

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 phyloquinone (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 ed-

ucation 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 K₁ (phylloquinone: PK) and vitamin K₂ (menaquinone: MK-n). Green leafy vegetables contain the highest content of PK and significantly contribute to the total vitamin K intake. Vitamin K₂ 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/m².

Fasting blood samples were obtained and sera were kept frozen at -80°C until measurement. The concentra-

tion 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) method²⁸ 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 Eiyō-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'-TGTTCTCTACGTCATGCTGGCCAG-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-

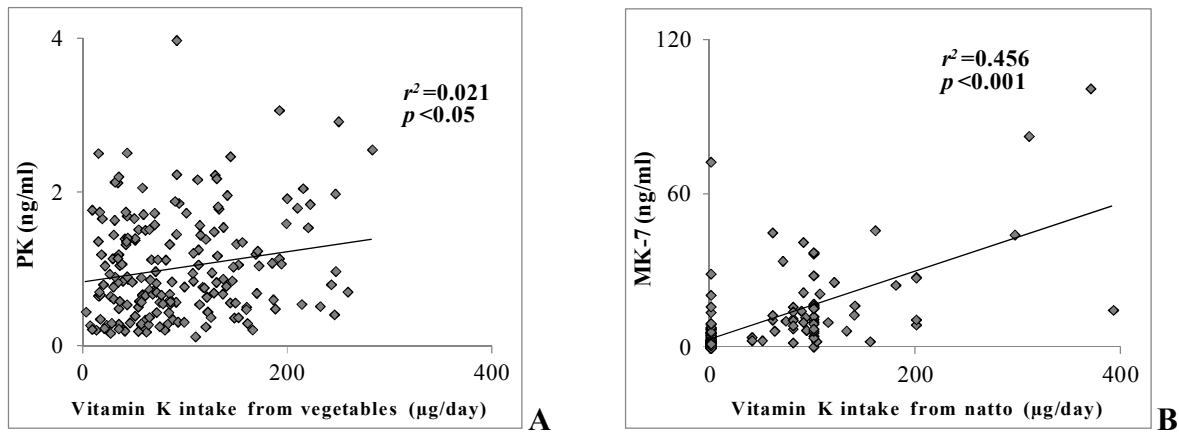


Figure 1. Association between vitamin K intake from vegetables and the concentration of serum PK (A), and association between vitamin K intake from natto and the concentration of serum MK-7 (B). There were significant negative correlations between the two parameters (A: $y=0.002x + 0.8375$, $r^2=0.021$,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

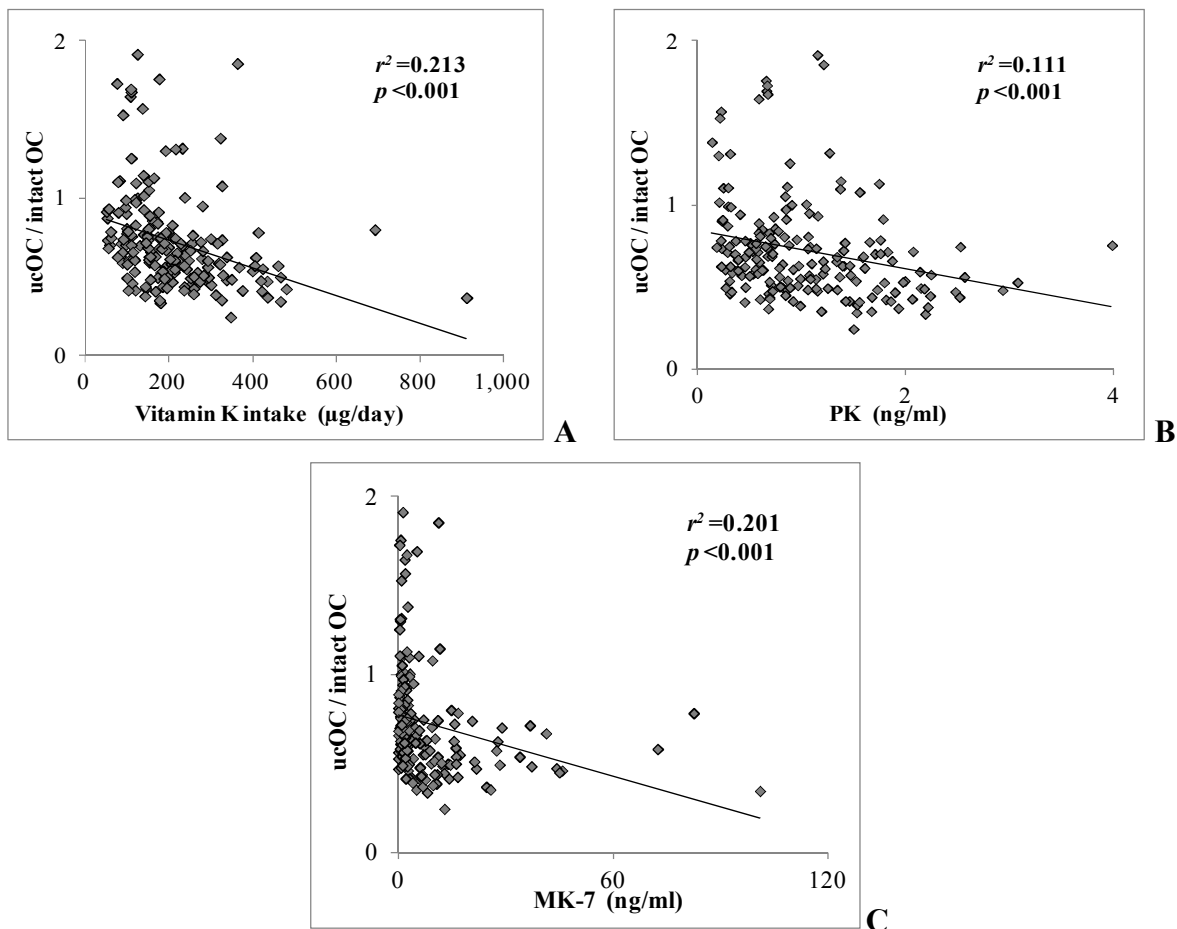


Figure 2. Association between the ratio of ucOC to intact OC and total vitamin K intake (A), and the concentration of PK (B) or MK-7 (C). There were significant negative correlations between the two parameters (A: $y=-0.0009x + 0.909$, $r^2=0.213$, $p<0.001$, B: $y=-0.1169x + 0.8464$, $r^2=0.111$, $p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

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.

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.

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 fracture³¹, 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-

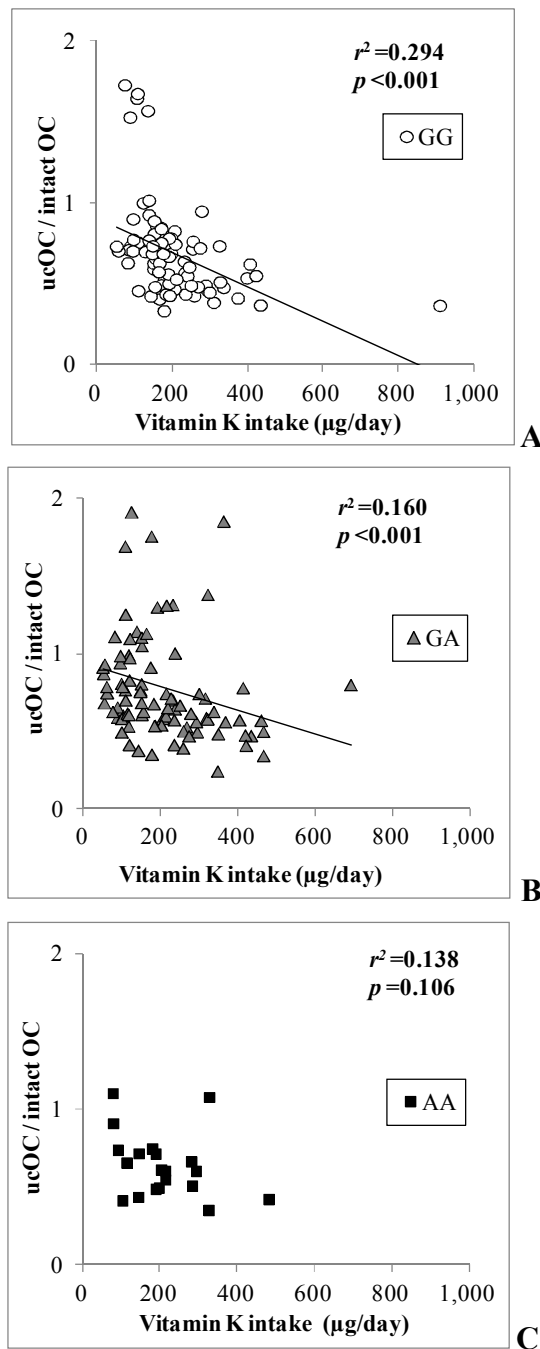


Figure 3. Association between the total vitamin K intake and ratio of ucOC to intact OC. Grouped by GGCX genotype, there was a significant negative correlation between the two parameters in 325R (GG-type) homozygotes (A) ($y=-0.0011x + 0.9105$, $r^2=0.294$, $p<0.001$) and heterozygotes (GA-type) (B)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

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

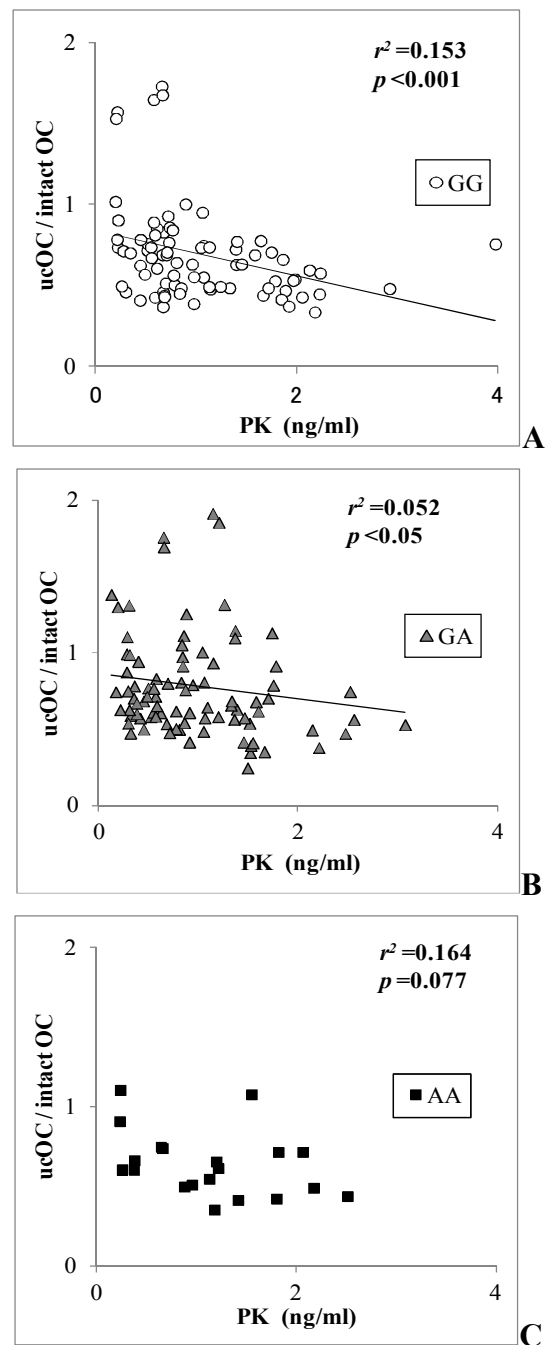


Figure 4. Association between the concentration of serum PK and ratio of ucOC to intact OC. Grouped by GGCX genotype, there was a significant negative correlation between the two parameters in 325R (GG-type) homozygotes (A) ($y=-0.139x + 0.8344$, $r^2=0.153$, $p<0.001$) and heterozygotes (GA-type) (B)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

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

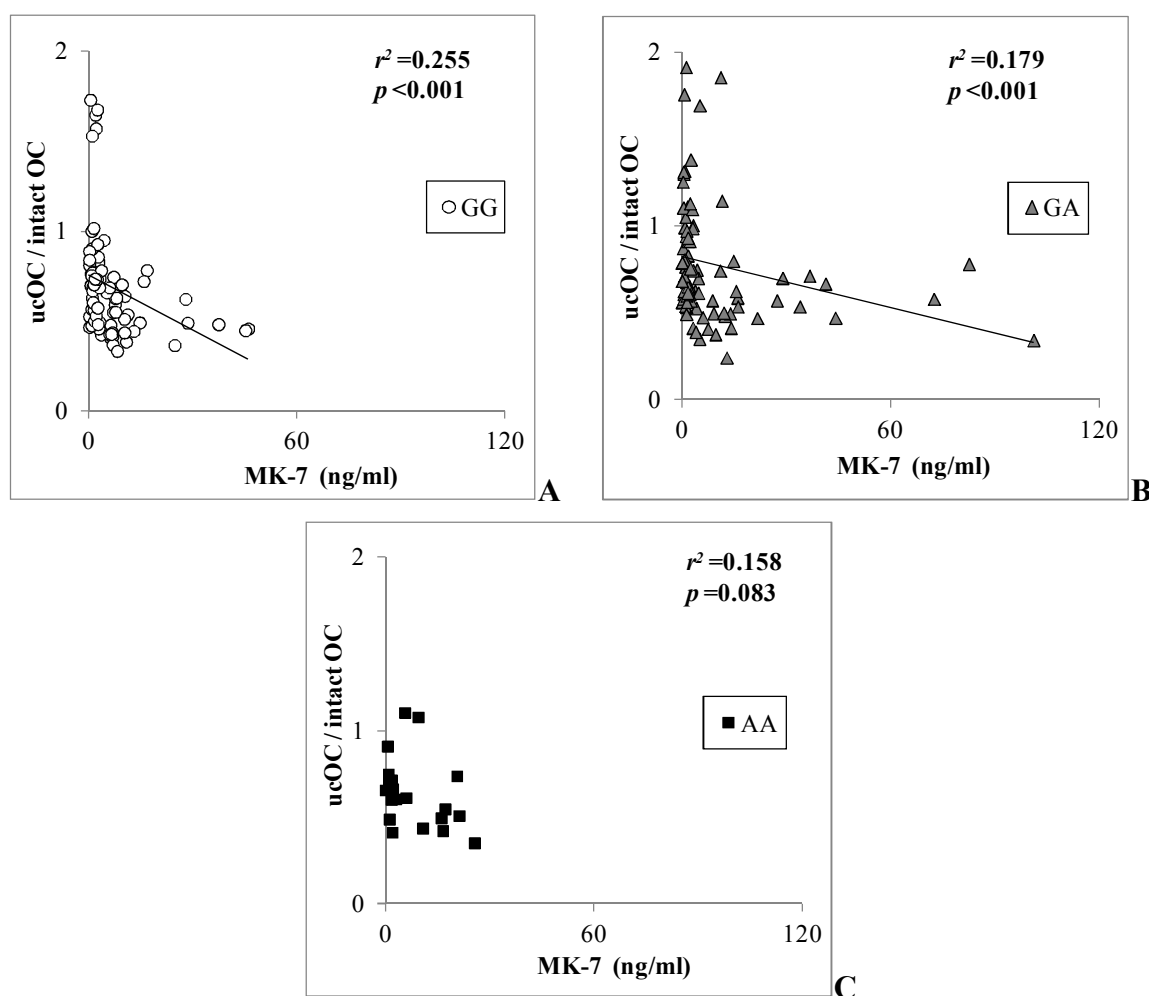


Figure 5. Association between the concentration of serum MK-7 and ratio of ucOC to intact OC. Grouped by the GGCX genotype, there was a significant negative correlation between the two parameters in 325R (GG-type) homozygotes (A) ($y = -0.0101x + 0.7533$, $r^2 = 0.255$, $p < 0.001$) and heterozygotes (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)