Nutritional Effects of \( \gamma \)-Glutamyl Carboxylase Gene Polymorphism on the Correlation between the Vitamin K Status and \( \gamma \)-Carboxylation of Osteocalcin in Young Males

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Summary  Vitamin K is a cofactor for \( \gamma \)-glutamyl carboxylase (GGCX), which is an essential enzyme for the \( \gamma \)-carboxylation of vitamin K-dependent proteins such as osteocalcin (OC). Associations among dietary vitamin K intake, vitamin K status, and bone metabolism have not been thoroughly investigated. Recently, it has been reported that single nucleotide polymorphisms of GGCX (R325Q, 974G>A) were associated with age-related bone loss and the kinetic affinity for the substrate. In the present study, we investigated the associations among dietary vitamin K intake, the level of serum vitamin K, and the ratio of undercarboxylated OC (ucOC) to intact OC. The subjects were 60 healthy young male volunteers (mean age, 22.6 y; standard deviation, 1.6). Dietary nutrient intake was assessed by consecutive individual 3-d food records taken before the day of blood examinations. Serum concentrations of vitamin K (phyloquinone: PK, menaquinone 4: MK-4, and menaquinone 7: MK-7), ucOC, and intact OC were measured. All subjects were genotyped for polymorphism (R325Q) presence. Dietary vitamin K intake from vegetables was significantly correlated with the level of serum PK, and vitamin K intake from fermented beans, natto, was also significantly correlated with the level of serum MK-7. The ratio of ucOC to intact OC showed a negative association with the total vitamin K intake \( (r=-0.331, p=0.010) \) and serum MK-7 \( (r=-0.394, p=0.002) \). Interestingly, grouped by the GGCX genotype, a significant interaction between the ratio of ucOC to intact OC with serum MK-7 was observed in 325R homozygotes \( (r=-0.572, p=0.003) \), but not in heterozygotes, nor in 325Q homozygotes. This is the first report to suggest the effects of the single nucleotide polymorphism R325Q in the GGCX gene on the correlation between the level of serum MK-7 and \( \gamma \)-carboxylation of serum OC.

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

Osteoporosis was re-defined as a skeletal disorder characterized by compromised bone strength predisposing a person to an increased risk of fracture (1). The disease results from complex interactions between genetic and environmental factors. To reduce the risk of osteoporosis, it seems to be important to maximize peak bone mass during youth. Nutrition is one of the most important environmental factors in the prevention of osteoporosis, so nutritional education targeted at accelerating and maintaining bone health is indispensable for young people.

Recently, vitamin K has been suggested to play a role in the improvement of bone metabolism (2). Vitamin K is a cofactor for the vitamin K-dependent carboxylase, known as gamma-glutamyl carboxylase (GGCX), which facilitates the post-translational carboxylation of glutamyl residues in selected proteins (3–7). Three vitamin K-dependent proteins (osteocalcin, matrix Gla protein, and protein S) are found in bone; osteocalcin (OC) is the most abundant (8–10). OC is produced in osteoblasts, and fully carboxylated osteocalcin binds to the calcium ion of hydroxyapatite (11). 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 (12–15). In previous studies, elevated
ucOC has been associated with an increased risk of hip fracture in elderly people (16–19).

In nature, vitamin K is found in two forms. Vitamin K1 (phyloquinone; PK) is the primary dietary source of vitamin K in Western countries and is found in leafy, green vegetables (20). Vitamin K2 (menaquinone; MK-n) comprises a family of naphthoquinones with differing numbers of isoprenoid residues, and meat, egg, and dairy foods contain menaquinone-4 (MK-4). Japanese fermented beans, Bacillus natto (referred to as natto), contain large amounts of menaquinone-7 (MK-7) synthesized by bacteria (21). A previous report found a significant positive correlation between the level of MK-7 in serum and the habit of eating natto in postmenopausal women (22).

Recent advances in genetic research have made it possible to clarify polymorphisms of many bone metabolism-related genes (23–25). Kinoshita et al. reported a significantly higher association between the single nucleotide polymorphism (SNP) GGCX (R325Q, 974G>A) (rs699664, archived in the dbSNPs at http://www.ncbi.nlm.nih.gov/) and bone mineral density (BMD) among postmenopausal women (23). Dietary interventions based on genotypic knowledge may comprise a useful strategy for the prevention of osteoporosis. However, the role of SNP of the GGCX gene in the relationship between dietary vitamin K intake and the serum vitamin K status has not been investigated.

In this study, we examined the GGCX genotypes, dietary vitamin K intake interactions, and vitamin K metabolism in healthy young males. Our study may help to elucidate novel additional strategies for nutritional education concerning bone metabolism.

METHODS

Subjects. This study protocol was approved by the Institutional Review Board of the Japan Women’s University, and written informed consent was obtained from all study subjects. Young males living in Tokyo, Japan, were recruited. Participants were excluded if they had metabolic disease. The study population consisted of 60 healthy Japanese males aged 22.6 ± 1.6 y (mean ± SD), with a height of 172.8 ± 6.0 cm, weight of 63.2 ± 7.1 kg, and BMI of 21.1 ± 2.1.

Dietary assessment. Dietary nutrient intakes were measured by 3-d food records taken before the day of blood examinations. Trained personnel reviewed the food records, and the nutrient content was determined with the use of Elyo-Kun software (Kenpakusha, Japan). We estimated phyloquinone (PK) intake from vegetables and menaquinone-7 (MK-7) intake from natto, which is a Japanese fermented bean.

Measurement of the concentrations of serum bone markers, PK, MK-4, and MK-7. Fasting blood samples were obtained and serum and plasma were kept frozen at −80° C until measurement. The serum concentration of vitamin K (PK, MK-4, and MK-7) was measured using the new liquid chromatography-atmospheric pressure chemical ionization-tandem mass-mass spectrometry (LC-APCI-MS/MS) method (33). The concentration of undercarboxylated osteocalcin (ucOC), as a sensitive marker of vitamin K deficiency, was measured with the new electro-chemiluminescence immunoassay (Sanko Junyaku Co., Ltd., Ibaraki, Japan) as described by Tsugawa et al. (34). The specific antibody to ucOC was purchased from Takara Shuzo Co., Ltd. (Kyoto, Japan). Serum-intact OC was measured by immuno-radiometric assay (Mitsubishi Kagaku Bio Clinical Laboratories Inc., Tokyo, Japan). A bone formation marker, bone-specific alkaline phosphatase (BAP), was determined by enzyme immunoassay (Sumitomo Seiyaku Co., Ltd., Osaka, Japan).

Genotyping for molecular variants in the GGCX gene. All subjects were genotyped for GGCX polymorphism (R325Q, 974G>A) (dbSNP: rs699664). 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 polymerase chain reaction (PCR) (forward primer: 5’-TCTCTACTATCTATGCGGACG-3’; reverse primer: 5’- TGGTGTAGAAATGAGAATAGGGCA-3’). GGCX polymorphism was determined by direct sequencing using the thermo sequence Cy 5.5 dye terminator cycle sequencing kit (Amersham Biosciences Corp., Piscataway, NJ) with a Gene Rapid sequencer (Amersham Biosciences Corp.).

Statistical analysis. Values are shown as mean ± SD, and Spearman rank correlation coefficients were calculated to analyze the relationship between two parameters. 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 SPSS13.0J (SPSS Inc., IL, USA).

RESULTS

Descriptive characteristics of the study subjects, the serum vitamin K status, and bone markers are shown in Table 1. In these subjects, the mean (±SD) total dietary vitamin K intake was 212.2 ± 140.2 µg/d. Vitamin K from vegetables, which are the main PK source, was 70.9 ± 52.8 µg/d, and that from natto, which is the main MK-7 source, was 58.3 ± 105.1 µg/d.

As shown in Fig. 1, dietary vitamin K intake from vegetables (PK) was significantly correlated with the concentration of serum PK (r=0.337, p=0.009). The dietary vitamin K intake from natto (MK-7) was also significantly correlated with serum MK-7 (r=0.663, p<0.001) (Fig. 2).

The ratio of ucOC to intact OC is considered a sensitive measure of the vitamin K status of bone. As shown in Fig. 3, the ratio of ucOC to intact OC showed a significant negative association with the total vitamin K intake (r=−0.331, p=0.010).

Furthermore, we investigated the relation between the ratio of ucOC to intact OC and serum vitamin K status. Although serum PK and MK-4 were not significantly associated with the ratio of ucOC to intact OC, serum MK-7 was significantly negatively associated with the ratio of ucOC to intact OC in the total subjects.
Table 1. Dietary vitamin K and calcium intakes, serum vitamin K status, and bone markers.

<table>
<thead>
<tr>
<th></th>
<th>Mean ± SD</th>
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</thead>
<tbody>
<tr>
<td>Vitamin K Intake (µg/d)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>212.2 ± 140.2</td>
</tr>
<tr>
<td>From vegetables</td>
<td>70.9 ± 52.8</td>
</tr>
<tr>
<td>From natto</td>
<td>58.3 ± 105.1</td>
</tr>
<tr>
<td>Calcium intake (mg/d)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>595.9 ± 285.8</td>
</tr>
<tr>
<td>From dairy products</td>
<td>234.2 ± 215.2</td>
</tr>
<tr>
<td>From vegetables</td>
<td>57.6 ± 38.0</td>
</tr>
<tr>
<td>Serum</td>
<td></td>
</tr>
<tr>
<td>PK (ng/mL)</td>
<td>0.56 ± 0.34</td>
</tr>
<tr>
<td>MK-4 (ng/mL)</td>
<td>0.07 ± 0.05</td>
</tr>
<tr>
<td>MK-7 (ng/mL)</td>
<td>6.97 ± 13.30</td>
</tr>
<tr>
<td>Intact OC (ng/mL)</td>
<td>7.8 ± 3.6</td>
</tr>
<tr>
<td>ucOC (ng/mL)</td>
<td>6.8 ± 3.7</td>
</tr>
<tr>
<td>ucOC/intact OC</td>
<td>0.95 ± 0.41</td>
</tr>
<tr>
<td>BAP (U/mL)</td>
<td>32.4 ± 10.3</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SD.

Fig. 1. Association between vitamin K intake from vegetables and the concentration of serum PK in all subjects. There was a significant correlation between the two parameters ($y = 0.002x + 0.411$, $r = 0.337$, $p = 0.009$).

Fig. 2. Association between vitamin K intake from natto and the concentration of serum MK-7 in all subjects. There was a significant correlation between the two parameters ($y = 0.061x + 2.525$, $r = 0.663$, $p < 0.001$).

Fig. 3. Association between total vitamin K intake and the ratio of ucOC to intact OC in all subjects. There was a significant negative correlation between the two parameters ($y = -0.001x + 1.117$, $r = -0.331$, $p = 0.010$).

Fig. 4. Associations between the concentration of serum vitamin K and the ratio of ucOC to intact OC in all subjects. (A) Associations between the concentration of serum PK and the ratio of ucOC to intact OC. (B) Associations between the concentration of serum MK-4 and the ratio of ucOC to intact OC. (C) Associations between the concentration of serum MK-7 and the ratio of ucOC to intact OC. There was a significant negative correlation between serum MK-7 and the ratio of ucOC to intact OC ($y = -0.016x + 1.025$, $r = -0.394$, $p = 0.002$).

(r = -0.394, $p = 0.002$) (Fig. 4).

The distribution of GGCX gene polymorphism (R325Q, 974G>A) did not deviate from the Hardy-Weinberg expectations ($p > 0.05$). The allele frequencies were 0.650 for the G allele and 0.350 for the A allele. Twenty four subjects showed the 325R (GG-type)
Table 2. Serum vitamin K status and bone markers.

<table>
<thead>
<tr>
<th>GG CX group</th>
<th>n</th>
<th>PK (ng/mL)</th>
<th>MK-4 (ng/mL)</th>
<th>MK-7 (ng/mL)</th>
<th>Intact OC (ng/mL)</th>
<th>ucOC (ng/mL)</th>
<th>ucOC/Intact OC</th>
<th>BAP (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG</td>
<td>24</td>
<td>0.52±0.27</td>
<td>0.06±0.04</td>
<td>6.18±10.59</td>
<td>7.3±2.2</td>
<td>6.2±2.4</td>
<td>0.90±0.39</td>
<td>30.0±7.0</td>
</tr>
<tr>
<td>GA</td>
<td>30</td>
<td>0.60±0.40</td>
<td>0.08±0.05</td>
<td>7.37±16.04</td>
<td>8.4±4.7</td>
<td>7.6±4.6</td>
<td>1.03±0.44</td>
<td>34.5±12.9</td>
</tr>
<tr>
<td>AA</td>
<td>6</td>
<td>0.55±0.34</td>
<td>0.07±0.04</td>
<td>8.18±8.51</td>
<td>7.9±3.6</td>
<td>5.5±1.5</td>
<td>0.73±0.20</td>
<td>31.9±3.9</td>
</tr>
</tbody>
</table>

Each value represents the mean±SD.


Table 3. Dietary vitamin K and calcium intake.

<table>
<thead>
<tr>
<th>GG CX group</th>
<th>n</th>
<th>Vitamin K intake (μg/d)</th>
<th>Calcium intake (mg/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total From natto From vegetables</td>
<td>Total From dairy products From vegetables</td>
</tr>
<tr>
<td>GG</td>
<td>24</td>
<td>197.0±104.1 45.3±83.5 61.1±45.5</td>
<td>557.9±220.1 227.4±187.6 54.8±37.8</td>
</tr>
<tr>
<td>GA</td>
<td>30</td>
<td>229.4±171.8 73.6±125.8 76.3±58.1</td>
<td>630.9±334.0 238.9±241.6 60.3±39.7</td>
</tr>
<tr>
<td>AA</td>
<td>6</td>
<td>187.6±85.2 33.8±59.2 82.9±54.8</td>
<td>572.6±280.4 237.3±214.2 54.6±34.7</td>
</tr>
</tbody>
</table>

Each value represents the mean±SD.

Fig. 5. Association between the level of serum MK-7 and the ratio of ucOC to intact OC. Grouped by GG CX genotype, there was a significant negative relation in 325R (GG-type) homozygotes (A) (r = 0.572, p = 0.003), but not in heterozygotes (GA-type) (B) (r = 0.260, p = 0.166), nor 325Q (AA-type) homozygotes (C) (r = 0.143, p = 0.787).

DISCUSSION

Vitamin K is a cofactor for GG CX that facilitates the conversion of specific glutamyl residues to γ-carboxyglutamyl residues in bone-specific protein, such as OC. The physiological role of OC in bone metabolism has not yet been elucidated, but 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 (16–19). In this study, we examined the correlation between dietary vitamin K intake and vitamin K metabolism in healthy young males.

Green leafy vegetables contain the highest content of PK and significantly contribute to the total vitamin K intake. Natto, Japanese fermented beans, contains large amounts of MK-7 synthesized by bacteria (21), and previous reports have demonstrated that the level of serum MK-7 was significantly higher in those who ate natto frequently in Japanese postmenopausal women (22).
Previous studies have reported a significant correlation between PK intake from vegetables and plasma phylloquinone levels in postmenopausal Caucasian women (35) and healthy adults (36). In the present study, we determined the concentrations of serum PK, MK-4, and MK-7 using the LC-APCI-MS/MS technique, which is a very sensitive method for minimum detectable levels (33). By means of this effective method, we clarified a significant positive correlation between the vitamin K intake from vegetables (PK), estimated from the 3-d food records, and serum PK concentration \((r=0.337, p=0.009)\) (Fig. 1). This result also indicated that a weight-recording method of the dietary intake of PK is reliable for estimating the dietary intake of PK related to serum PK. As shown in Fig. 2, we also clarified that dietary MK-7 intake from natto was significantly correlated with the serum MK-7 concentration \((r=0.663, p=0.001)\).

The pharmacological administration of MK-4 (45 mg/d) had positive effects on a decreasing serum ucOC concentration and the prevention of bone fractures (37). As shown in Table 1, the concentration of serum MK-4 was very low compared with PK or MK-7 in young males. Almost the same data were obtained by Tsugawa et al. (34). They reported that the plasma concentration of MK-4 (0.07 ng/mL) was the lowest compared with PK (1.52 ng/mL) and MK-7 (4.96 ng/mL) in healthy women aged 30–49 y \((n=52)\) (34).

In the present study, we demonstrated that serum MK-7 was associated with the ratio of ucOC to intact OC, but not with serum PK or MK-4 in healthy young males. Tsugawa et al. demonstrated a significant negative correlation between the plasma concentrations of PK or MK-7 and the ratio of ucOC to intact OC in women aged >50 y, but not in those aged 30–49 y, and suggested that there was a difference in the vitamin K requirement for \(\gamma\)-carboxylation depending on age (34).

GGCX is a 758-residue integral membrane protein that appears to have 3 trans-membrane domains near its amino terminus (38). The GGCX gene is located on chromosome 2, 2p12 (39), and spans about 13 kb containing 15 exons (40). Various mutations in the GGCX gene have been discovered. L394R is the first reported missense mutation in the GGCX gene in 4 affected members of inbred kindred with a deficiency of vitamin K-dependent blood coagulation factors by Brenner et al. (41). This mutated carboxylase protein expressed in Drosophila cells was stable but demonstrated a threefold-reduced activity compared with wild-type carboxylase, confirming that the L394R mutation results in a defective carboxylase.

Recently, Kinoshita et al. reported that polymorphism of the GGCX gene \([R325Q, 974G>A]\) was associated with age-related bone metabolism in postmenopausal women \((n=500, \text{age: } 73.6\pm5.7)\) (23). In a subpopulation older than 75 y \((n=207)\), there was a significantly higher BMI-adjusted Z-score in those showing the 325Q (974A) GGCX gene homozygote (AA-type) among genotypes, but not in the younger subgroup \((\leq75\text{ y})\) (23). These results suggested that this variation in the GGCX gene may be an important determinant of age-related bone loss in humans.

Population diversity was reported using the dbSNPs of NCBI (http://www.ncbi.nlm.nih.gov/), whereby this polymorphism in the GGCX gene was found in various populations. The genotypes frequencies in GGCX R325Q (974G>A) of a European population \((n=60)\) were 0.575 for G and 0.425 for A, respectively (dbSNPs of NCBI). For African Americans \((n=23)\), these were 0.304 for G and 0.696 for A, respectively (dbSNPs of NCBI). In an Asian population \((n=45)\), the frequencies were 0.667 for G and 0.333 for A, respectively (dbSNPs of NCBI). In the present study, the frequencies were almost identical to those of the Asian population.

In addition, our study suggested that GGCX genotypes have an effect on the serum vitamin K status in healthy young males. Interestingly, a significant negative relation between serum MK-7 and the ratio of ucOC to intact OC was observed in 325R homozygotes (GG-type) \((r=-0.572, p=0.003)\), but not in heterozygotes (GA-type) \((r=-0.260, p=0.166)\), nor 325Q homozygotes (AA-type) \((r=-0.143, p=0.787)\) (Fig. 5B). These results indicate that the requirement of vitamin K for \(\gamma\)-carboxylation may be different depending on the GGCX genotypes. It was demonstrated that expression of the R325Q GGCX gene using COS-7 cells showed that the protein translated from 325Q (974A) had a significantly lower Km value \((p=0.029)\) compared with that of cells bearing the 325R (974G) gene (23). 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 specificity, modulation of activity, and may also contribute to regulatory effects on gamma-carboxylation.

Our present findings indicate the possibility that gene-nutritional factor-related interactions potentially modulate osteoporosis risk. We suppose that the higher Km value of 325R (974G) would mean that a higher intake of vitamin K may cancel the effects of this genotype (23), although the reason why a significant negative relationship between serum MK-7 and the ratio of ucOC to intact OC was observed is unclear. An adequate nutritional strategy is necessary for people with high-risk genotypes, and our data may be valuable to establish novel strategies of nutritional education for the prevention and treatment of osteoporosis. As there are the limitations of this association study due to the small sample size and the lack of extensive functional studies, further investigations are necessary to determine the recommended dietary allowance of vitamin K for the maintenance of adequate \(\gamma\)-carboxylation.

REFERENCES
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