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REGULAR ARTICLE

A1330V polymorphism of the *low-density lipoprotein receptor-related protein 5* gene and bone mineral density in Japanese male workers

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Abstract
Objectives Both genetic and lifestyle factors have been shown to influence bone mineral density (BMD). We investigated the correlations between BMD and *low-density lipoprotein receptor-related protein 5* (*LRP5*) A1330V (rs3736228) polymorphism, exercise, smoking, and alcohol intake in Japanese male workers.
Methods The subjects were 829 male employees (aged 20–59 years) of a large-scale integrated manufacturing facility in Japan. BMD was measured at the nondominant radius by dual-energy X-ray absorptiometry. Lifestyle information was obtained by a questionnaire at the same time, and genomic DNA was isolated from peripheral leukocytes.
Results Mean ± standard deviation (SD) BMD was 0.557 ± 0.059 g/cm^2. The genotype frequencies of *LRP5* gene polymorphism were 51, 42, and 7% for *AA*, *AV*, and *VV*, respectively. Analysis of variance and post hoc Tukey test indicated that mean BMD was significantly lower in subjects with *VV* genotype than in those with *AA* genotype (0.540 ± 0.048 versus 0.562 ± 0.062 g/cm^2). According to multiple linear regression analysis, *LRP5* A1330V polymorphism was an independent determinant of BMD, after adjusting for age, body mass index (BMI), and lifestyle variables. Exercise (past or current) also influenced BMD.
Conclusions These findings suggest that *LRP5* A1330V polymorphism and exercise may influence BMD in Japanese male workers.

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Introduction

Osteoporosis is usually considered to be a typical problem of postmenopausal or elderly women, but it is also prevalent in men. In fact, approximately 30% of hip fractures occur in men [1], and the mortality associated with fracture is considerably higher in men than in women [2]. Recently, the American College of Physicians recommended that physicians should assess risk factors for osteoporosis in older men and measure the bone mineral density (BMD) of men at increased risk for osteoporosis [3]. Thus, male osteoporosis has now been recognized as an important public health issue. Because of the long-term emphasis on osteoporosis in women, however, the cellular and molecular basis of male osteoporosis is still poorly understood [4]. Since osteoporosis is clinically silent until fracture occurs [5], men at risk of osteoporosis and those with the disease need to be identified.

Diagnosis of osteoporosis is based on assessment of BMD, which is influenced by both genetic and lifestyle factors (exercise, smoking, alcohol, etc.) [6]. Low BMD is an important risk factor for osteoporosis and has high heritability [7]. In recent years, genomewide association studies have emerged as a powerful tool for identifying genes related to many common human disorders and phenotypes. A recent multicenter collaborative genomewide association study on 314,075 single-nucleotide polymorphisms (SNPs) in 8,557 individuals showed that rs3736228 (A1330V) in the *low-density lipoprotein receptor-related protein 5* (*LRP5*) gene on chromosome 11 was strongly

associated with the BMD of women and men in Europe, since the expression of the risk allele decreased the BMD by 0.13 SD [8]. Other studies [9–16] have also suggested that *LRP5* A1330V polymorphism is associated with BMD, and that individuals with the *AV* or *VV* genotype have lower BMD than those with the *AA* genotype. Better understanding of the relation between BMD and *LRP5* A1330V polymorphism, as well as the influence of lifestyle factors, may allow us to identify a high-risk group for osteoporosis and lead to more effective prevention of this condition and improvement of public health. The purpose of the present cross-sectional study is to investigate the correlations among BMD, *LRP5* A1330V polymorphism, and lifestyle factors (exercise, smoking, and alcohol intake) in Japanese male workers.

Methods

Subjects and methods

The subjects were 829 male employees aged 20–59 years (mean 37.8 ± 6.7 years) who worked at a large-scale integrated manufacturing facility in Japan (Table 1). The criteria for entry into this study were no previous diagnosis of osteoporosis, no systemic diseases, and no medications known to influence bone or calcium metabolism. Height, weight, BMD, urinary deoxypyridinoline (DPD), and serum bone alkaline phosphatase (BAP) were measured during a comprehensive health check. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared. Lifestyle information, such as past history of exercise, current exercise, smoking status, and alcohol intake, was obtained with a self-reporting questionnaire [17]. Each lifestyle factor was divided into 2 categories as follows: past history of exercise (regular exercise until the age of 20 years): no versus yes; current exercise: ≤2 days/week versus ≥3 days/week; smoking status: no versus yes; and alcohol intake: no versus yes. The study protocol was approved by the ethics committee of Kumamoto University, and all subjects provided written informed consent.

Bone metabolism markers

BAP levels were measured using an Alkaphase B kit (enzyme immunoassay; Metra Biosystems). Urinary DPD levels were determined using commercially available pyridinium cross-links with a high-performance liquid chromatography (HPLC) kit (enzyme immunoassay; Metra Biosystems, Mountain View, CA, USA). Urinary concentrations of creatinine were determined using a colorimetric assay performed with a creatinine test kit (Wako, Osaka, Japan). DPD excretion was expressed as a ratio of urinary creatinine concentration. DPD has been validated as a useful marker of bone resorption, while BAP is a marker of bone formation [18].

Table 1 Profile of the subjects (829 men)

Age (years)[a]	37.8 ± 6.7
Height (cm)[a]	169.9 ± 6.0
Weight (kg)[a]	67.8 ± 10.1
BMI (kg/m^2)[a]	23.4 ± 3.1
BMD (g/m^2)[a]	0.557 ± 0.059
Bone metabolism markers	
DPD (nmol/mmol Cr)[b]	3.5 (10.0)
BAP (U/L)[b]	24.3 (80.7)
Lifestyle	
Past history of exercise (%)	
Yes	80.0
No	20.0
Current exercise (%)	
≤2 days/week	89.9
≥3 days/week	10.1
Smoking status (%)	
Yes	58.1
No	41.9
Alcohol intake (%)	
Yes	68.1
No	31.9

Past history of exercise was determined from exercise habits until the age of 20 years

BMI body mass index, *BMD* bone mineral density, *DPD* deoxypyridinoline, *BAP* bone alkaline phosphatase

[a] Values are mean ± SD

[b] Values are median (interquartile range)

Measurement of bone mineral density

BMD was measured at the distal 1/3 site of the radius on the nondominant side by dual-energy X-ray absorptiometry (DXA, Osteometer DTX200) according to the manufacturer's protocol [coefficient of variation (CV) for precision error <1.0% in vivo]. Quality control was carried out in accordance with the manufacturer's instructions.

DNA extraction and genotyping

Genomic DNA was extracted from peripheral blood leukocytes using a DNA Extractor WB Kit (Wako Pure Chemical Industries, Osaka, Japan) according to the manufacturer's instructions.

A real-time quantitative polymerase chain reaction assay was performed using a Step One Sequence Detector (Applied Biosystems). *LRP5* A1330V (rs3736228) polymorphism

was genotyped with a Custom TaqMan Genotyping Assay (Applied Biosystems) according to the manufacturer's protocol.

Statistical analysis

Statistical analysis was performed with SPSS 18.0 software, and the results are presented as mean ± standard deviation (SD) or median (interquartile range) as appropriate. The chi-square test was employed to verify Hardy–Weinberg equilibrium of genotype frequencies. DPD and BAP values did not show a normal distribution in the Kolmogorov–Smirnov test. Pearson's correlation analysis was used to examine the influence of age, height, weight, and BMI on BMD. Spearman's rank correlation analysis was used to examine the influence of DPD and BAP levels on BMD. Analysis of variance (ANOVA) and the post hoc Tukey test were used to assess the differences in the mean values of age, height, weight, BMI, and BMD among subjects with different polymorphisms. Kruskal–Wallis test was used to examine the differences in DPD, BAP, past history of exercise, current exercise, smoking status, and alcohol intake among subjects with different polymorphisms. The stepwise method of multiple linear regression analysis was performed to detect variables with an independent influence on BMD. To examine multicollinearity of the regression model, we calculated the variance inflation factor. (A variance inflation factor exceeding 10 was defined as indicating that collinearity was problematic.) Age, BMI, *LRP5* A1330V polymorphism, past history of exercise, current exercise, smoking status, and alcohol intake were selected among subjects with variance inflation factor <10, i.e., height and weight were excluded. Statistical significance was accepted at $P < 0.05$.

Results

Table 1 presents a profile of the subjects. Mean age was 37.8 ± 6.7 years, and mean BMD was 0.557 ± 0.059 g/cm^2 (Table 1).

Correlations were analyzed to investigate factors with an influence on BMD. As a result, BMD was positively correlated with age ($r = 0.08$, $P < 0.05$), height ($r = 0.10$, $P < 0.001$), weight ($r = 0.38$, $P < 0.001$), and BMI ($r = 0.38$, $P < 0.001$), but was negatively correlated with DPD ($r = -0.08$, $P < 0.05$) and BAP ($r = -0.14$, $P < 0.001$) (Table 2).

The characteristics of the subjects stratified according to *LRP5* A1330V polymorphism are shown in Table 3. *LRP5* A1330V polymorphism showed a distribution that followed Hardy–Weinberg equilibrium ($P = 0.30$). The allele frequency of *LRP5* A1330V polymorphism (51% *AA*, 42% *AV*, and 7% *VV*) was similar to that reported previously in a Japanese population (46% *AA*, 45% *AV*, and 9% *VV*) [15]. ANOVA and post hoc Tukey test showed that mean BMD was significantly lower in subjects with *VV* genotype than in those with *AA* genotype. There were no significant differences in mean values of age, height, weight, and BMI and the median values of DPD and BAP among the three *LRP5* A1330V genotypes. In addition, Kruskal–Wallis test indicated no intergroup differences in past history of exercise, current exercise, smoking status, and alcohol intake (Table 3). Table 4 shows the results obtained by multiple linear regression analysis that assessed various factors for an influence on BMD. As a result, age, BMI, *LRP5* A1330V polymorphism, past history of exercise, current exercise, smoking status, and alcohol intake were selected among subjects with variance inflation factor <10, i.e., height and weight were excluded. The parameters showing an independent association with BMD were BMI (coefficient = 0.007, $P = 0.000$), past history of exercise (coefficient = 0.014, $P = 0.005$), *LRP5* A1330V polymorphism (coefficient = −0.007, $P = 0.018$), and current exercise (coefficient = 0.014, $P = 0.030$). The variance inflation factors in this regression model were all <1.06, and the adjusted R^2 value was 16% (Table 4).

Table 2 Correlation between various parameters and bone mineral density among 829 men

	r	*P*
Age (years)[a]	0.08	<0.05
Height (cm)[a]	0.10	<0.001
Weight (kg)[a]	0.38	<0.001
BMI (kg/m^2)[a]	0.38	<0.001
DPD (nmol/mmol Cr)[b]	−0.08	<0.05
BAP (U/L)[b]	−0.14	<0.001

BMI body mass index, *DPD* deoxypyridinoline, *BAP* bone alkaline phosphatase

[a] Pearson correlation

[b] Spearman's rank correlation

Discussion

In this study, we found that Japanese male workers with *VV* genotype had significantly lower BMD than those with *AA* genotype. In addition, we found that *LRP5* A1330V polymorphism was an independent determinant of BMD of Japanese male workers after adjusting for age, BMI, and other lifestyle variables by multiple linear regression analysis. The significant association between *LRP5* A1330V polymorphism and BMD detected in this study agrees with the results of previous studies performed in Japanese women [15], Caucasian men [12–14], and Caucasian

Table 3 Characteristics of subjects stratified according to *low-density lipoprotein receptor related protein 5* (*LRP5*) A1330V polymorphism

Valuable	*AA* ($n = 424$)	*AV* ($n = 346$)	*VV* ($n = 59$)	*P*
Age (years)[a]	38.0 ± 6.9	37.6 ± 6.4	37.7 ± 7.3	0.76
Height (cm)[a]	169.8 ± 6.3	170.1 ± 5.7	169.8 ± 5.9	0.71
Weight (kg)[a]	68.0 ± 10.5	67.9 ± 9.8	65.0 ± 9.4	0.09
BMI (kg/m^2)[a]	23.6 ± 3.2	23.5 ± 3.1	22.6 ± 3.1	0.07
BMD (g/m^2)[a]	0.562 ± 0.062	0.555 ± 0.057	0.540 ± 0.048*	0.02
Bone metabolism markers				
DPD (nmol/mmol Cr)[b]	3.6 (7.2)	3.4 (10.0)	3.4 (5.1)	0.18
BAP (U/L)[b]	24.3 (80.7)	24.4 (77.6)	24.9 (35.3)	0.64
Lifestyle				
Past history of exercise (%)				
Yes	80.6	79.5	79.7	0.93
No	19.4	20.5	20.3	
Current exercise (%)				
≤2 days/week	89.8	89.8	91.4	0.93
≥3 days/week	10.2	10.2	8.6	
Smoking status (%)				
Yes	57.9	56.7	67.8	0.28
No	42.1	43.3	32.2	
Alcohol intake (%)				
Yes	67.1	69.8	64.9	0.64
No	32.9	30.2	35.1	

Past history of exercise was determined from exercise habits until the age of 20 years

Data were analyzed by analysis of variance and post hoc Tukey test or Kruskal–Wallis test

Age, height, weight, BMI, and BMD were analyzed by analysis of variance and post hoc Tukey test

DPD, BAP, past history of exercise, current exercise, smoking status, and alcohol intake were analyzed by Kruskal–Wallis test

BMI body mass index, *BMD* bone mineral density, *DPD* deoxypyridinoline, *BAP* bone alkaline phosphatase

* $P < 0.05$ compared with *AA* genotype (post hoc Tukey test)

[a] Values are mean ± SD

[b] Values are median (interquartile range)

women [16], which have indicated that allelic variation of *LRP5* A1330V polymorphism contributes to regulation of BMD. However, none of the previous studies have evaluated Japanese male population. To our knowledge, this is the first report of an association between *LRP5* A1330V polymorphism and BMD in Japanese men. *LRP5* A1330V polymorphism is probably a useful genetic marker for predicting BMD in Japanese men.

LRP5 is a coreceptor in the canonical Wnt signaling pathway [19, 20], which is involved in differentiation of osteoblast precursors into mature osteoblasts, which in turn increases bone formation [21]. This Wnt signaling pathway also induces upregulation of osteoprotegerin (OPG) expression and downregulation of receptor activator of nuclear factor κB ligand (RANKL) expression in osteoblasts, resulting in inhibition of bone resorption [21]. A recent study demonstrated that the Wnt signaling pathway regulates BMD through LRP5 [20]. In addition, a previous study has provided extensive genetic and functional data indicating that the *LRP5* gene and the Wnt signaling pathway have a substantial influence on bone formation and the risk of osteoporosis and that LRP5 signaling is essential for normal morphology, normal development, and bone health [22]. In vitro, a previous study investigated functional differences between LRP5 A1330 and V1330 in HEK293T cells with or without co-expression vectors for Wnt3a [23]. The results showed that Wnt3a-induced T-cell factor/lymphoid enhancer factor (Tcf-Lef) activity was significantly reduced in cells containing LRP5–V1330 compared with those containing the wild-type allele [LRP5–A1330 versus LRP5–V1330, 1.900 ± 0.064 versus 1.597 ± 0.101; mean ± standard error (SE)]. In the case of cells not treated with Wnt3a, Tcf-Lef activity of cells with LRP5–V1330 was not significantly changed compared with that of those with LRP5–A1330 (LRP5–A1330 versus LRP5–V1330, 1 ± 0.056 versus 0.975 ± 0.015; mean ± SE). In addition, the association between the *LRP5* A1330V polymorphism and total-body BMD was

Table 4 Multiple linear regression analysis on variables associated with bone mineral density among 829 men

	Coefficient	Standard error	*t*	*P*
Age (years)	0.000	0.000	1.0	0.337
BMI (kg/m^2)	0.007	0.001	10.9	0.000
LRP5 A1330V polymorphism[a]	−0.007	0.003	−2.4	0.018
Past history of exercise[b]	0.014	0.005	2.8	0.005
Current exercise[c]	0.014	0.006	2.2	0.030
Smoking status[d]	0.006	0.004	1.5	0.143
Alcohol intake[e]	0.008	0.004	1.9	0.056

Multiple linear regression analysis, adjusted $R^2 = 0.16$

Past history of exercise was determined from exercise habits until the age of 20 years

BMI body mass index, *LRP5 low-density lipoprotein receptor related protein 5*

[a] *LRP5* A1330V polymorphism was rated as 0 (*AA* genotype), 1 (*AV*genotype) or 2 (*VV* genotype)

[b] Past history of exercise was rated as 0 (no), or 1 (yes)

[c] Current exercise was rated as 0 (≤2 days/week) or 1 (≥3 days/week)

[d] Smoking status was rated as 0 (no) or 1 (yes)

[e] Alcohol intake was rated as 0 (no) or 1 (yes)

replicated in 739 postmenopausal women (*AA* versus *VV*; $P = 0.0026$). Although the exact mechanism by which *LRP5* A1330V polymorphism influences BMD is not fully understood, this polymorphism might have an effect on Wnt signaling [23]. Our result that *LRP5* A1330V polymorphism is associated with BMD implies the need for further studies on the functions of LRP5 and the polymorphisms associated with bone metabolism.

In this study, exercise (past or current) was also a significant independent determinant of the BMD. Past history of exercise was assessed from exercise habits until the age of 20 years in this study. We categorized current exercise on the basis of frequency (≤2 days/week versus ≥3 days/week) because the American College of Sports Medicine has developed guidelines for exercise to improve bone health and their recommendation is exercise from 3 to 5 times per week [24]. There is some evidence that exercise has a beneficial effect on BMD [24, 25], and the most beneficial effect of exercise on BMD might be obtained during growth [24, 26]. We previously reported on the relation between lifestyle and BMD in some of the present subjects [19], and our results suggested that exercise influenced the BMD of male workers. After taking genetic factors into account, we found that exercise (past or current) had an independent influence on BMD, suggesting that exercise may be useful to protect against osteoporosis even in persons who have genetic susceptibility to a low BMD. A large-scale prospective study will be needed to define the relation between BMD and physical activity, as well as the influence of *LRP5* A1330V polymorphism.

Alcohol intake was not an independent determinant of BMD after adjustment for age, BMI, *LRP5* A1330V polymorphism, past history of exercise, current exercise, and smoking. The effect of alcohol on BMD remains unclear [6]. A dose-dependent effect of alcohol on bone metabolism has also been reported [27]. A recent meta-analysis revealed that persons consuming more than half to one drink per day had a lower risk of hip fracture than abstainers, and persons consuming more than two drinks per day had a higher risk [28]. However, the precise mechanism by which moderate intake of alcohol alters bone metabolism is still unknown. We did not assess the alcohol consumption of drinkers in the present study. The amount and frequency of alcohol consumption may be important factors determining the influence of alcohol on BMD.

Smoking was also not an independent determinant of BMD after adjustment for age, BMI, *LRP5* A1330V polymorphism, past history of exercise, current exercise, and alcohol intake. Some previous studies have shown negative correlation between history of smoking and BMD in elderly men [29, 30]. However, we could not find a relationship between smoking and bone loss in our study. This discrepancy could have occurred because the duration of smoking in our younger subjects was shorter than that of the subjects in other studies. Clearly, further prospective studies will be required to define the relationship between bone loss and smoking.

In this study, BMD was negatively correlated with DPD and BAP, which are bone metabolism markers. Rogers et al. investigated the value of measurements of biochemical markers for the prediction of bone loss rates in 60 postmenopausal Caucasian women aged 49–62 years. Rogers found a significant negative correlation (Spearman rank correlation) between the measured biochemical markers and the rate of change in BMD (BAP: $r = -0.43$, $P = 0.0009$; DPD: $r = -0.35$, $P = 0.0076$) [31]. In addition, a previous study in Japanese women aged 45 years or older with elevated levels of serum osteocalcin (OC), bone-specific alkaline phosphatase (bone ALP), type I collagen crosslinked C-terminal telopeptide (CTX), or free and total (tDPD) forms of immunoreactive deoxypyridinoline showed that bone loss during the follow-up period in these women was significantly greater than that in women with lower levels of these markers [32]. However, these previous studies targeted women. Only one previous study [33] has examined the association between bone metabolism markers and BMD in Japanese men. Yoshikawa investigated age-related changes of BMD in 443 Japanese adult males (mean baseline age 54.3 ± 10.4 years; mean

follow-up 4.8 years) in association with bone metabolic markers [33]. They found that the annual percentage change of BMD was $0.405 \pm 1.56\%$ at the lumbar spine and $-0.249 \pm 1.12\%$ at the femoral neck, and that these changes were correlated with DPD ($r = -0.323$ and -0.439, respectively) [32]. Bone strength reflects the influence of two main factors: BMD and bone quality [34]. Bone quality depends on bone architecture, mineralization, turnover, and accumulation of microfractures, while biochemical markers of bone turnover are considered to reflect the extent of bone formation or bone resorption [35]. When high levels of DPD and BAP are detected, metastatic bone tumor, metabolic bone disease, or abnormal calcium metabolism can be suspected, but these parameters were not significantly associated with *LRP5* A1330V polymorphism in the present study. Because the examined bone metabolism markers are useful indicators for changes in BMD in relatively short period, the absence of correlation between these markers and *LRP5* polymorphism would indicate that the correlation between BMD and this polymorphism may be established at younger age. Further studies about the association between bone metabolism markers and BMD, as well as the influence of *LRP5* A1330V polymorphism, are required.

In conclusion, *LRP5* A1330V polymorphism and exercise may influence BMD in Japanese male workers. These findings suggest that investigation of *LRP5* A1330V polymorphism may be useful for identifying individuals who are susceptible to osteoporosis, while exercise may be useful for protecting Japanese male workers from osteoporosis even if they have genetic susceptibility to low BMD.

Conflicts of interest None.

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