore, the concentrations of serum MK-4 were not determined in the present study.

Table 3. Dietary vitamin K intakes (μg/day)

Genotype groups	n	total	from vegetables	from natto
GG	80	208 ± 117	94 ± 62	28 ± 49
GA	89	207 ± 122	89 ± 65	44 ± 84
AA	20	206 ± 102	95 ± 55	38 ± 51

Each value represents the mean ± SD.
There were no significant differences among these genotype groups.

Schurgers *et al* compared the absorption and efficacy of the oral intake of PK and MK-7 in healthy subjects between 25-35 years old, and they demonstrated that both PK and MK-7 taken in the form of an oil solution were absorbed well, with peak serum concentrations at 4 hours after intake.[30] A previous study reported a significant negative correlation between the incidence of hip fracture and consumption of natto, containing large amounts of MK-7.[23] Moreover, a low plasma PK concentration was found to be associated with a high incidence of bone fracture [31], and PK supplementation reduced the serum ucOC concentration in healthy young and elderly adults.[15] The physiological role of OC in bone metabolism has not yet been elucidated; however, the level of ucOC is considered a sensitive measure of the vitamin K status of bone, and a high concentration of ucOC has been associated with a risk of hip fracture. Tsugawa *et al* reported that the plas-

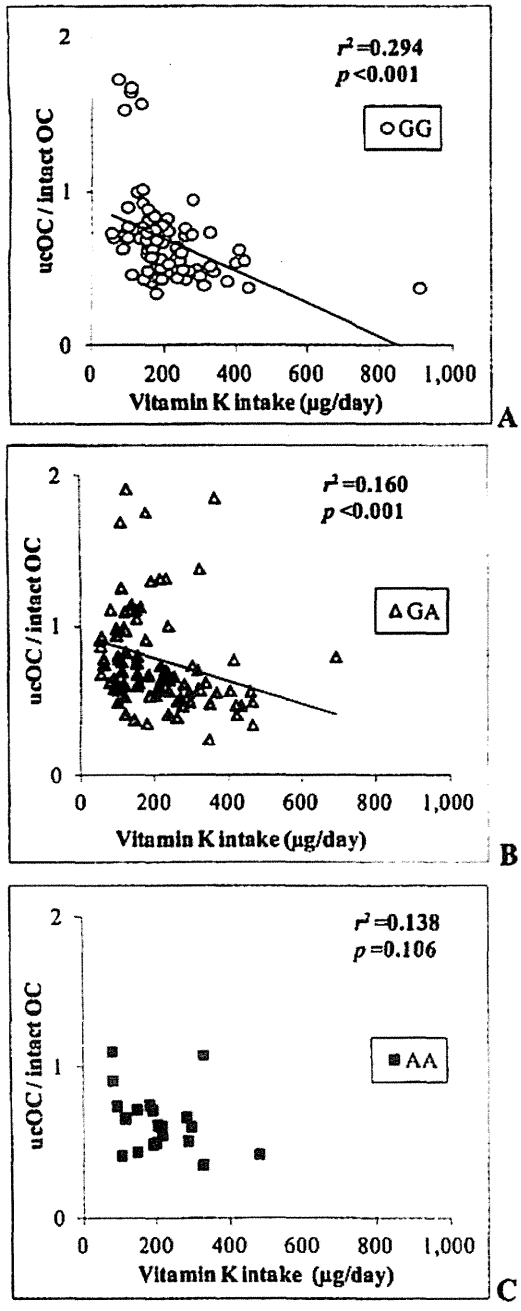

Figure 3. Association between the total vitamin K intake and ratio of ucOC to intact OC. Grouped by GGCX genotype, there was a significant negative correlation between the two parameters in 325R (GG-type) homozygotes (A) (y=-0.0011x + 0.9105, r^2=0.294, p<0.001) and heterozygotes (GA-type) (B) (y=-0.0008x + 0.9372, r^2=0.160, p<0.001), but not 325Q (AA-type) homozygotes (C) (r^2=0.138, p=0.106).

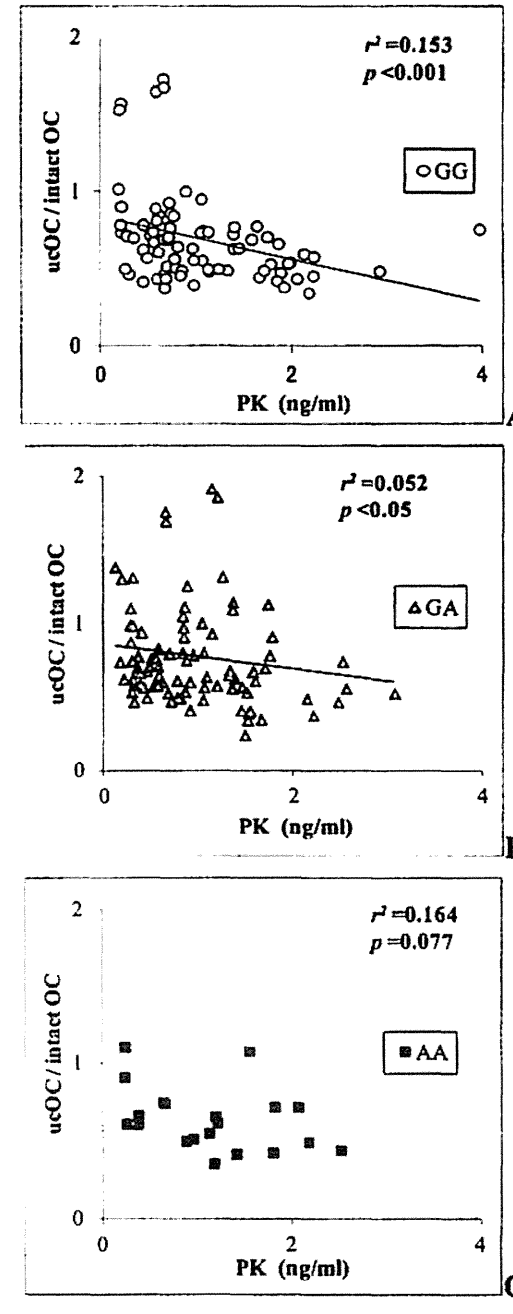

Figure 4. Association between the concentration of serum PK and ratio of ucOC to intact OC. Grouped by GGCX genotype, there was a significant negative correlation between the two parameters in 325R (GG-type) homozygotes (A) (y=-0.139x + 0.8344, r^2=0.153, p<0.001) and heterozygotes (GA-type) (B) (y=-0.081x + 0.8592, r^2=0.052, p<0.05), but not 325Q (AA-type) (C) homozygotes (r^2=0.164, p=0.077).

ma PK or MK-7 concentration required to minimize ucOC concentration was highest in the group aged 70 years, and decreased progressively in younger age groups.[27]

According to the Japanese Dietary Reference Intakes (DRIs) by the Ministry of Health, Labour, and Welfare, an adequate intake (AI) of vitamin K per day is estimated to be 75 μg for men and 60 μg for women, aged 18-29 years.[32] The total vitamin K intake was 207±117 μg/day in our study, and this level of vitamin K intake is similar to the average in young adults aged 20-29 years (201±171 μg/day) based on a national nutrition survey in Japan.[33]

As the AI of the vitamin K intake in Japanese DRIs was calculated for maintaining normal blood coagulation, it recommends that a higher intake of vitamin K (250-300 μg/day) is needed to prevent osteoporosis.[23] It was shown that bone metabolism requires more vitamin K than blood coagulation, and the requirement of vitamin K intake for favorable bone health was 155-188 μg/day in adolescents.[34]

In the present study, we suggested the effect of GGCX gene polymorphism on the association between serum vitamin K and gamma-carboxylation of osteocalcin in young adults. The GGCX gene is located on chromosome

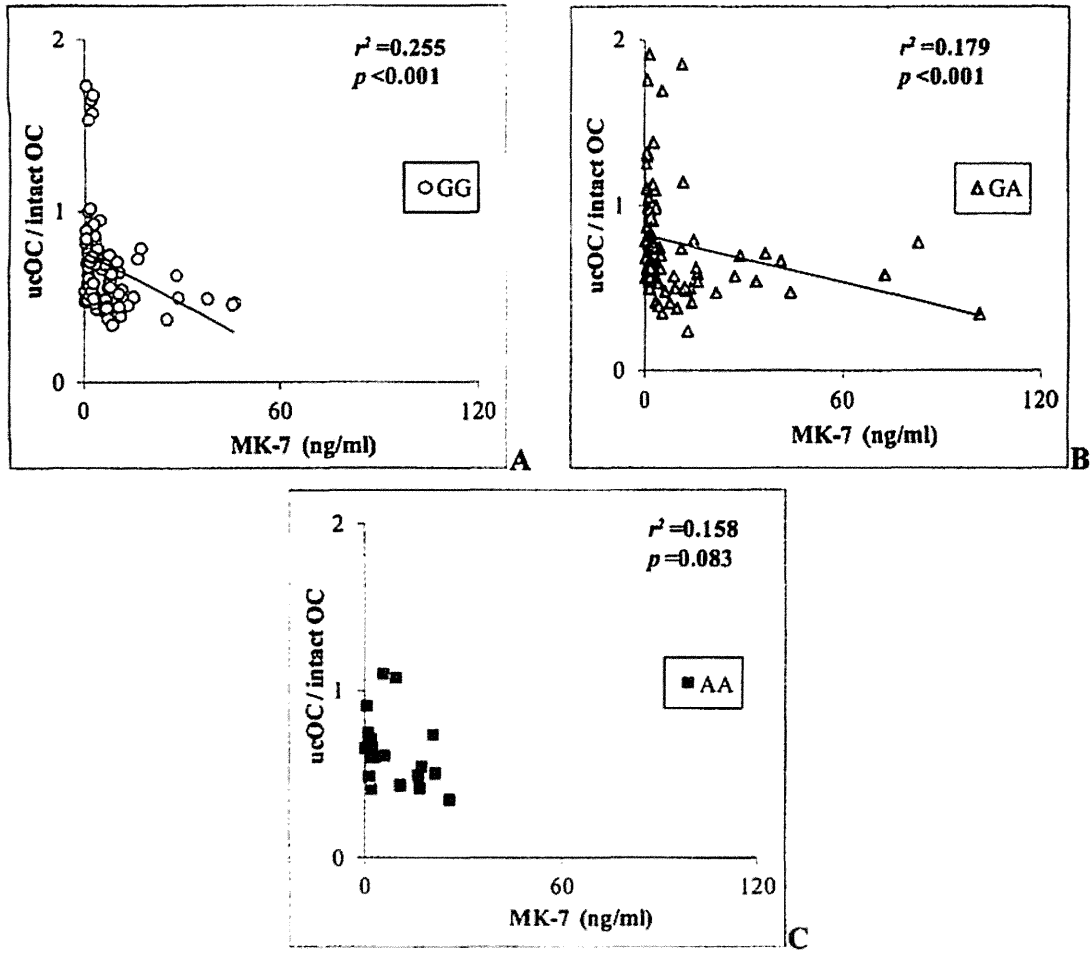

Figure 5. Association between the concentration of serum MK-7 and ratio of ucOC to intact OC. Grouped by the GGCX genotype, there was a significant negative correlation between the two parameters in 325R (GG-type) homozygotes (A) (y=-0.0101x + 0.7533, r^2=0.255, p<0.001) and heterozygotes (GA-type) (B) (y=-0.0049x + 0.8244, r^2=0.179, p<0.001), but not 325Q (AA-type) (C) homozygotes (r^2=0.158, p=0.083).

2, 2p12, and consists of 15 exons, with a size of about 13 kb.[5] The GGCX gene encodes a 758-residue integral membrane protein and appears to have three transmembrane domains near its amino terminus.[35] Amino acid substitution of residue 325 (Arg/Gln) may affect enzymatic activity directly. Kinoshita *et al* reported that there was a higher adjusted z score in those with the AA-type than in those with the GG- or GA-type in a subpopulation older than 75 years.[24] The AA-type has a lower Km value than the GG-type using COS-7 cells, and has a higher carboxylase activity than the GG-type.[24] Previously, we reported that the serum MK-7 concentration showed a significant negative correlation with the ratio of ucOC to intact OC in the GG-type (r=-0.572, p<0.01), but not in the GA-type (r=-0.260, p=0.166), nor in the AA-type.[25] In the present study, we revealed that there was a significant negative correlation with the ratio of ucOC to intact OC and concentration of PK in the GG-type (r^2=0.153, p<0.001) and GA-type (r^2=0.052, p<0.05), but not in the AA-type (Figure 4).

In addition, total vitamin K intake showed a significant negative correlation with the ratio of ucOC to intact OC in the GG-type (r^2=0.294, p<0.001) and GA-type (r^2=0.160, p<0.001), but not in the AA-type (Figure 3). These results suggest that the requirement of vitamin K for gamma-carboxylation may be different depending on the GGCX genotype. We propose that the higher Km value of 325R (GG-type) means that a higher intake of vitamin K may cancel out the effects of the genotype.[24]

However, assessments of cigarette smoking, alcohol consumption, and physical activity were not possible in this sample. Additionally, limitations of the study due to its sample size are acknowledged. The lack of statistical significance in the AA-type is likely to be caused by the so-called type-II error due to the markedly smaller number of subjects in this group.

Although there are limitations due to the small sample size in the AA-type, we indicated the possibility that nutritional factor-related interactions potentially modulate the osteoporosis risk. An adequate nutritional strategy is necessary for people with high-risk genotypes {325R (GG-type) or 325R/Q (GA-type)}, and our data may be valuable to establish novel strategies of nutritional education for the prevention and treatment of osteoporosis. Further investigations of the genotype are necessary to determine the recommended dietary allowance of vitamin K for the maintenance of adequate gamma-carboxylation.

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AUTHOR DISCLOSURES

There are no competing interests for all authors.

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Original Article

Effects of gamma-glutamyl carboxylase gene polymorphism (R325Q) on the association between dietary vitamin K intake and gamma-carboxylation of osteocalcin in young adults

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γ-麩胺醯羧化酶基因多型性(R325Q)對於年輕成人飲食中維生素 K 攝取和骨鈣素 γ-羧化相關性之影響

前言：γ-麩胺醯羧化酶(GGCX)基因的單核苷酸多型性(SNP)與骨骼礦物質密度(BMD)之相關性已被證實。本篇研究探討，在日本的健康年輕受試者中，其 GGCX 多型性(974G>A)對於維生素 K 攝取、血清中維生素 K 濃度和羧化不全骨鈣素(ucOC)與完整骨鈣素(OC)比值之間關聯性的影響。方法：共有 189 位健康年輕成人進行基因多型性檢測，並測量其血清中維生素 K、OC、ucOC 濃度和飲食中營養素攝取量。結果：飲食中攝取來自蔬菜的維生素 K 與血清中維生素 K1(PK；葉綠醌)有顯著相關；而攝取來自發酵豆類-納豆的維生素 K 也與血清中維生素 K2(MK-7；甲萘醌-7)有顯著相關。此外，從飲食中攝取的總維生素 K 和 ucOC 與 OC 比值有顯著負相關。值得注意的是，將 GGCX 基因型分組時發現，同型結合子(GG-type)和異型結合子(GA-type)兩組的 ucOC 與 OC 比值和維生素 K 攝取有顯著交互作用($p<0.001$)。以上結果顯示，適當的營養策略對於具有高風險基因型(GG-或 GA-type)的人是必要的。結論：本研究證實 GGCX 基因中的 SNP(974G>A)多型性對於飲食維生素 K 攝取與血清骨鈣素 γ-羧化相關性之效應。本資料對於規劃預防骨質疏鬆症之策略也許會有幫助。

關鍵字：維生素 K 攝取、維生素 K1(PK)、維生素 K2(MK-7)、單核苷酸多型性(SNP)、γ-麩胺醯羧化酶(GGCX)

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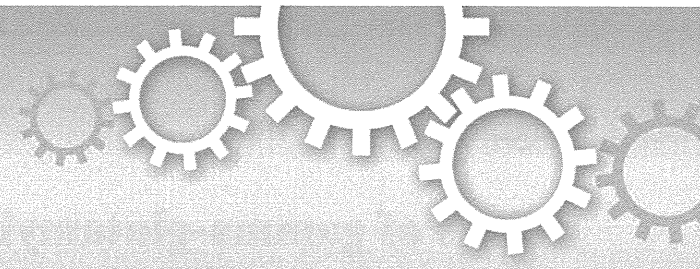

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Relationship between Low Free Testosterone Levels and Loss of Muscle Mass

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We assessed longitudinal relationships between baseline testosterone and muscle mass changes in Japanese men. Data were collected from community-dwelling 957 adult men who participated in a longitudinal study of ageing biennially from 1997–2010. Appendicular muscle mass (AMM) was measured with dual-energy X-ray absorptiometry at baseline and follow-up examinations. The cut-off point of sarcopenia was defined as a skeletal muscle index (AMM/height^2) < 6.87 kg/m^2. Total testosterone (TT) and free testosterone (FT) were measured with a radioimmunoassay. The calculated FT (cFT) was determined with a formula using albumin, TT, and sex hormone-binding globulin levels. We analyzed 4,187 or 2,010 cumulative data points using generalized estimating equations. Low TT was not associated with sarcopenia. Low cFT (odds ratio = 2.14, 95% confidence interval: 1.06–4.33) and FT (odds ratio = 1.83, 95% confidence interval: 1.04–3.22) were associated with sarcopenia. Low FT may be a predictor of risk for muscle loss in Japanese men.

Sarcopenia is the degenerative loss of skeletal muscle mass and strength associated with ageing[1]. Sarcopenia accelerates the frailty syndrome and leads to deterioration of activities of daily living and quality of life[2,3]. Development of preventative measures for sarcopenia is essential for extending a healthy life expectancy. The European Working Group on Sarcopenia in Older People assumed that muscle loss is a required component of sarcopenia diagnosis and suggested that muscle loss is a symptom of deterioration in muscle strength and physical performances[4]. Estimation of the risks for muscle loss appears to be necessary for developing steps to prevent sarcopenia.

Several cross-sectional studies have reported an association between serum levels of testosterone (T) and muscle mass in men[5,6]. Appendicular muscle mass was correlated with the serum level of free T (FT) in non-Hispanic white men aged 65–97 years[5]. Low appendicular muscle mass was observed in French men in the group with the lowest serum level of FT[6]. Additionally, androgen deprivation therapy for prostate cancer induces a decrease in muscle mass[7]. These studies suggest that the age-related decline in T is a trigger for muscle loss during ageing. Although T is associated with muscle mass, few longitudinal epidemiological studies have been published showing that circulating T levels are associated with prospective decreases in muscle mass[8]. In particular, muscle mass differs among races/ethnicities[9]. Circulating T levels also differ by race/ethnicity or the environment[10–12]. Demonstrating an association between muscle decrease and a decline in T appears to be necessary in each race/ethnicity.

The aim of this study was to determine whether circulating T levels predict muscle loss in middle-aged and elderly Japanese men. We assessed muscle loss with ageing using 10-year follow-up examinations and dual-energy X-ray absorptiometry (DXA) in middle-aged and elderly Japanese men. We also measured serum levels of T and evaluated the association between prospective muscle loss and the T levels in community-living middle-aged and elderly Japanese men using longitudinal analysis.

Results

Table 1 presents the elementary statistics of the participants at baseline according to sarcopenia status. Of the total of 957 men, 249 participants (26.0%) had a diagnosis of sarcopenia at baseline. Participants with sarcopenia were significantly older than those without sarcopenia (each, $p < 0.0001$). Body height ($p = 0.0018$), body weight

Table 1 | The characteristics of participants at the baseline examination. Means ± SE. The p values were obtained using the t-test for continuous data and the chi-square test (Fisher's exact test) for categorical data

	Normal (n = 708)	Sarcopenia (n = 249)	p
Age (years)	58.1 ± 0.4	63.1 ± 0.8	<0.0001
≥60 years (n)	314 (44.4%)	168 (67.5%)	<0.0001
Body height (cm)	164.9 ± 0.2	163.4 ± 0.4	0.0018
Body weight (kg)	65.0 ± 0.3	53.9 ± 0.4	<0.0001
Body mass index (kg/m^2)	23.9 ± 0.1	20.2 ± 0.1	<0.0001
% of body fat	21.8 ± 0.2	20.2 ± 0.3	<0.0001
Appendicular muscle mass (kg)	21.0 ± 0.1	17.2 ± 0.1	<0.0001
Skeletal muscle index (kg/m^2)	7.7 ± 0.02	6.4 ± 0.02	<0.0001
Albumin (mg/ml)	44.4 ± 0.2	44.3 ± 0.2	0.6894
Total testosterone (ng/ml)	5.0 ± 0.06	5.3 ± 0.1	0.0431
Sex hormone binding globulin (nmol/l)†	54.3 ± 1.3	71.4 ± 2.5	<0.0001
Calculated free testosterone (pg/ml)†	78.6 ± 1.1	68.9 ± 1.8	<0.0001
Free testosterone (pg/ml)	13.4 ± 0.1	12.3 ± 0.3	0.0002
Total energy intake (kcal/day)	2363.6 ± 14.2	2170.3 ± 22.7	<0.0001
Total protein intake (g/day)	88.9 ± 0.6	80.9 ± 1.0	<0.0001
Vitamin D intake (μg/day)	10.3 ± 0.2	9.6 ± 0.4	0.1502
Leisure-time physical activity (METs × hour/day)	2.2 ± 0.1	2.2 ± 0.2	0.9518
Current smoker (n)	236 (33.3%)	111 (44.6%)	0.0015
With medical history			
Stroke (n)	26 (3.7%)	10 (4.0%)	0.8063
Heart disease (n)	86 (12.2%)	33 (13.3%)	0.6491
Cancer (n)	17 (2.4%)	13 (5.2%)	0.0281
Diabetes (n)	59 (8.3%)	34 (14.0%)	0.0148
Osteoporosis (n)	4 (0.6%)	10 (4.0%)	<0.0001
Rheumatoid arthritis (n)	41 (5.8%)	18 (7.2%)	0.4171

†cFT and SHBG levels obtained from 327 normal men and 128 men with sarcopenia.

($p < 0.0001$), Body mass index ($p < 0.0001$), and percent of body fat ($p < 0.0001$) were significantly lower in the sarcopenia group than in the normal group. Appendicular muscle mass (AMM) and skeletal muscle index (SMI) were also significantly lower in the sarcopenia group than in the normal group (each, $p < 0.0001$). Total T (TT; $p = 0.0431$) and sex hormone binding globulin (SHBG; $p < 0.0001$) were significantly higher in the sarcopenia group than in the normal group. cFT and FT were significantly lower in the sarcopenia group than in the normal group ($p < 0.0001$, $p = 0.0002$, respectively). Total energy and protein intake were significantly lower in the sarcopenia group than in the normal group ($p < 0.0001$). No significant differences in albumin, vitamin D intake, and leisure-time physical activity were noted between the normal and sarcopenia groups. The ratio of current smokers ($p = 0.0015$) in the sarcopenia group was significantly higher than in the normal group. No differences in the ratios of stroke, heart disease, and rheumatoid arthritis history were noted. The ratios of cancer ($p = 0.0281$), diabetes ($p = 0.0148$), and osteoporosis ($p < 0.0001$) history in the sarcopenia group were significantly higher than in the normal group.

Table 2 presents the frequencies according to sarcopenia and the T level status at baseline. No differences in the ratio of sarcopenia between the normal TT group and the low TT group were observed. The ratio of sarcopenia in the low cFT group was significantly higher than that in the normal cFT group ($p = 0.0353$). The ratio of sarcopenia in the low FT group was also significantly higher than that in the normal FT group ($p = 0.0002$).

Among the 4,187 cumulative samples, the numbers of samples in the normal and sarcopenia groups were 3,084 (73.7%) and 1,103 (26.3%), respectively. The numbers of participants with low TT in the normal muscle status group (n = 3,084) and the sarcopenia group (n = 1,103) were 141 (4.6%) and 67 (6.1%), respectively ($p = 0.0487$). The numbers of participants with low FT in the normal muscle status group (n = 3,084) and the sarcopenia group (n = 1,103) were 103 (3.3%) and 87 (7.9%), respectively ($p < 0.0001$). Among the 2,010 cumulative samples that were analyzed for cFT, the numbers of samples in the normal and sarcopenia groups were 1,460 (72.6%) and 550 (27.4%), respectively. The numbers of participants with low cFT in the normal muscle status group (n = 1,460) and the sarcopenia group (n = 550) were 56 (3.8%) and 40 (7.3%), respectively ($p = 0.0013$).

The results from the generalized estimating equations (GEE) analyses, controlling for the effects of repeated observations within

Table 2 | The testosterone levels and sarcopenia status at the baseline examination. The p values were obtained using the chi-square test

	Normal (Skeletal muscle index ≥ 6.87 kg/m^2)		Sarcopenia (Skeletal muscle index <6.87 kg/m^2)	
	n	%	n	%
Total testosterone				
Normal (≥2.9 ng/ml)	677	74.5	232	25.5
Low (<2.9 ng/ml)	31	64.6	17	35.4
Calculated free testosterone				
Normal (≥46.3 pg/ml)	313	73.0	116	27.0
Low (<46.3 pg/ml)	14	53.9	12	46.1
Free testosterone				
Normal (≥7.7 pg/ml)	681	75.3	224	24.7
Low (<7.7 pg/ml)	27	51.9	25	48.1

Table 3 | Longitudinal relationships between baseline testosterone levels and sarcopenia. The cumulative data were analyzed with generalized estimating equations. Moderator variables: Crude model: none; Model 1: baseline age; Model 2: age, leisure-time physical activity, nutrition intake (total energy, total protein, vitamin D), medical history (stroke, heart disease, cancer, diabetes, osteoporosis, rheumatoid arthritis), and smoking habit at baseline

	Odds ratio (95% confidence intervals)		
Total testosterone	Normal (≥2.9 ng/ml)	Low (<2.9 ng/ml)	p value
n	3979	208	
Crude model	1.00 (Reference)	1.6178 (0.9486 – 2.7592)	0.0774
Model 1	1.00 (Reference)	1.4790 (0.8606 – 2.5416)	0.1566
Model 2	1.00 (Reference)	1.5717 (0.9004 – 2.7434)	0.1116
Calculated free testosterone	Normal (≥46.3 pg/ml)	Low (<46.3 pg/ml)	p value
n	1914	96	
Crude model	1.00 (Reference)	2.6503 (1.3182 – 5.3285)	0.0062
Model 1	1.00 (Reference)	2.1396 (1.0555 – 4.3370)	0.0349
Model 2	1.00 (Reference)	2.1432 (1.0617 – 4.3262)	0.0334
Free testosterone	Normal (≥7.7 pg/ml)	Low (<7.7 pg/ml)	p value
n	3997	190	
Crude model	1.00 (Reference)	2.8915 (1.7116 – 4.8846)	<0.0001
Model 1	1.00 (Reference)	1.9416 (1.1046 – 3.4129)	0.0211
Model 2	1.00 (Reference)	1.8296 (1.0391 – 3.2215)	0.0364

participants and confounding factors, are presented in Table 3. No significant association of TT levels with sarcopenia was observed in any model. The association of the cFT and FT levels with sarcopenia was significant in all models. The odds ratios of sarcopenia in low cFT participants compared to that in normal cFT participants were 2.65 (95% confidence interval [CI], 1.32–5.33; $p = 0.0062$) in the crude model, 2.14 (95% CI, 1.06–4.34; $p = 0.0349$) in model 1, and 2.14 (95% CI, 1.06–4.33; $p = 0.0334$) in model 2. The odds ratios of sarcopenia in low FT participants compared to that in normal FT participants were 2.89 (95% CI, 1.71–4.88; $p < 0.0001$) in the crude model, 1.94 (95% CI, 1.10–3.41; $p = 0.0211$) in model 1, and 1.83 (95% CI, 1.04–3.22; $p = 0.0364$) in model 2.

Discussion

The etiology of sarcopenia is assumed to be multi-factorial, including factors such as ageing, diseases, nutritional deprivation, and inactivity[4]. Few epidemiologic studies have been published about sarcopenia in Japanese people, and the risk factors for sarcopenia are not understood[9]. In this study, significant associations between muscle loss and FT, regardless of whether FT was calculated or measured, remained after adjustment for age, medical history, nutrition intake, and physical activity. Low FT levels appeared to be independently associated with muscle loss in middle-aged and elderly Japanese men, regardless of these factors. Our result is in line with previous studies that reported a relationship between low FT and low muscle mass in men[5,6]. The observed association between muscle loss and FT in this study appears to have biological plausibility. T stimulates protein synthesis and inhibits protein degradation in muscle cells[13,14]. T also increases satellite cell replication and activation in older men[15]. In this study, no significant association between TT levels and muscle loss were observed. However, recent longitudinal cohort studies have reported that elderly American people with higher baseline TT levels have a low risk of decline in appendicular lean mass[8]. Although a progressive decrease in TT levels with ageing is observed in middle-aged and elderly American men[16,17], the TT levels do not change during ageing in Japanese men[21,22]. The decrease in TT may occur at a later stage when hypogonadism has advanced in Japanese men[21]. FT levels may be a good marker for the loss of muscle mass in Japanese men.

In Japanese men, preventing the decline in FT may prevent the loss of muscle mass during ageing. In this cohort, participants in the low cFT group (<46.3 pg/ml) had approximately a 2.1- to 2.7-fold risk of muscle loss compared to those in the normal cFT group (≥46.3 pg/ml) (Table 3). Participants in the low FT group (<7.7 pg/ml) also had approximately a 1.8- to 2.9-fold risk of muscle loss compared to those in the normal FT group (≥7.7 pg/ml) (Table 3). The serum levels of FT decrease by approximately 50%, from the 20 s through the 70 s in Japanese men[14]. The Japanese Urological Association defined the reference value for androgen replacement therapy as a serum level of 8.5 pg/ml FT as measured with radioimmunoassay (RIA)[21]. Thus, the FT level associated with the risk of muscle loss in this cohort was lower than the reference value for androgen replacement therapy for Japanese men. Improvement in circulating FT levels with appropriate therapies, such as androgen replacement therapy or lifestyle interventions, may reduce the risk of muscle loss during ageing.

The effect of ageing on sarcopenia in Japanese men appear to be large. The prevalence of sarcopenia increased significantly with age[1]. In this study, the baseline age of men in sarcopenia group was statistically older than men in normal group (Table 1). The odds ratio of sarcopenia calculated by the model 1 which were controlled for the baseline age were smaller than those by the crude model (Table 3). The muscle loss might have been affected by the age-related accumulation of the various factors, such as a muscle fiber apoptosis or a mitochondrial dysfunction[4].

Approximately 1% to 2% of T in the blood exists as FT[21]. However, the FT values using RIA are much lower than cFT values[22,23]. In fact, serum FT levels were one-fifth to one-sixth of those of cFT in this study (Table 1). The odds ratio of sarcopenia determined by GEE appeared to have been influenced by these results. The odds ratios of sarcopenia determined by cFT were higher than those of FT, except for in the unadjusted crude model (Table 3). The risk of sarcopenia may be underestimated when FT measured by RIA is an index.

Interestingly, appendicular muscle loss was significantly associated with low levels of FT. These results suggest that a threshold level of FT exists for muscle loss, rather than a dose-response relationship. In the previous cross-sectional and longitudinal studies of French and American men, no dose-response relationships were reported between T and muscle mass[6,8]. A minimal serum level of FT may be needed to preserve muscle mass in men, regardless of race/ethnicity.

This study has significant strengths. The longitudinal design of our analyses lends strength to our inferences. Our study that the same individuals were followed over time provided evidence of a causal association between low level of endogenous FT and the appendicular muscle loss. We adjusted our analyses for potential confounders, including age, physical activities, nutrition intake, medical history, and smoking habit. This is the first population study to evaluate the relationship between sarcopenia and circulating T levels.

This study has several limitations. The first limitation is that the odds ratios of the muscle loss were determined based on serum levels of T at baseline. Although T decreases during ageing, the rate of the decline in T varies depending on different environments and lifestyles among individuals[10,11]. Further studies with longitudinal measurements of T may clarify an association between the decrease in T and muscle loss during ageing. Second, women, who have little T compared with men[18], were not examined in this study. In women, serum FT levels also decrease during ageing[18]. Total lean mass is associated with bioavailable T in postmenopausal women[19]. Further studies are needed to determine the role of androgens in preserving muscle mass in women.

In summary, using the longitudinal design of the cohort, we evaluated the association between loss of muscle mass and decline in FT in community-living, middle-aged and elderly Japanese men with a 10-year follow-up duration. Our data confirm that a low FT level is a significant predictor of a risk for loss of appendicular muscle. The findings in this study may be beneficial for developing methods to prevent sarcopenia in Japanese men.

Methods

Participants. The participants in this study were from the National Institute for Longevity Sciences-Longitudinal Study of Aging (NILS-LSA), which involves ongoing population-based biennial examinations of a cohort of approximately 2,300 persons. The participants in the NILS-LSA were randomly selected from resident registrations and stratified by both decade of age and sex. The NILS-LSA is a comprehensive and interdisciplinary study to observe age-related changes and consists of various gerontological and geriatric measurements, including medical examinations, blood chemical analysis, body composition, anthropometry, nutritional analysis, psychological tests, physical function, and physical activity[20]. Those who did not consent to have blood samples taken and those who did not complete the measurement of muscle mass with DXA were excluded. Participants with a current medical history of Parkinson's disease and androgen preparation users were also excluded. The baseline participants of this study were 957 men aged 40–79 years who completed the first-wave examinations of NILS-LSA between November 1997 and April 2000. Of these, 777 (81.2%) took part in the second-wave examination between April 2000 and May 2002, 689 (72.0%) took part in the third-wave examination between May 2002 and May 2004, 638 (66.7%) participated in the fourth-wave examination between May 2004 and July 2006, 590 (61.7%) took part in the fifth-wave examination between July 2006 and July 2008, and 536 (56.0%) participated in the sixth-wave examination between July 2008 and July 2010. The mean number of repeat visits was 3.2. The total number of visits, including repeat visits, was 4,187; participants from whom the data were derived were 40–88 years of age and took part in the NILS-LSA between November 1997 (the first wave) and July 2010 (the sixth wave).

The study protocol was approved by the Ethics Committee of the National Center for Geriatrics and Gerontology, and written informed consent was obtained from all participants.

Blood sampling and measurement of T. Blood samples were taken between 0800 and 0900 h, separated immediately by centrifugation at 2000 × *g* for 15 min, and sera were frozen and stored in a deep freezer (−80°C). Samples were transferred to the laboratory (SRL Inc., Tokyo, Japan) for TT, FT, SHBG, and albumin measurement.

The serum levels of TT (ng/ml) and FT (pg/ml) were also measured with a RIA using commercially available kits (Diagnostic Products Corporation, Los Angeles, CA, USA). The inter-assay coefficients of variation (CV) were less than 15% for both kits, according to the manufacturer's information. In 455 men who were randomly selected by decade of age at the time of the first-wave examinations, SHBG (nmol/l) was also measured with RIA using a commercially available kit (Diagnostic Products Corporation). The CV was less than 8.5% according to the manufacturer's information. Serum albumin (mg/ml) was measured with nephelometry.

For measuring FT, the detection precision of the equilibrium dialysis was better than that of RIA[21]. However, equilibrium dialysis is not used in Japan, because equilibrium dialysis is difficult to perform, not automated, and largely inaccessible to most clinicians[21]. Thus, the calculated FT (cFT) was derived from serum levels of albumin, TT, and SHBG in 455 male participants[22,23]. In this study, the coefficient of correlation between cFT and FT was 0.80438 ($n = 455$; $p < 0.0001$).

Definition of sarcopenia. Appendicular muscle mass (AMM, kg) and fat mass were assessed with DXA (QDR-4500; Hologic, Bedford, MA, USA). AMM is equal to the appendicular fat-free mass minus bone mineral contents, and is assumed to be an index of the amount of muscle mass.

We used the SMI to evaluate sarcopenia[1]. The SMI was calculated by AMM divided by height squared (kg/m^2). Sarcopenia was defined as muscle mass minus 2 standard deviations below the mean for young adult healthy people[1]. In this study, we set the cut-off point of sarcopenia as SMI < 6.87 kg/m^2. The SMI of 6.87 kg/m^2 was muscle mass minus 2 standard deviations below the mean for young adult healthy people in the Japanese men[9]. Sanada et al.[9] also measured appendicular muscle mass with DXA using the same model (QDR-4500; Hologic) we used in this study. The participants were divided into two groups based on DXA results at baseline and follow-up examinations: the sarcopenia group (SMI < 6.87 kg/m^2) and the normal group (SMI ≥ 6.87 kg/m^2).

Other parameters. Body height and weight were measured using a digital scale. Body mass index (kg/m^2) was calculated by weight divided by height squared. Medical history, smoking habit, and use of medications were assessed with questionnaires, which were confirmed by a physician at the medical examinations. All prescribed and non-prescribed medications used during the previous 2 weeks were documented and brought by the participants; the physicians confirmed and coded them. Trained interviewers used a questionnaire and asked the participants about the frequency and exercise intensity (metabolic equivalents: METs) of their physical activity habits during leisure time over the past 12 months[24]. The means per day for leisure-time physical activity (metabolic equivalents; METs × h/day) were calculated. Nutritional intake was assessed with a 3-day diet record[25]. Foods were weighed separately on a scale before cooking or portion sizes were estimated. Participants used a disposable camera to take photographs of meals before and after eating. Registered dietitians used the photographs to complete missing data and telephoned participants to resolve any discrepancies or to obtain further information when necessary. The average over the 3 days for 119 nutrient intake periods was calculated. The means per day for total energy intake (kcal/day), total protein intake (g/day), and vitamin D intake (μg/day) were calculated from the 3-day dietary record.

Statistical analysis. Statistical testing was performed using the Statistical Analysis System release 9.3 (SAS Institute Inc., Cary, NC, USA). A probability level less than 0.05 was considered significant. The results are shown as the means ± standard error (SE). Differences in continuous and class variables between the normal and sarcopenia groups were assessed with t-tests and chi-square tests, respectively. To assess differences in the medical history of osteoporosis between the normal and sarcopenia groups, Fisher's exact test was used because the minimum expected cell size was less than five.

Cumulative data were analyzed using GEE, which take into account the dependency of repeated observations within participants; this is an important feature that is necessary for longitudinal analyses. An additional advantage of GEE is that participants are included regardless of missing values. Thus, participants who were lost to follow-up after early wave examination were also included in the analyses. GEE models were fitted using the GENMOD procedure of SAS. The GENMOD procedure fits generalized linear models. The correlation structure was specified to be autoregressive.

The serum T levels were modeled as dichotomized variables in GEE analyses. In this study, the cut-off values of T were established based on the serum level of FT, because under the current circumstances in Japan, hypogonadism is diagnosed using the serum level of FT. The FT decreases during ageing, whereas the TT levels do not change during ageing in Japanese men[21,22]. In addition, measurement of SHBG cannot be performed for the diagnosis of hypogonadism in Japan, because SHBG measurement is not included in the gonadal function tests covered by health insurance. The participants were divided into two groups based on the serum level of FT in the baseline examination: the low level group (<7.7 pg/ml) and the normal level group (≥7.7 pg/ml). The FT of 7.7 pg/ml, which was minus 2 standard deviations below the mean for healthy Japanese men aged 40–49 years, was approximately equal to the 5th percentile of participants in this study[21]. Thus, the cut-off values of TT and cFT were defined as the 5th percentile of serum levels (TT 2.9 ng/ml; cFT 46.3 pg/ml) in participants.

Analyses were carried out with an unadjusted crude model and several adjusted models, controlling for different combinations of confounding variables: age was taken as a moderator variable in model 1; age, leisure-time physical activity, nutrition intake (total energy, total protein, vitamin D), medical history (stroke, heart disease, cancer, diabetes, osteoporosis, rheumatoid arthritis), and smoking habit were considered moderator values in model 2.

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Author contributions

A.Y. designed the study, carried out the statistical analyses and wrote the manuscript. R.O., R.K., I.K. and T.O. participated in data collection and analysis. F.A. and H.S. revised the manuscript and managed the overall project.

Additional information

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ORIGINAL ARTICLE

Age-related changes in skeletal muscle mass among community-dwelling Japanese: A 12-year longitudinal study

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Aim: The present study aimed to evaluate age-related changes in skeletal muscle mass among community-dwelling middle-aged and elderly Japanese.

Methods: This 12-year longitudinal study of a community-dwelling population in Japan included 15 948 examinations of 1962 men and 1990 women. We assessed appendicular muscle mass (AMM) using dual X-ray absorptiometry and calculated the skeletal muscle index (SMI) using the AMM divided by height squared (kg/m^2). Low muscle mass was defined as muscle mass minus two standard deviations below the mean for young healthy adults. Leg extension power (watts) was measured as an index of muscle function. Longitudinal data of skeletal muscle mass were analyzed using a general linear mixed-effect model.

Results: The prevalence of low muscle mass at the first wave of examinations was 27.1% in men and 16.4% in women. Longitudinal analysis showed that skeletal muscle mass decreased with aging during the 12-year study period except in middle-aged men, and to a greater extent in elderly men (*P* for trend, <0.001). Skeletal muscle mass decreased slightly, but significantly, in women. Although a cross-sectional analysis showed that SMI did not differ with age in women, leg extension power per leg muscle mass and grip strength per arm muscle mass as indices of muscle quality were significantly lower in older women (*P* for trend, <0.001 for both).

Conclusion: Age-related decreases in muscle mass were trivial, especially in women, but the quality of muscle decreased with aging in both sexes. **Geriatr Gerontol Int 2014; 14 (Suppl. 1): 85–92.**

Keywords: aging, epidemiology, longitudinal study, sarcopenia, skeletal muscle.

Introduction

Aging is associated with a progressive loss of neuromuscular function that often leads to progressive disability and loss of independence along with a reduced quality of life among the elderly.[1–6] The loss of skeletal muscle mass and strength with biological and pathological aging is now commonly described as sarcopenia.[1] This decline of skeletal muscle is thought to be inevitable even among healthy older adults. The European Working Group on Sarcopenia in Older People (EWGSOP) assumed that muscle loss is a required component for a diagnosis of sarcopenia, as well as low muscle strength and/or low physical performance.[6]

However, the rate at which community-dwelling populations lose skeletal muscle mass with aging is unclear, because accurate assessments of muscle mass can be challenging. Skeletal muscle mass can be determined by anthropometric measurements, bioelectrical impedance analysis and dual X-ray absorptiometry (DXA),[7,8] and DXA is the most effective method recommended for clinical practice.[7] However, DXA is usually impractical for epidemiological surveys, because it is costly and it involves exposure to radiation, although minimal.

The definition of low muscle mass (sarcopenia by muscle mass) proposed by Baumgartner in the Population of New Mexico Elder Health Survey has been widely applied.[9] This definition uses the ratio between appendicular skeletal muscle mass (ASM) of the upper and lower limbs (kg) and height squared (m^2; ASM/

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