Relation of Bone Mass to Vitamin D Receptor Gene Polymorphism and Lifestyle Factors in Japanese Female College Students

Yoichi Tachi1, Yuri Sakamoto10, Kaoruko Iida2 and Pao-Li Wang3

1) Laboratory of Nutritional Physiology, Tokyo Kasei University, Tokyo, Japan
2) Department of Nutrition and Food Science, Graduate School of Humanities and Science, Ochanomizu University, Tokyo, Japan
3) Department of Innovation in Dental Education, Osaka Dental University, Osaka, Japan

Abstract: Osteoporosis, caused by loss in bone mass, has become a major concern in Japan. Several studies have indicated a relationship between bone mass and gene polymorphisms; however, the effect of lifestyle habits is yet to be explored. The aim of the present study was to identify vitamin D receptor (VDR) gene polymorphism and lifestyle factors associated with bone mass in Japanese female college students. The subjects comprised 317 female college students aged between 20 and 24, all of whom were living in the Kanto region at the time of the study. Bone mass measurement was based on qualitative ultrasound (QUS). The subjects were asked what type of sport they used to play in their junior high school and high school days. Gene polymorphism for the restriction enzyme TaqI digestion site (rs731236) were detected by real-time PCR using TaqMan probes. No significant difference in the bone mass was observed between the VDR polymorphisms (TT versus TC + CC). The present study did not demonstrate any correlation between VDR polymorphism and bone mass. Similarly, no correlation was observed between nutritional intake and bone mass. Meanwhile, the results of multiple linear regression analysis ascertain that high BMI levels and the experience of high-impact exercise during junior high school or high school have an independent positive effect on bone mass. These results suggest that BMI and exercise are highly likely to be important factors in increasing bone mass.

Key words: Bone mass, Vitamin D receptor, Gene polymorphism, Multiple linear regression analysis

Introduction

Japan is facing an issue of super-aging society, with the life expectancy reaching approximately 87 years for women and 81 years for men. Amid such circumstances, the prevention of osteoporosis is an urgent issue, given that the condition is leaving many elderly people bedridden after an osteoporosis-derived fracture, considerably aggravating their quality of life. At present, the number of Japanese osteoporosis patients is estimated to be around 12.8 million, with women accounting for about 80%. The most effective tool for assessing the fracture risk of osteoporosis patients is to measure bone mass. People normally have the peak amount of bone mass around age 20, after which it is maintained at stable levels before starting to decrease around age 50. Women, in particular, reach menopause around 50 years of age, which makes it inevitable for them to lose bone mass to a certain extent. Several studies have indicated that it is important for women to acquire high levels of maximum bone mass around age 20 to prevent osteoporosis. Factors affecting bone mass include sex, age, genes, exercise, and nutrition. In recent years, some notable advances have been made in the field of genetics. Among the genes that may be associated with the development of osteoporosis, polymorphism of the vitamin D Receptor (VDR) gene has been most widely studied. While some studies have indicated a correlation between VDR gene polymorphisms and bone mass (osseous-assessment index: OSI), others have argued against any correlation. One problem inherent in these studies is that they tend to focus solely on gene polymorphisms while disregarding the impact of lifestyle habits, such as the intake of nutrients and impact of exercise. In particular, regarding Japanese women, little data is available on the correlation between VDR polymorphisms and lifestyle habits: the intake of nutrients and impact of exercise. Therefore, the aim of the present study was to identify VDR gene polymorphism and lifestyle factors associated with bone mass in Japanese female college students.

Materials and Methods

Subjects

The subjects comprised 368 female college students aged between 20 and 24, all of whom were living in the Kanto region at the time of the study. After briefed on the study objective both verbally and in writing, the subjects were allowed to express their consent in writing of their own volition. The implementation of this study was approved by the ethics committee of Tokyo Kasei University (Approval number: ITAH26-04). Of the 368 subjects, 42 who had received treatment at obstetrics and gynecology for conditions such as menstrual disorder and 9 who had provided inadequate questionnaire data were excluded. This left 317 students who were subjected to the final analysis.

Bone mass measurement

Bone mass measurement was based on qualitative ultrasound (QUS). The right calcaneus bone of each subject was measured with an AOS...
100SA system (Hitachi Co. Lid. Tokyo, Japan). First, the speed of sound (SOS) and transmission index (TI) of the site were measured. Then, based on the formula TI × SOS, the osteo sono-assessment index (OSI) was calculated. OSI is a general index of overall bone strength, including elasticity and flexibility. The present study adopted OSI as an index of bone mass. All OSI values referenced in this manuscript are ones in which the original values were divided by 10².

**Table 1. Strength of the exercise**

| high impact group | low impact group |
|-------------------|------------------|
| karate            | aikido           |
| soccer            | rhythmic         |
| jogging           | gymnastics       |
| tennis            | walking          |
| skipping rope     | swimming         |
| basketball        | aerobics         |
| ballet            | skating          |
| volleyball        | bone             |
| handball          | cross-country    |
| beach volleyball  | gymnastics       |
| marathon          | kendo            |
| lacrosse          | table tennis     |
| running           | kendo            |
| track and field   | table tennis     |

**Questionnaire about exercise**

The subjects were asked what type of sport they used to play in their junior high school and high school days. Those who used to practice several sports were requested to specify all of them. Based on the intensity of physical exercise, the subjects were divided into the high-impact and low-impact groups. Table 1 lists the specific types of sport the respondents were engaged in. The authors had already published this data in a previous report. This classification was in accordance with a report by Hara et al., which was predicated on a number of international studies and hence was believed to be highly credible.

**Current nutrient intake revealed by a survey on food-consumption frequency**

The subjects’ current nutrient intake was surveyed using Food Frequency Questionnaire Based on Food Groups (FFQG) Ver.3.0 software, an add-in software of Excel Eiyou-kun (Kenpakusha Co. Lid. Tokyo, Japan). The daily intake of three nutrients (calcium, vitamin D, and vitamin K) was calculated per 1,000 kcal.

**DNA extraction and genotyping**

DNA was extracted from saliva samples using an Oragene® DNA extraction kit (DNA Genotek Co. Lid., Ottawa, Canada) in accordance with the manufacturer’s protocol. The final concentration of the extracted DNA samples was coordinated at less than 100 ng/µl. The extracted DNA samples were stored at -20°C. Gene polymorphism for the restriction enzyme TagI digestion site (rs731236) were detected by real-time PCR using TaqMan probes. The real-time PCR was performed using Cycler PCR Reaction Mix and Thermal Cycler Dice Time System Lite (Takara Bio Co. Lid. Shiga, Japan). A 256 base pair (bp) fragment was generated by PCR with primers located on intron 8 and exon 9. The primer sequences were 5'-AGCTTCTGTTGCCCTTCTTTCC-3' (forward) and 5'-TTAGCTCATGCTGCACTCA-3' (reverse). The FAM-labelled cycling probe T had an antisense sequence of 5'-(FAM) TGGCCTC(A) AT(Eclipse)-3', whereas the ROX-labelled cycling probe C had an antisense sequence of 5'-(ROX) GGCCTC(G)AT(Eclipse)-3'. The 2 × Cycler PCR reaction mix, 10 µM PCR forward primer, 10 µM PCR reverse primer, 5 µM FAM-labelled cycling probe T, and 5 µM ROX-labelled cycling probe C were mixed with the DNA samples coordinated at less than 100 ng/µl. The solutions were then subjected to 45 cycles of 95°C for 5 seconds, 55°C for 10 seconds, and 72°C for 20 seconds. Genotypes were determined using software packaged with Thermal Cycler Dice Time System Lite. The results of the software determination were verified in two ways: digestion by the restriction enzyme TagI and sequencing of the products. Three base sequences exist for VDR gene polymorphism (rs731236): TT, TC, and CC. From the software-determined samples, two to five were picked from each type to ascertain the results. Fig. 1 shows the image of PCR products digested by the restriction enzyme TagI. In some previous studies, TC and CC were represented as Tt and tt, respectively, but they signify the same types. The authors purified PCR products prior to digestion by the restriction enzyme TagI. Since the 256 bp bands of the PCR products were sliced out and purified using Wizard® SV Gel and PCR Clean-Up System (Promega Co. Lid.), the image does not show primer bands. Fig. 2 shows the confirmed sequences of the VDR gene polymorphism (rs731236).

**Statistical processing**

All values were shown as mean ± SD. Student’s t-test was performed to compare two groups after the normal distribution of the data was confirmed. To reveal factors that independently affect bone mass (OSI), multiple linear regression analysis was performed.
Table 2. Characteristics of the subjects among 317 women

|                   | TT (78%) | TC + CC (22%) | p    |
|-------------------|----------|---------------|------|
| Bone mass (OSI)   | 2.7 ± 0.2| 2.7 ± 0.3     | 0.872|
| Age (years)       | 20.1 ± 0.4| 20.2 ± 0.2    | 0.540|
| BMI (kg/m²)       | 20.4 ± 1.9| 20.4 ± 2.3    | 0.978|
| Calcium (mg/1,000 kcal/day) | 270.5 ± 75.4 | 254.1 ± 62.7 | 0.097|
| Vitamin D (µg/1,000 kcal/day) | 2.5 ± 1.1 | 2.4 ± 1.1 | 0.508|
| Vitamin K (µg/1,000 kcal/day) | 91.5 ± 31.9 | 88.2 ± 36.1 | 0.468|

Table 4. Multiple linear regression analysis on variables associated with bone mass among 317 women

|                          | β      | SE    | p     |
|--------------------------|--------|-------|-------|
| VDR gene polymorphism (rs731236) | 0.00603 | 0.033 | 0.857 |
| BMI (kg/m²)              | 0.04514 | 0.007 | <0.01*|
| Calcium (mg/1,000 kcal/day) | 0.00003 | <0.001 | 0.873|
| Vitamin D (µg/1,000 kcal/day) | -0.01343 | 0.014 | 0.329|
| Vitamin K (µg/1,000 kcal/day) | 0.00037 | <0.001 | 0.440|
| High-impact exercise during junior high school or high school | 0.06437 | 0.028 | <0.05* |

Table 3. Characteristics of the subjects stratified according to vitamin D receptor gene polymorphism (rs731236)

- **Discussion**

No correlation was observed between the VDR polymorphism (rs731236) and bone mass (OSI). Likewise, no correlation was present between the nutritional intake and bone mass (OSI). Meanwhile, the results of multiple linear regression analysis confirmed that high-impact exercise during junior high school or high school had a positive impact on bone mass (OSI) independent of the other factors. These results were consistent with the findings from a previous study that the authors conducted in the past targeting another group of people. This another group studied the relationship between female bone mass and exercise. The positive influence of exercise on bone mass (OSI) has been corroborated by many reports. As expected, exercise appears to be a critical factor in improving bone mass (OSI).

The results of multiple linear regression analysis indicate that BMI has an independent positive impact on bone mass (OSI). High levels of BMI mean that body weight is heavy relative to height. The heavier one weighs, the more load is placed on the bones like exercise, thus augmenting bone mass. In a previous study targeting a different group of subjects, we observed a positive correlation between BMI and bone mass. Several previous studies have suggested similar correlation between body BMI and increased bone mass. However, increasing BMI solely to increase bone mass is not advisable. It is common knowledge that high BMI levels are attributed to lifestyle-related diseases. A lesson to be learned from these findings is that non-exercisers with low BMI are predisposed to decreased bone mass.

While some reports suggest association between VDR polymorphisms and bone mass (OSI), other argue against any correlation. With varying results being obtained from Japanese subjects, further investigation will be necessary. However, almost all results suggesting a correlation between VDR polymorphisms and bone mass (OSI) are obtained from studies involving a small number of samples. By contrast, most studies, including the present study, refuting the correlation between VDR polymorphisms and bone mass (OSI) have involved a relatively large number of subjects. To yield more accurate results in the future research, conducting larger-scale studies involving more subjects will be essential.

The vitamin D receptor plays several vital roles in vivo, such as maintaining normal bone metabolism. Previous studies have reported that VDR polymorphisms are associated with preposition to various diseases, and bone metabolism disorder is responsible for a variety of conditions, including periodontal disease, abnormal immune functions, and...
incidence of cancer. The VDR gene is located on chromosome 12, and the TaqI polymorphism (rs731236) exists on exon 9. Although this domain does not directly encode amino acids, reports have suggested that this polymorphism is correlated with the difference in the length of the poly-A tail of the 3'-untranslated region. This may be the cause of functional differences. However, with some points remaining unclear, the underlying mechanism has not been completely elucidated. It is hoped that future studies will shed light on how VDR gene polymorphisms are responsible for certain diseases. The present study aimed to clarify a portion of the entire bone metabolism mechanism. The authors are confident that the results of this study will contribute to dentistry and life science as a whole.

In conclusion, the present study did not demonstrate any correlation between VDR polymorphism (rs731236) and bone mass (OSI). Similarly, no correlation was observed between nutritional intake and bone mass (OSI). Meanwhile, the results of multiple linear regression analysis ascertain that high BMI levels and the experience of high-impact exercise during junior high school or high school have an independent positive effect on bone mass (OSI). These results suggest that BMI and exercise are highly likely to be important factors in increasing bone mass (OSI).

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Conflict of Interest

The authors have declared that no COI exists.

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