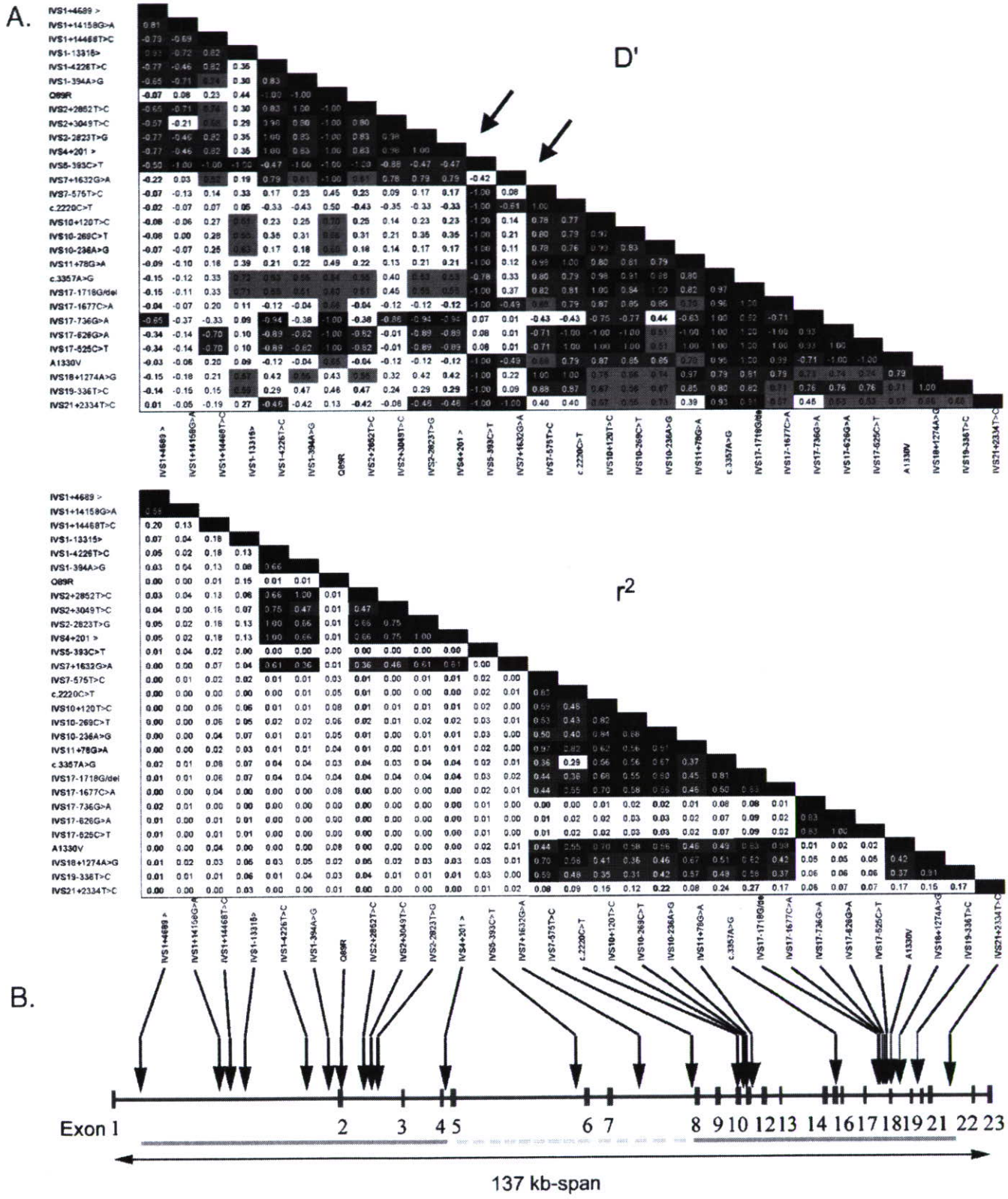

Fig. 1. Linkage disequilibrium analysis among 29 SNPs within the *LRP5* locus. (A) Indices of linkage disequilibrium D' (upper half) and r^2 (lower half), calculated from the predicted haplotype frequencies and presented as separate tables. D' values greater than 0.5 and r^2 values greater than 0.1 are highlighted by gray-to-black gradients. Arrows indicate a possible hot spot for recombination. (B) Schematic diagram of the *LRP5* gene showing locations of the 23 exons. Locations of the 29 tested SNPs are indicated by downward arrows.

Table 4
Contingency table analysis on Group-A subjects

Group-A	*n*	A1330V			*P* value[a]
		AA	AV	VV	
Z-score > 3.0	8	5	2	1	NA
Z-score < −3.0	4	1	3	0	
Z-score > 2.0	33	22	8	3	0.025 *
Z-score < −2.0	34	13	14	7	
Z-score > 1.0	91	52	33	6	0.046 *
Z-score < −1.0	139	61	63	15	

NA: not applicable.
[a] *P* values are calculated in chi-square test for trend.
* $P < 0.05$.

Haplotyping, LD analysis, and statistical analysis

Maximum likelihood haplotype frequencies among the 582 chromosomes of 291 subjects from Group-B were estimated by an EM algorithm using SNPAlyze v3.1 (DYNA-COM, Chiba, Japan). The LD for all possible two-way combinations of SNPs was tested with *D*, *D′*, and r^2 [26,27]. After defining LD blocks and tag-SNPs in each block, each individual diplotype was estimated by SNPAlyze v3.1 software.

Quantitative associations between genotypes and adjusted BMD values (g/cm^2) were examined by analysis of variance (ANOVA), with regression analysis as a post hoc test using Instat 3 software (GraphPad Software, San Diego, CA). The three genotypic categories of each SNP were converted into incremental values (0, 1, and 2), which represent the number of chromosomes carrying the major allele. Significant association was defined when the given *P* value of the ANOVA *F*-test was less than 5% ($P < 0.05$). Similarly, quantitative association was tested for major 5- and 4-haplotypes (frequency >5%) defined for each LD block in the *LRP5* gene locus. Chi-square tests were used to ascertain Hardy–Weinberg equilibrium among genotypes ($P > 0.05$). Multiple linear regression analysis was applied for examining potential combined effects of Q89R and A1330V alleles, using Instat 3 software. Distribution analysis of the genotype frequencies among phenotypically divided groups, i.e., BMD *Z*-scores > 1.0 ($n = 91$) and *Z*-scores < −1.0 ($n = 139$) was conducted by chi-square tests as in our previous experiments [21]. Predictive analysis of protein function for the two missense coding SNPs was conducted using the SIFT (Sorting Intolerant From Tolerant) program (http://blocks.fhcrc.org/sift/SIFT.html) [28]. When the given score was less than 0.05, the alteration was deemed to be intolerant (i.e., deleterious).

Results

To identify the genetic ground involved in a previously detected association of an intronic *LRP5* polymorphism with low BMD in Group-A subjects [15], we first searched for a causative mutation anywhere among the 23 exons of the gene. However, among 12 subjects who had extremely high or low BMD *Z*-scores (>3.0 or <−3.0), no mutations were detected apart from two already known missense variations (Q89R and A1330V). We analyzed the effect of these amino acid alterations, using predictive computer program SIFT [28]; this program estimated a deleterious effect of 1330-V (score = 0.03) and possible mild effect of 89-R (score = 0.19). Although the lowest score was predicted for another missense variation V667M (score = 0.01), this variant was not detected in our test population. Based on the notion that strong candidate for the causative polymorphism would be missense variation, we investigated association of these SNPs among the entire Group-A subjects.

To analyze the effects on BMD, all the extended 387 subjects were genotyped for these two SNPs as well as previously tested IVS17-1677C > A (Table 2). No association of the Q89R genotypes was detected for BMD *Z*-score ($r = 0.03$, NS). However, we detected significant correlation of the A1330V genotype with spinal BMD *Z*-score ($r = 0.11$, $P = 0.034$); homozygous carriers of the minor T-allele (V/V) had the lowest BMD *Z*-scores (−0.47 ± 1.47, $n = 35$), heterozygous individuals (A/V) were intermediate (−0.35 ± 1.38; $n = 174$), and homozygous carriers of the C-allele (A/A) had the highest BMD *Z*-scores (−0.03 ± 1.62; $n = 178$) (Tables 2 and 3). The result was consistent with previously detected results for the intron SNP IVS17-1677C > A [15], as indicated (Table 2). We noticed that these SNPs were in strong linkage disequilibrium (see Fig. 1). Consistent results were achieved in a distribution analysis of phenotypically divided subjects (high BMD *Z*-scores: $n = 91$ and low BMD *Z*-scores: $n = 139$), using 2 × 3 chi-square tests for detecting trends ($P = 0.046$) (Table 4). A similar result was obtained by analyzing subjects with more prominent phenotypes (*Z*-scores > +2.0: $n = 33$, and *Z*-scores < −2.0: $n = 34$; $P = 0.025$) (Table 4).

No significant differences were detected in physical characteristics (body weight, height, or BMI) between groups genotypically classified according to A1330V alleles. Although we detected a significant correlation of A1330V with levels of intact osteocalcin in serum ($P = 0.004$), this might be affected by a correlation detected for age distribution ($P = 0.01$) (Table 3).

To examine the reproducibility of that correlation, 384 adult Japanese women recruited from general Japanese population were analyzed (Group-B) on adjusted values of radial BMD by ANOVA with linear regression. As a result, significant correlation of the A1330V genotype with adjusted BMD was replicated (Tables 2 and 5). Homozygous carriers of the minor

Table 5
Physical and clinical characteristics of the subjects in Group-B (cohort subjects)

Group-B subjects ($n = 384$)	A1330V			*P* value *
	AA	AV	VV	
Number	195	155	34	–
Age (Years)	58.4 ± 8.72 (34–69)	58.3 ± 8.14 (32–69)	58.9 ± 9.93 (32–69)	NS
Weight (kg)	53.2 ± 9.01 (34.8 ± 84.8)	55.1 ± 8.73 (28.8–89.0)	55.9 ± 8.7 (44.6–80.0)	0.027
BMI (kg/m^2)	23.3 ± 3.63 (16.2–37.7)	23.9 ± 3.61 (14.7–38.5)	24.6 ± 3.29 (17.5–31.3)	0.018
Height (cm)	151.2 ± 6.04 (130.5–169.0)	151.7 ± 5.41 (139.0–166.0)	150.6 ± 6.65 (140.0–163.4)	NS
Radial BMD (g/cm^2)	0.402 ± 0.082 (0.222–0.635)	0.398 ± 0.084 (0.178–0.613)	0.390 ± 0.079 (0.270–0.566)	NS
Adjusted BMD (g/cm^2)	0.405 ± 0.053 (0.275–0.551)	0.395 ± 0.059 (0.225–0.554)	0.385 ± 0.041 (0.298–0.448)	0.019

* *P* values are calculated for regression analysis with ANOVA *F*-test.

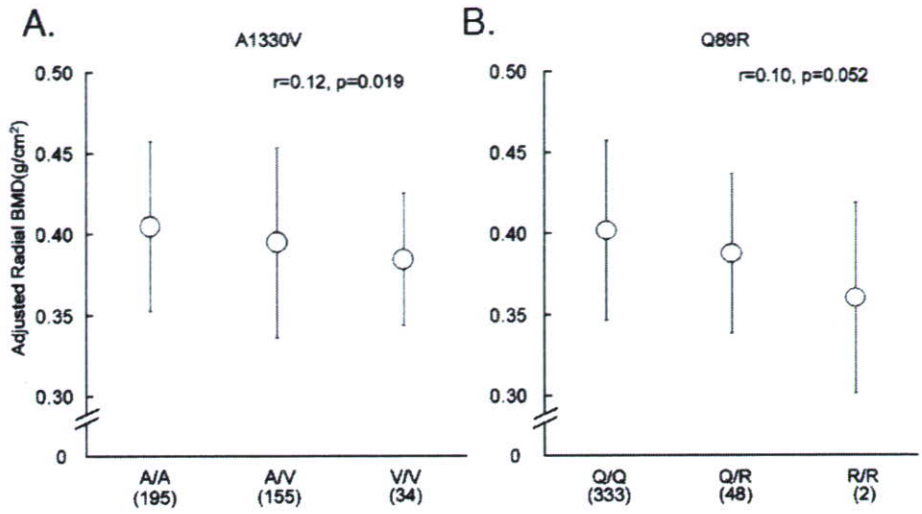

Fig. 2. Reproducible association of two missense *LRP5* variations (Q89R and A1330V) with adjusted BMD. (A) Plots of adjusted BMD values for three genotypically classified subgroups by A1330V among 384 subjects from the general population. (B) Plots of adjusted BMD values for three genotypically classified subgroups by Q89R. Open circles indicate mean values and error bars indicate standard deviations. Correlations between the number of minor allele possessed and the adjusted BMD were tested by linear regression analysis.

T-allele (V/V) had the lowest adjusted BMDs (0.385 ± 0.041 g/cm^2, $n = 34$); heterozygous individuals (A/V) were intermediate (0.395 ± 0.059 g/cm^2; $n = 155$); and homozygous C-allele carriers (A/A) had the highest adjusted BMDs (0.405 ± 0.053 g/cm^2; $n = 195$), indicating a possible codominant effect of the minor T-allele (corresponding to the second nucleotide for a valine codon, gTg) on lowering adjusted BMD levels (Fig. 2A; $r = 0.12$, $P = 0.019$; $n = 384$). Interestingly in these subjects, we observed a tendency for association of Q89R minor G-allele (corresponds to arginine; cGg) to low adjusted BMD, although this tendency did not quite reach statistical significance (Fig. 2B; $r = 0.10$, $P = 0.052$). We also examined if any patient characteristics correlated with the genotype; however, no significant differences were detected except in body weight ($r = 0.11$, $P = 0.03$) or BMI ($r = 0.12$, $P = 0.02$). A contribution of A1330V to control of body mass appeared to be a possibility (Table 5). For analysis of possible combined effects of Q89R and A1330V on BMD determination, multivariate linear regression analysis estimated a fitting equation explained about 2% of BMD variances ($r^2 = 0.023$, $P = 0.01$). However only the A1330V genotype contributed significantly to the equation ($P = 0.004$).

We then analyzed LD in the LRP5 locus by genotyping these subjects for informative 29 SNPs (minor allele frequencies >0.05) (Table 1). Average distance between neighboring SNPs was 4.6 kb (median, 3.0 kb; range, 33–21,779 bp). Genotype, allelic frequency, and heterozygosity were clarified successfully for each SNP, and no deviation was detected from Hardy–Weinberg equilibrium. Indices of pairwise LD (D' and r^2) were calculated by estimating the maximum-likelihood haplotypes and their frequencies from 293 Group-G subjects genotyped for all 29 SNPs. These procedures detected two LD blocks separated by a region of about 11 kb, from intron 5 to intron 7 (Fig. 1). In the second block, the indices of pairwise LD between the A1330V and IVS17-1677C > A variants were remarkably high ($D' = 0.99$, $r^2 = 0.98$). More important, the two missense SNPs, Q89R (c.266A > G) and A1330V (c.3989C > T), appeared to localize in different LD blocks, although the indices of pairwise LD between the two SNPs were slightly higher than the neighboring intronic SNPs ($D' = 0.64$, $r^2 = 0.07$). By analyzing haplotype frequency estimated by all the belonging SNPs for each LD block (11 and 18, respectively), representative 4 and 5 SNPs were selected (Tables 6 and 7). By estimating diplotype in each individual,

Table 6
Representative haplotypes for LD block-1

Hap-No	IVS1 + 4689C > G	IVS1 + 14158G > A	IVS1 + 14468T > C	IVS2 + 2852T > C	Frequency (%)	SUM (%)
#1–1	0	0	0	0	37.6	
#1–2	1	1	0	0	26.4	
#1–3	1	1	1	0	15.0	
#1–4	1	1	1	1	6.5	
#1–5	0	1	0	0	6.3	
#1–6	1	0	1	0	2.1	
#1–7	1	0	0	0	2.1	
#1–8	0	0	1	0	1.0	
#1–9	0	0	0	1	0.9	98.0

0 and 1 represent major and minor alleles, respectively.

Table 7
Representative haplotypes for LD block-2

Hap-No	IVS7-575T > C	c.3357A > G	IVS 17-626G > A	A1330V	IVS21 + 2334T > C	Frequency (%)	SUM (%)
#2–1	0	0	0	0	0	53.0	
#2–2	1	1	0	1	0	13.9	
#2–3	1	1	0	1	1	9.2	
#2–4	0	1	0	0	0	3.8	
#2–5	1	1	0	0	0	3.5	
#2–6	0	1	0	1	0	3.4	
#2–7	1	0	0	0	0	3.2	
#2–8	0	1	1	0	1	2.9	
#2–9	0	1	0	1	1	1.8	
#2–10	0	1	1	0	0	1.6	
#2–11	0	1	0	0	1	1.4	97.6

0 and 1 represent major and minor alleles, respectively.

association was examined for 9 haplotypes (5 and 4 haplotypes for each block). Significant association was represented by haplotype-#2–3 (C-G-G-V-C) for the second LD block ($r = 0.15$, $P = 0.0041$); homozygous and heterozygous haplotype#3 carriers had lower adjusted BMD (0.384 ± 0.049, $n = 6$ and 0.379 ± 0.057, $n = 57$) compared to non-carriers (0.402 ± 0.053, $n = 299$).

Discussion

In the study reported here, we investigated a possible contribution of *LRP5* polymorphisms to determination of bone-mineral density in Japanese women. A search for mutations, an LD analysis, and a study for association between two candidate SNPs and adjusted BMD values in two independently collected groups revealed reproducible association of the *LRP5* variations with BMD levels. During the preparation and submitting this manuscript, five independent groups reported similar results. One reported consistent correlation of A1330V with radial BMD in adult Japanese women [16], and the other reported a similar tendency of Q89R to be associated with hip BMD in young Korean males [17]. Although another study, involving Caucasians, reported a c.2047G > A variation (V667M: nucleotide numbering is from the cap site of mRNA in that report [18]), that variant appeared to be so rare in our test subjects that we excluded it from our investigations. Recent two articles further supported consistent association of A1330V, although both reports emphasized association of other intron SNPs in strong LD with A1330V [29,30]. We propose that *LRP5* variations may be important determinants of BMD in the general population, and believe that the A1330V polymorphism is the most likely LRP5 determinant of bone mass as well, at least in adult Japanese women.

Although the molecular basis for the function of LRP5 in determining BMD is not fully clarified, evidence is accumulating. Several mutations in the *LRP5* gene have been identified as causing inherited diseases with bone phenotypes, including OPPG and autosomal-dominant types of osteopetrosis [8–11]. Functional molecular analyses, including gene targeting experiments and analysis of mutated LRP5 products, have revealed a mechanistic basis for bi-directional phenotypic expression, where a key is the Wnt pathway and binding of its antagonists to the YWTD motif in the extracellular domains of this co-receptor [4,10,31]. So far, however, the cellular and in vivo mechanisms causing reduction of bone mass are not well understood. Therefore, functional differences focusing on the alanine residue at 1330 and the LDLR class-A domain should be investigated, and binding affinity for Wnt ligands or other co-binding regulators like dickkopfs (Dkks) [6] should be examined. In our experiments, a predictive informatics program (SIFT [28]) for functional alterations caused by the two missense variations in *LRP5* estimated an intolerable alteration of alanine to valine at A1330V (score = 0.03) but not for glutamine to arginine in Q89R (score = 0.19). Those estimates were consistent with our assumption from association analysis that the effect of Q89R might be weaker than that of A1330V. However, a cross-sectional study conducted elsewhere had indicated a selective influence of a V667M variant on vertebral bone mass among Caucasians where the SIFT program predicted an intolerant effect of 667M (score = 0.01). Longitudinal human studies, or an in vivo animal study, may clarify the developmental period(s) during which *LRP5* variants affect BMD most strongly.

The feasibility of a candidate-gene association study generally depends on control of confounding factors resulting from population bias, unreliability of diagnosis or phenotypic evaluation, interaction with environmental factors, or deficiencies in statistical power [32–34]. In our study, a reliable measurement of BMD, a quantitative trait with significant heredity, was evaluated with adjustment for age and BMI among unbiased population with no evidence of the stratification. Moreover, the reproducibly demonstrated association of the 1330 V variant with low BMD among independent groups of subjects, in other studies and ours [16,17], seems to provide comprehensive evidence for a functional contribution of this variant, even though in all of the studies cited, the significance levels were moderate without adjustment for multiple testing [16–18]. Supportive data from linkage analysis includes a quantitative trait locus (QTL) for spinal BMD at 11q12–13 that has been identified in general populations [35]. A possible combined effect of the multiple coding SNP(s) tested in our

multiple regression analysis should be re-evaluated in a larger cohort study.

The existence of overlapping mechanisms for lipid metabolism, body mass regulation, and bone metabolism is a classically discussed issue [36–38]. These processes are under systemic control of leptin and neuro-endocrinological systems [37–39], but are influenced by apolipoprotein E polymorphisms [40], subject to developmental controls of mesenchymal-cell differentiation by transcription factors like PPAR-gamma [41], and affected by statins [42]. In addition, the Wnt-LRP system may affect regulation of both bone and body mass [43]. Our data support that notion because body weight and BMI, as well as adjusted BMD, correlated with A1330V genotypes among 384 adult women from the general Japanese population. Other effector molecules in the Wnt signaling system might contribute as well.

In summary, our data suggest a functional effect of polymorphic variants in a candidate osteoporosis-susceptibility gene, *LRP5*, whose common polymorphisms significantly correlated with bone mineral density of women recruited from two independent populations. Because osteoporosis is a multifactorial disease, other genes, especially genes acting through the Wnt pathway, may have to be examined for potential contributions to disease susceptibility. That information will help to clarify the complex mechanism of BMD determination in vivo, and may explain, at least in part, the pathogenesis of postmenopausal osteoporosis. Such studies should provide a novel viewpoint for establishing suitable treatment designs and preventive strategies for the disease.

Acknowledgments

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ORIGINAL ARTICLE

Association of a single nucleotide polymorphism in *Wnt10b* gene with bone mineral density

Takahiko Usui,[1] Tomohiko Urano,[1,2] Masataka Shiraki,[3] Yasuyoshi Ouchi[1] and Satoshi Inoue[1,2,4]

[1]*Department of Geriatric Medicine and* [2]*Department of Coca-Cola Anti-Aging Medicine, Graduate School of Medicine, University of Tokyo, Tokyo,* [3]*Research Institute and Practice for Involutional Diseases, Nagano, and* [4]*Research Center for Genomic Medicine, Saitama Medical School, Saitama, Japan*

Background: Wnt signaling pathway regulates bone mineral density (BMD) through the lipoprotein receptor-related protein (LRP)5, a receptor of this signaling. Recently, we and several groups have shown that genetic variations at the *LRP5* gene locus are associated with osteoporosis. These data suggest that genetic variations in Wnt signaling genes may affect the pathogenesis of osteoporosis. To explore whether the Wnt signaling molecules are involved in the maturation of osteoblasts, we analyzed the expression levels of Wnt signaling genes, including LRP5, LRP6 and Wnt10b, in rat primary osteoblasts. Then, we studied an association of a single nucleotide polymorphism (SNP) in *Wnt10b* gene with BMD.

Methods: Expression levels of LRP5, LRP6 and Wnt10b mRNA were analyzed during the culture course of rat primary osteoblasts by real-time reverse transcription polymerase chain reaction (RT-PCR). Association of the *Wnt10b* gene polymorphism at 1059C/T (His353His), that is the only coding SNP found in J-SNP database with BMD, was examined in 221 postmenopausal Japanese women.

Results: LRP5, LRP6 and Wnt10b mRNA were detected during the differentiation of rat primary osteoblasts. As an association study of the SNP in the *Wnt10b* gene, the subjects without the T allele (CC; $n = 59$) had significantly higher total body and lumbar BMD than the subjects bearing at least one T allele (TT + TC; $n = 162$) (total body, $P = 0.0091$; lumber spine, $P = 0.0052$).

Conclusion: Wnt10b mRNA was expressed and regulated in rat primary osteoblasts. A genetic variation at the *Wnt10b* gene locus is associated with BMD, suggesting an involvement of the *Wnt10b* gene in the bone metabolism. SNP of Wnt signaling genes would serve to facilitate early diagnosis, treatment and prevention of osteoporosis.

Keywords: bone mineral density (BMD), LRP5, LRP6, osteoporosis, single nucleotide polymorphism (SNP), Wnt10b.

Introduction

Osteoporosis is a skeletal disorder characterized by low bone mineral density (BMD) and micro-architectural deterioration of bone tissue leading to an increased risk of fracture.[1] BMD is a complex trait that is influenced by both genetic and environmental factors. Heritability studies in twins and family studies have shown that genetic factors account for 50–90% of the variance in BMD.[2–6] In studies on osteoporosis-related genes, significant associations of the vitamin D receptor (*VDR*) gene,[7] estrogen receptor α (*ERα*) gene,[8] collagen type Iα1 (*COLIA1*) gene[9] and low density lipoprotein receptor-related protein 5 (*LRP5*) gene[10] polymorphisms with

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Correspondence: Dr Satoshi Inoue MD PhD, Department of Coca-Cola Anti-Aging Medicine, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, 113-8655, Japan. Email: inoue-ger@h.u-tokyo.ac.jp

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BMD in postmenopausal women have already been described. Identification of candidate genes, of which polymorphisms affect bone mass, will be useful for early detection of individuals who are at risk for osteoporosis and early institution of preventive measures.

The Wnt proteins represent a large group of secreted signaling proteins that are involved in cell proliferation, differentiation and morphogenesis.[11] Wnt proteins activate signal transduction through Frizzled which acts as receptors for Wnt proteins[12] and induce stabilization of cytoplasmic β-catenin protein. Meanwhile, low-density lipoprotein (LDL) receptor-related protein 5 and 6 (LRP5/6) were also found to be required for Wnt co-receptors.[13,14] Recent reports demonstrated that the Wnt signaling pathway regulates bone density through the LRP5.[15–18] We and several groups have shown that their is a significant association between BMD and polymorphisms in the *LRP5* gene.[10,19–21] We also have shown that a genetic variation of the *sFRP4* gene,[22] which is an inhibitor of Wnt signaling, affects the BMD among postmenopausal Japanese women. These data suggest that the single nucleotide polymorphism (SNP) in other Wnt signaling genes may affect the BMD.

Although these and other studies suggest that endogenous Wnt signaling regulates osteoblastogenesis and bone formation, Wnt molecules that are responsible for activation of this pathway in bone cells have to be determined. Recently, Wnt10b has demonstrated to regulate bone formation *in vivo*.[23] In this report, FABP4-Wnt10b mice, which overexpress Wnt10b in bone marrow, have shown increased bone.[23] It has also shown that $Wnt10b^{-/-}$ mice have decreased trabecular bone and serum osteocalcin.[23] These data suggest that Wnt10b may be a promising Wnt molecule as a determinant of BMD through the regulation of osteogenesis.

In the present study, we examined the expression of the Wnt10b in rat primary osteoblasts and the association of a polymorphism in the *Wnt10b* gene with BMD in Japanese women to investigate possible contribution of the Wnt10b in bone metabolism.

Materials and methods

Cell culture

Rat primary osteoblasts were isolated from calvaria of 5-day-old neonatal rats by enzymatic digestion as described previously[24] with some modification. Briefly, calvaria were minced and incubated at 37°C for 20 min in magnesium-free phosphate-buffered saline containing 0.1% collagenase and 0.2% dispase. The enzymatic digestion was repeated twice. The second digestion was performed for 70 min. Cells isolated at second digestion were cultured in α-minimum essential medium (MEM) containing 10% fetal bovine serum (FBS) and antibiotics (100 IU/mL penicillin and 100 mg/mL streptomycin). Cells at the second passage were used for experiments.

Total RNA isolation and cDNA synthesis

Osteoblasts were cultured in 6-cm dishes with α-MEM containing 10% FBS, 50 μg/mL ascorbic acid and 5 mmol/L β-glycerophosphate for 3, 5, 8, 11, 13, 15 or 18 days. Total RNA were extracted from these cells using a ToTALLY RNA Kit (Ambion, Austin, TX, USA). cDNA was synthesized from 1 μg of total RNA of primary osteoblasts using a first strand cDNA synthesis kit (Amersham, Chicago, IL, USA).

SYBR green real time PCR

Primers were designed using PRIMER EXPRESS 1.0 software (Applied Biosystems, Foster City, CA, USA). Definitive primers were: rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward 5′-GGC ACAGTCAAGGCTGAGAAT-3′, reverse 5′-TCGCGC TCCTGGAAGATG-3′, rat alkaline phosphatase (ALP) forward 5′-TGACCACCACTCGGGTGAA-3′, reverse 5′-GCATCTCATTGTCCGAGTACCA-3′, rat LRP5 forward 5′-TGGATGGGCGTCAGAACA-3′, reverse5′-TGGGAGAGGTCAGCATGGA-3′, rat LRP6 forward 5′-AGCGTCCTCAAGCAGCTCTTC-3′, reverse5′-CGATGGTGGTGGGTTCAAA-3′ and rat Wnt10b forward 5′-TCTCTCGGGATTTCTTGGATTC-3′, reverse 5′-TGTTGTGGATCCGCATTCTC-3′. Quantitative polymerase chain reaction (PCR) was carried out using a 2× master mix composed from the SYBR Green PCR Core Reagents (Applied Biosystems) and 50 nmol/L primers. PCR reactions were performed using an ABI Prism 7000 system (Applied Biosystems) with the following sequence: 2 min at 50°C, 10 min at 95°C and 40 cycle of 15 s at 95°C and 1 min at 60°C. ALP, LRP5, LRP6 or Wnt10b signals were normalized to GAPDH signals.

Subjects

Genotypes were analyzed in DNA samples obtained from 221 healthy postmenopausal Japanese women. We chose postmenopausal women who were older than 50 years from volunteers (mean age ± SD; 61.8 ± 6.6). All women were non-related volunteers who lived in the Chubu district of Japan and provided informed consent before this study. Exclusion criteria included endocrine disorders and a metabolic bone disease other than primary osteoporosis such as hyperthyroidism, hyperparathyroidism, diabetes mellitus, liver disease, renal disease or unusual gynecological history. Women taking medicine related to bone metabolism such as active vitamin D, vitamin K, a vitamin K antagonist, estrogen, bisphosphonate, corticosteroids, anticonvulsants and

heparin sodium were also excluded. Ethical approval for the study was obtained from ethics committees of University of Tokyo and Research Institute and Practice for Involutional Diseases.

Measurement of BMD and biochemical markers

The lumbar-spine BMD and total body BMD (g/cm^2) of each participant were measured by dual-energy X-ray absorptiometry using fast-scan mode (DPX-L; Lunar, Madison, WI, USA). We measured serum concentration of calcium (Ca), ALP, intact-osteocalcin (I-OC, ELISA; Teijin, Tokyo, Japan), intact parathyroid hormone (PTH), calcitonin (CT) and $1,25(OH)_2D_3$. We also measured urinary ratios of urinary deoxypyridinoline (DPD, high-performance liquid chromatography method) to creatinine. The BMD data were recorded as "Z scores"; that is, deviation from the weight-adjusted average BMD for each age. Z scores were calculated using installed software (Lunar DPX-L) on the basis of data from 20 000 Japanese women.

Determination of a single nucleotide polymorphism in the Wnt10b gene

Because there was only one coding SNP in the *Wnt10b* gene in the Japanese-SNP database (J-SNP), we examined association of this SNP in the *Wnt10b* gene at 1059C/T (His353His) with BMD in 221 postmenopausal Japanese women. We also extracted this variation in the Wnt10b gene from the Assays-on-Demand SNP Genotyping Products database (Applied Biosystems) and, according to its localization on the gene, denoted it 1059C/T. We determined the 1059C/T polymorphism of the *Wnt10b* gene using the TaqMan (Applied Biosystems) PCR method.[22] To determine the *Wnt10b* SNP we used Assays-on-Demand SNP Genotyping Products C_7470505_1 (Applied BioSystems), which contains sequence-specific forward and reverse primers and two TaqMan MGB probes for detecting alleles. During the PCR cycle, two TaqMan probes competitively hybridize to a specific sequence of the target DNA and the reporter dye is separated from the quencher dye, resulting in an increase in fluorescence of the reporter dye. The fluorescence levels of the PCR products were measured with the ABI PRISM 7000, resulting in clear identification of three genotypes of the SNP.

Statistical analysis

Comparisons of Z scores and biochemical markers between the group of individuals possessing one or two chromosomes of the T-allele and the group with only C-allele encoded at that locus were subjected to statistical analysis (Student's *t*-test; StatView-J 4.5). A *P*-value less than 0.05 was considered statistically significant.

Results

Wnt10b mRNA expression is regulated during the course of primary osteoblast differentiation

At the inception of this study, we measured the Wnt10b mRNA levels during the course of differentiation in rat primary osteoblasts. In the presence of ascorbic acid and β-glycerophosphate, primary osteoblasts proceed to differentiation normally with the deposition of a collagenous extracellular matrix that mineralizes.[25,26] The continual maturation of the osteoblasts was reflected by the increase of ALP mRNA (Fig. 1A). The Wnt10b mRNA was detected at day 2 and then decreased in primary osteoblasts (Fig. 1B). Inversely, the LRP5 mRNA increased persistently during the time-course of osteoblastic differentiation until day 28 (Fig. 1C). The levels of LRP6 mRNA were almost parallel to those of LRP5 mRNA (Fig. 1D).

Association of the Wnt10b gene polymorphism with BMD

We examined a *Wnt10b* polymorphism at 1059C/T (His353His) in postmenopausal Japanese women, using the TaqMan methods. Among 221 postmenopausal women, 42 were TT homozygotes, 120 were CT heterozygotes, and 59 were CC homozygotes. The genotype distribution was found to be in the Hardy–Weinberg equilibrium.

We compared Z scores for BMD of total body and lumbar spine between the subjects bearing at least one T allele (TT + TC) and subjects without the T allele (CC). Those with the T allele had significantly lower Z scores for total body BMD (Z score; 0.24 ± 0.99 vs 0.65 ± 1.11; $P = 0.0091$) (Figs 2A and 1A) and lumbar spine BMD (Z score; -0.42 ± 1.35 vs 0.16 ± 1.41; $P = 0.0052$) (Fig. 2B). The background and biochemical data were not statistically different between these two groups (Table 1).

Discussion

During the course of primary osteoblast differentiation, Wnt10b mRNA levels showed gradual decrease and sustained at certain levels during the observation period. Recent reports demonstrated that during the course of adipogenetic differentiation in 3T3L1 cells, Wnt10b rapidly falls to an undetectable level by the first 0–1 day.[27,28] The differential expression of Wnt10b in osteoblasts and adipocytes may imply a different role of Wnt10b in the cell differentiation. The increase of LRP5 and LRP6 expression was accompanied by the increase of ALP expression, which is a marker of osteoblast differentiation.[29] A previous report also demonstrated that BMP2 induced the osteoblastic differentiation markers,

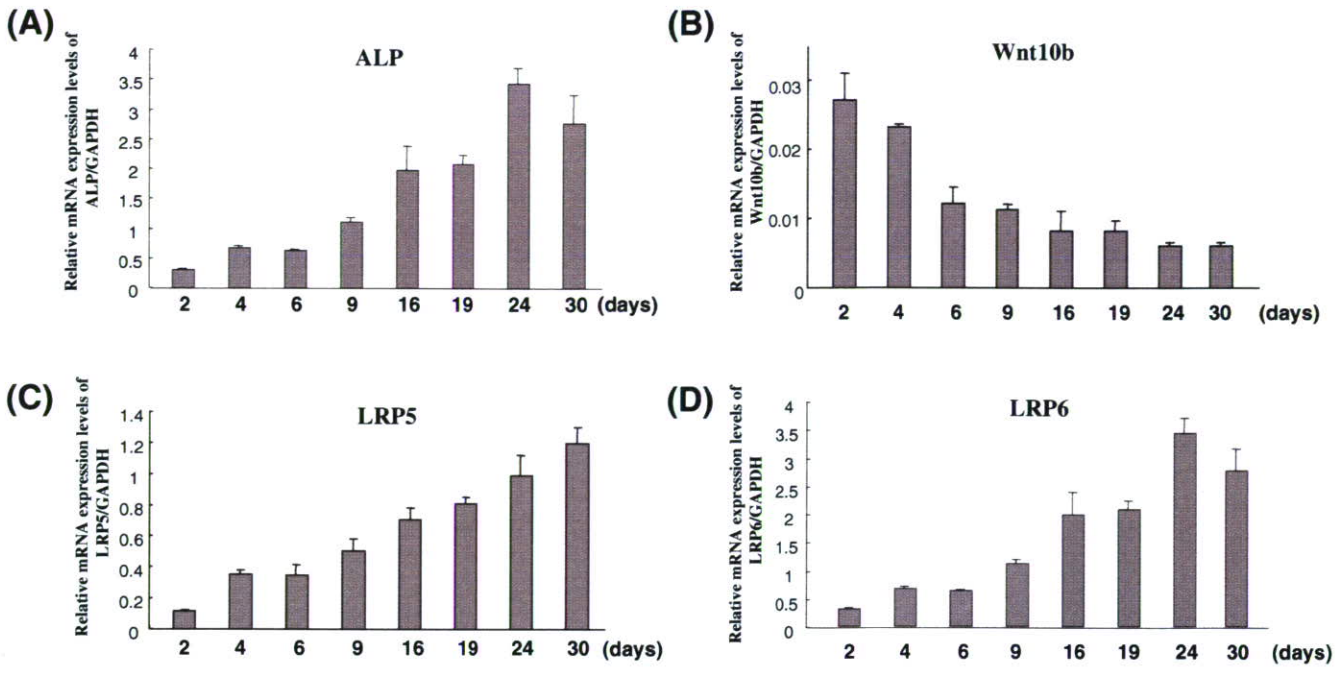

Figure 1 Expressions of the alkaline phosphatase (ALP), Wnt10b, lipoprotein receptor-related protein (LRP)5 and LRP6 mRNA during culture course of rat primary osteoblasts were analyzed by real-time reverse transcription polymerase chain reaction (RT-PCR). Rat primary osteoblasts were cultured with α-minimum essential medium (MEM) containing 10% fetal bovine serum (FBS), 50 μg/mL ascorbic acid and 5 mmol/L β-glycerophosphate up to 18 days. At the indicated times, RNA were extracted and the expression levels of ALP (A), Wnt10b (B), LRP5 (C) and LRP6 (D) were analyzed by real-time RT-PCR, normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression ($n = 4$ for each group). Values are shown as means ± SD.

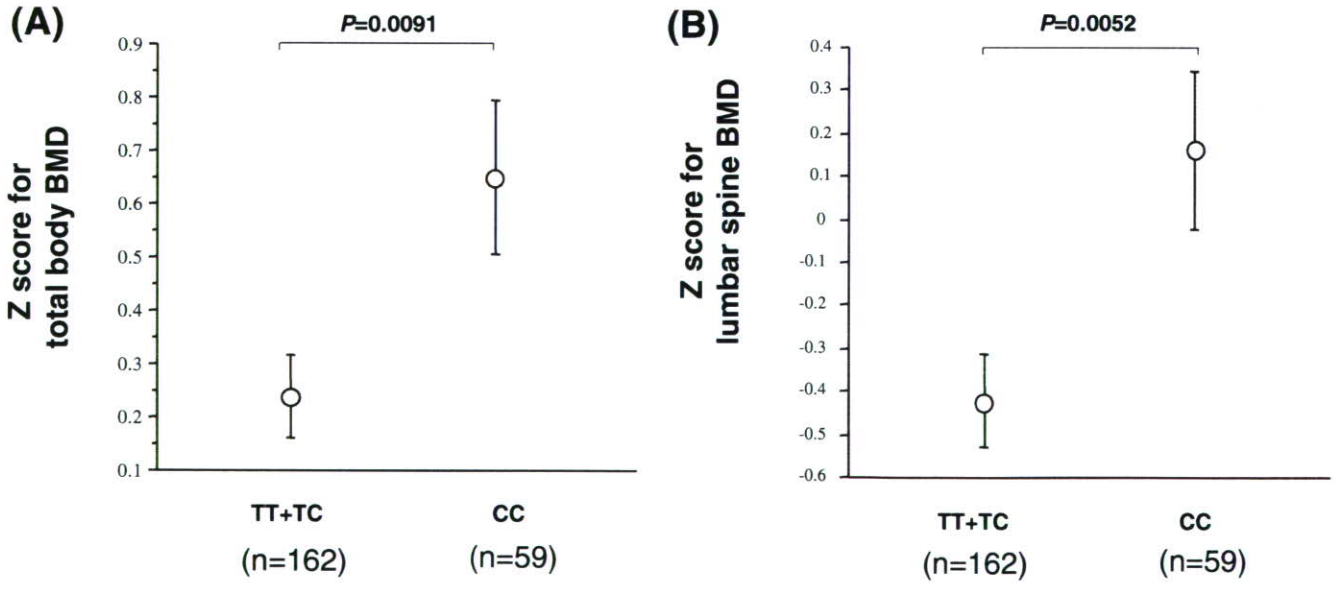

Figure 2 "Z score" values of total body and lumbar spine bone mineral density (BMD) in the groups of genotype TT + TC and genotype CC of the *Wnt10b* polymorphism in exon 6 (1059C/T). (A) Z score values for total BMD are shown for genotype TT + TC and for genotype CC. Values are expressed as mean ± SE. Numbers of subjects are shown in parentheses. (B) Z scores for lumbar BMD are shown in the same manner as (A).

followed by the increase of the LRP5 and LRP6 expression in ST2 cells.[15] Thus, the increase of LRP5 and LRP6 expression may have some roles in osteoblastic differentiation.

To our knowledge, the present study is the first to investigate the influence of a polymorphism of the Wnt10b gene on BMD. We demonstrated that the Japanese postmenopausal women who had one or two

Table 1 Comparison of background, bone mineral density and biochemical data between subjects bearing at least one T allele (TT + TC) and subjects with no T allele (CC) in the *Wnt10b* gene (1059C/T)

Items	Genotype (mean ± SD)		*P*-value
	TT + TC	CC	
No. of subjects	162	59	
Age (years)	61.4 ± 6.5	62.9 ± 6.7	NS
Height (cm)	152.3 ± 5.8	151.6 ± 5.8	NS
Bodyweight (kg)	52.2 ± 7.8	52.2 ± 7.0	NS
Lumber spine BMD (Z score)	−0.42 ± 1.35	0.16 ± 1.41	0.0052
Total body BMD (Z score)	0.24 ± 0.99	0.65 ± 1.11	0.0091
ALP (IU/L)	187.6 ± 58.8	182.3 ± 64.5	NS
I-OC (ng/mL)	8.2 ± 3.9	7.2 ± 3.2	NS
DPD (pmol/μmol/Cr)	6.3 ± 4.0	6.1 ± 3.3	NS
Intact PTH (pg/mL)	33.1 ± 10.7	36.4 ± 19.7	NS
Calcitonin (pg/mL)	23.0 ± 12.9	22.3 ± 7.9	NS
1,25 $(OH)_2D_3$ (pg/mL)	34.7 ± 10.2	35.2 ± 11.7	NS
% fat	32.6 ± 7.3	33.0 ± 7.0	NS
BMI	22.5 ± 3.1	22.7 ± 3.1	NS

ALP, alkaline phosphatase; BMD, bone mineral density; BMI, body mass index; DPD, deoxypyridinoline; I-OC, intact-osteocalcin; NS, not significant; PTH, parathyroid hormone. Statistical analysis was performed according to the method described in the text.

allele(s) of a synonymous change of C-T transition showed significant total body and lower lumbar BMD. Lower BMD in postmenopausal women could be considered as results of abnormally rapid bone loss and/or lower peak bone mass. The SNP analyzed in the present study would be useful as a genetic marker for low BMD and susceptibility to osteoporosis. Although the biological meanings of this polymorphism should be revealed by functional studies, several hypotheses could be proposed at present. First, this silent polymorphism may be linked with other mutations in exons, which contributes to the change of the Wnt10b protein function, such as in case of the *PADI4* polymorphisms in rheumatoid arthritis.[30] Second, the SNP may be linked with a mutation in regulatory elements affecting the levels of expression through variable transcriptional regulation, such as in case of the *LTA* exon 1 polymorphisms in myocardial infarction.[31] Third, this SNP in the *Wnt10b* gene may be linked with a mutation of another undefined gene adjacent to the *Wnt10b* gene that causes low BMD directly or indirectly, such as in case of the *ECM2* and *ASPN* polymorphisms in osteoarthritis.[32]

Because of the limited sample size and the number of SNP utilized in the present study, we need larger scale studies on this coding SNP and other polymorphisms in the Wnt10b gene in the future. The association study between multiple SNP and BMD using a statistical correction as well as functional analysis of SNP would be helpful.

The Wnt pathway has recently been implicated in the control of bone mass in adults in human and mice.[15–18] Activation of this pathway increases bone mass through a number of mechanisms including renewal of stem cells, stimulation of preosteoblast replication, induction of osteoblastgenesis, and inhibition of osteoblast and osteocyte apoptosis.[33] Taken together, these studies suggest that endogenous Wnt signaling plays an important role in osteogenesis and bone formation. However, the Wnt that are involved directly in the bone metabolism have to be identified among 19 members of the Wnt family. Recently, it was demonstrated that expression of Wnt10b in bone marrow increased bone mass and strength in mice.[22] Taking together with these data, our present finding of an association of a polymorphism in *Wnt10b* gene with BMD suggests that Wnt10b may be a specific ligand responsible for BMD among several Wnt.

In conclusion, our findings suggest that the *Wnt10b* gene may be a genetic determinant of BMD in postmenopausal women as is the case with its related co-receptor, *LRP5*. Examining the variation in the *Wnt10b* gene will hopefully enable us to elucidate one of mechanisms of involutional osteoporosis. Furthermore, the variation may be a potential genetic susceptibility factor that need to be further evaluated with regard to the condition of other metabolisms in which the Wnt signaling have been clearly implicated, including cholesterol, glucose and fat metabolisms.[34,35]

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A functional single nucleotide polymorphism in the vitamin-K-dependent gamma-glutamyl carboxylase gene (Arg325Gln) is associated with bone mineral density in elderly Japanese women

Hiroyuki Kinoshita [a], Kimie Nakagawa [b], Ken'ichiro Narusawa [c], Masae Goseki-Sone [d], Mariko Fukushi-Irie [d], Lena Mizoi [d], Hideyo Yoshida [e], Toshio Okano [b], Toshitaka Nakamura [c], Takao Suzuki [e], Satoshi Inoue [a], Hajime Orimo [f], Yasuyoshi Ouchi [a], Takayuki Hosoi [g,h,*]

[a] *Department of Geriatric Medicine, Graduate School of Medicine, University of Tokyo, Japan*
[b] *Department of Hygienic Sciences, Kobe Pharmaceutical University, Japan*
[c] *Department of Orthopedic Surgery, School of Medicine, University of Occupational and Environmental Health, Japan*
[d] *Department of Food and Nutrition, Japan Women's University, Japan*
[e] *Epidemiology and Health Promotion Research Group, Tokyo Metropolitan Institute of Gerontology, Japan*
[f] *Health Science University, Japan*
[g] *Endocrinology Section, Tokyo Metropolitan Geriatric Hospital, Japan*
[h] *Department of Advanced Medicine, National Center for Geriatrics and Gerontology, Japan*

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Abstract

The vitamin-K-dependent gamma-glutamyl carboxylase (GGCX) carboxylates vitamin-K-dependent proteins including bone Gla protein (osteocalcin) and matrix Gla protein, which play important roles in bone metabolism. Therefore, GGCX polymorphism might explain in part individual susceptibility to osteoporosis. In the present study, polymorphisms in the exons of this gene were screened in Japanese elderly women and a non-synonymous single nucleotide polymorphisms (SNP) were found; c.8762 G>A; (Arg325Gln). When the kinetic parameters of GGCX325-Gln and GGCX325-Arg were compared *in vitro*, Vmax/Km was significantly higher for GGCX325-Gln (944.4±9.21 pmol/30 min/mg/mM FLEEL) than for GGCX325-Arg (671.9+10.79 pmol/30 min/mg/mM FLEEL) ($p=0.018$). Then, association study of this polymorphism with forearm bone mineral density (BMD) of Japanese postmenopausal women ($n=500$, age 73.6±5.74) was conducted. As a result, the body mass index (BMI)-adjusted Z score in the subpopulation older than 75 years ($n=207$) was higher in those with 325-Gln (0.650±0.883, mean±SD) than those with 325-Arg/Gln or 325-Arg (0.133±0.650) ($p=0.0383$). This is the first report to demonstrate the different activities of GGCX between the common genotypes and their association with BMD.

Keywords: Vitamin-K-dependent gamma-glutamyl carboxylase; Single nucleotide polymorphism; Osteoporosis; Bone mineral density; Bone metabolism

Introduction

Osteoporosis is defined as a skeletal disorder characterized by compromised bone strength predisposing a person to an increased risk of fracture [1]. Because osteoporosis consequently leads to deterioration in activities of daily living (ADL) and quality of life (QOL), prevention and treatment are becoming more important in the current aging society.

Several risk factors for osteoporotic fractures have been established [2], which include the family history of fractures. Epidemiological studies also support the heritability of BMD [3], indicating the contribution of the genetic factors to the pathogenesis of osteoporosis. Therefore, the elucidation of genetic factors for this disease has been awaited. Recently, genetic factors for osteoporosis have been investigated with

* Corresponding author. Department of Advanced Medicine, National Center for Geriatrics and Gerontology, 36-3 Gengo, Morioka-cho, Ohbu-shi, Aichi 474-8511, Japan. Fax: +81 562 46 9595.
E-mail address: hosworld@cb3.so-net.ne.jp (T. Hosoi).

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polymorphisms of genes. Quite a few numbers of association studies have been done with so-called candidate gene approaches [4] and genome-wide approaches [5]. Polymorphisms of many genes were shown to have significant association with BMD. However, the contribution of each gene in determining BMD is small and the result is not always reproducible [6,7]. One of the ways to circumvent the problems and to make outcome biologically and clinically relevant would be to use polymorphisms whose functional variations can be studied.

The vitamin-K-dependent gamma-glutamyl carboxylase (GGCX or VKGC; EC 6.4.-.-) is a microsomal enzyme and is necessary for post-translational modification of vitamin-K-dependent proteins to exert their functions. The genomic structure of GGCX was elucidated in 1997 [8]. Concurrent with conversion of glutamate residue (Glu) to gamma-carboxyl glutamate residue (Gla), vitamin K hydroquinone is oxidized to vitamin K 2,3-epoxide by GGCX, then vitamin K 2,3-epoxide is reduced to vitamin K hydroquinone by vitamin K2,3-epoxide reductase (VKOR) [9]. In addition to vitamin-K-dependent coagulation factors, growth arrest-specific protein (gas6), proline-rich Gla protein-1 (PRGP-1), PRGP-2, bone Gla protein (BGP=osteocalcin) and matrix Gla protein (MGP) are also vitamin-K-dependent proteins [10]. BGP and MGP are abundant in bones [11] and assumed to play important roles in bone metabolism. Therefore, variation in the quality, i.e., carboxylation status, as well as the quantity of these proteins may contribute to variations in the susceptibility of individuals to osteoporosis and other skeletal disorder.

It is reported that the rare mutations of GGCX gene with amino acid substitution (Leu395Arg, Trp501Ser) cause consequential abnormal enzymatic activity, and these lead to vitamin-K-dependent protein defects and severe bleeding disorders [12,13]. However, any association between common variants of the GGCX gene with diseases has not been investigated. In addition, there have been no report comparing the function of products from the polymorphic genes. In this study, we compared the carboxylase activity of the products of the GGCX gene with each non-synonymous SNP and conducted association studies with forearm BMD according to the "common disease, common variant hypothesis" [14].

Materials and methods

Subjects

DNA samples were obtained from peripheral blood of 500 postmenopausal Japanese women living in an area of Japan. Mean ages with SD were 73.6± 5.7 years (range 65–90 years). None of the subjects were under the treatment with Warfarin.

All subjects were non-related volunteers and provided informed consent before the study. No participant had medical complications or was undergoing treatment for conditions known to affect bone metabolism, such as pituitary diseases, hyperthyroidism, primary hyperparathyroidism, renal failure, adrenal diseases, or rheumatic diseases, and none were receiving estrogen replacement therapy. The BMD of the radial bone (expressed in g/cm^2) of each participant was measured by dual energy X-ray absorptiometry (DXA) using DTX-200 (Osteometer Meditech Inc., Hawthorne CA, USA). *Z* scores were calculated using installed software (DTX-200) on the basis of Japanese women and adjusted for body mass index (BMI) utilizing regression analysis.

The ethics committee of the Tokyo Metropolitan Geriatric Hospital approved this study according to the Declaration of Helsinki.

SNPs screening and genotyping

DNA samples were extracted from leukocytes in peripheral blood. SNP screening in the exons of the GGCX gene was conducted with DNA samples of randomly chosen 20 subjects. All of the exons of the GGCX gene were amplified by polymerase chain reaction (PCR) with primers designed as reported previously [15]. Then, denaturing high performance liquid chromatography (DHPLC) with WAVE (Transgenomic Japan, Tokyo, Japan) was used to detect SNPs [16]. The detected variations in the PCR products were validated by direct sequencing utilizing Gene Rapid (Amersham Biosciences Corp, Piscataway, NJ). Then, the genotyping was performed by WAVE.

Construction of human GGCX cDNA (c.8762=A and c.8762=G) expression plasmid

Human GGCX cDNA (c.8762=A) in p*CMV*5 was obtained from American Type Culture Collection (ATCC Number 68666, GenBank M81592) (Manassas, VA). Site-directed mutagenesis was performed with a QuickChange XL Site-Directed Mutagenesis Kit (Stratagene, LA Jolla, CA) to prepare GGCX cDNA (c.8762=G) in p*CMV*5. A pair of mutagenesis primers, 5′-TCCTACTGCCCCC-GAAGGTTGCAACAA-3′ and 5′-TTGTTGCAACCTTCGGGGGCAG-TAGGA-3′ (underlined nucleotides are the mutagenesis target) was used for this process. Direct sequencing of the full length of each c.8762=G and c.8762=A was performed utilizing Gene Rapid (Amersham Biosciences Corp, Piscataway, NJ) to ascertain the sequence. Then, the ligation to pcDNA3.1/V5-His/lacZ was performed.

Preparation of microsome fraction from COS-7 cells

Microsomal fraction was prepared from the transfected COS-7 cells as reported previously [17]. COS-7 cells cultured for 24 h were transfected with pcDNA3.1/V5-His/lacZ-GGCX (c.8762=A; 325Gln) or pcDNA3.1/V5-His/lacZ-GGCX (c.8762=G; 325Arg) using the LipofectAMINE (Invitrogen Corp., Carlsbad, CA). The transfected cells were cultured for 5 days in 10% FBS D-MEM medium containing G418 300 μg/ml. The cells (5×10^7 cells) were washed with PBS (−) (calcium and magnesium free PBS), scraped and collected in PBS (−) containing 20% glycerol and 1× PIC (protease inhibitor cocktail: 2 mM dithiothreitol, 2 mM EDTA, 0.5 μg/ml leupeptin, 1 μg/ml pepstatin A, 2 μg/ml caprotinin). Cells were homogenized in a glass homogenizer (3 × 10 strokes) and centrifuged at 500×*g* for 7 min. The pellet was re-homogenized and washed 3 times with the same buffer. Pooled supernatants were centrifuged at 105,000×*g* for 1 h at 4°C to separate the microsomal fraction. The pellet was resuspended in PBS (−) containing 0.5% (w/v) CHAPS, 0.2% (w/v) phosphatidylcholine, 1× PIC and 20% (v/v) glycerol by sonication.

Carboxylase activity assays

Carboxylase activity was assayed by previously described methods [18–20]. FLEEL was purchased from Bachem (Philadelphia, PA). L-a-Phosphatidylcholine (type V-E) and CHAPS were from Sigma Aldrich Japan (Tokyo, Japan). Vitamin K_2 (menaquinone-4) was from Eisai Co., Ltd. (Tokyo, Japan). The peptide ProFIX19 which contains the sequence AVFLDHENANKILNRPKRY was synthesized by Genenet Co., Ltd. (Fukuoka, Japan). $NaH^{14}CO_3$ (specific activity, 58 mCi/mmol) was from Amarsham Biosciences Corp. (NJ).

The amount of $^{14}CO_2$ incorporated into exogenous substrates was measured in reaction mixtures at 125 μl containing substrate at the indicated concentration, 222 μM reduced vitamin K2 (vitamin KH_2), 16 μM propeptide ProFIX19, 1.4 mM $NaH^{14}CO_3$ (5 μCi), 25 mM MOPS (pH 7.0), 500 mM NaCl, 0.16% (w/v) phosphatidylcholine, 0.16% (w/v) CHAPS, 8 mM DTT and 0.8 M ammonium sulfate, unless stated otherwise. All of the assay components except for the microsomal fraction were prepared as a master mixture. $^{14}CO_2$ incorporation into peptide substrates for over 30 min was assayed in a scintillation counter. Stimulation experiments with vitamin KH_2 were performed at a constant concentration of the enzyme sample and substrate (3.6 mM FLEEL)

with increasing concentrations vitamin KH_2, as indicated. All assays were performed in quadruplicate.

Statistical analysis

The allele frequency, haplotype frequency and indices of linkage disequilibrium such as D, D' and r^2 were calculated by SNPAlyze ver3.2 Pro (DYNACOM, Tokyo, Japan). The Pearsons's goodness of fit test with one degree of freedom was used to examine Hardy–Weinberg equilibrium among GGCX genotypes.

The following analysis were performed by StatView 5.0 software (SAS Institute Inc., Cary, NC) and significance was defined as $p<0.05$. ANOVA was used to compare baseline characteristics (age, height, weight and BMI) among GGCX genotypes. The Mann–Whitney's U test was utilized to examine the effects of GGCX genotypes on BMI adjusted Z scores. The unpaired t-test was used to compare the carboxylase activity.

Results

SNP search and genotyping

Table 1 summarizes the SNPs detected in our SNP search. These were already registered in the dbSNP (http://www.ncbi.nlm.nih.gov/SNP/) (Table 1a) and each genotype showed Hardy–Weinberg equilibrium ($p>0.05$). The linkage disequilibrium coefficients, D' values, in c.8762 G>A, c.9167 C>T and c.9191 C>T were higher than 0.98, indicating that these three SNPs are in a strong linkage disequilibrium and constitute a haplotype block [21] (Table 1b). The genotype c.8762=AA perfectly corresponded to c.9167=TT, c.8762=AG to c.9167=CT and c.8762=GG to c.9167=CC, i.e., c.8762 G>A and c.9167 C>T were completely linked together. The SNP c.8762 G>A was a non-synonymous SNP, changing amino acid 325 Arg to Gln (Table 1a). Therefore, the genotypes were described as 325-Arg, 325-Arg/Gln, or 325-Gln afterward in this report. We focused on this non-synonymous polymorphism in further analyses. Genotype distribution in the subjects was as follows; 325-Arg=265, 325-Arg/Gln=203 and 325-Gln=32. The baseline characteristics (age, height, weight and BMI) were not significantly different among these genotypes (Table 2).

Table 1
Summary of GGCX SNPs

a. Detected SNPs

Name	Base position	Allele frequency	Amino acid change	dbSNP[a]
c.8762 G>A	8762 (exon8)	G:A=0.73:0.27	Arg325Gln	Rs699664
c.9167 C>T	9167 (exon9)	C:T=0.73:0.27	none (406Arg)	Rs2592551
c.9191 C>T	9191 (exon9)	C:T=0.93:0.07	none (414Thr)	Rs10179904

b. Tests for linkage disequilibrium between GGCX SNPs

SNP1	SNP2	SNP3	D	D'	r^2
c.8762 G>A	c.9167 C>T		0.1957	1.000 0	1.000 0
c.8762 G>A		c.9191 C>T	−0.0179	0.999 9	0.026 2
	c.9167 C>T	c.9191 C>T	−0.0179	0.999 9	0.026 2

The D, D', and r^2 were calculated by SNPAlyze ver3.2 Pro.
The D' value was an absolute value ($0 \leqq D' \leqq 1$).
[a] Numbers are from dbSNP database of NCBI (http://www.ncbi.nlm.gov/SNP/).

Table 2
Baseline characteristics of subjects

Genotype	325-Gln	325-Arg/Gln	325-Arg	p value
Number	32	203	265	
Age	74.1±5.9	73.1±5.7	73.9±5.7	0.344
Height (cm)	144.4±5.0	144.8±5.9	145.3±5.7	0.534
Weight (kg)	48.7±5.6	50.4±8.7	51.1±8.2	0.265
BMI (kg/m^2)	23.3±2.4	24.0±3.5	24.2±3.4	0.409

Data were shown as mean±SD.
The p values were calculated by ANOVA.

Carboxylase activity

Efficient transfection into COS-7 cells was confirmed by measuring luciferase activity from co-transfected internal control vector (pRL-*CMV*) (data not shown). Moreover, production of GGCX by transfected COS-7 cells was visualized

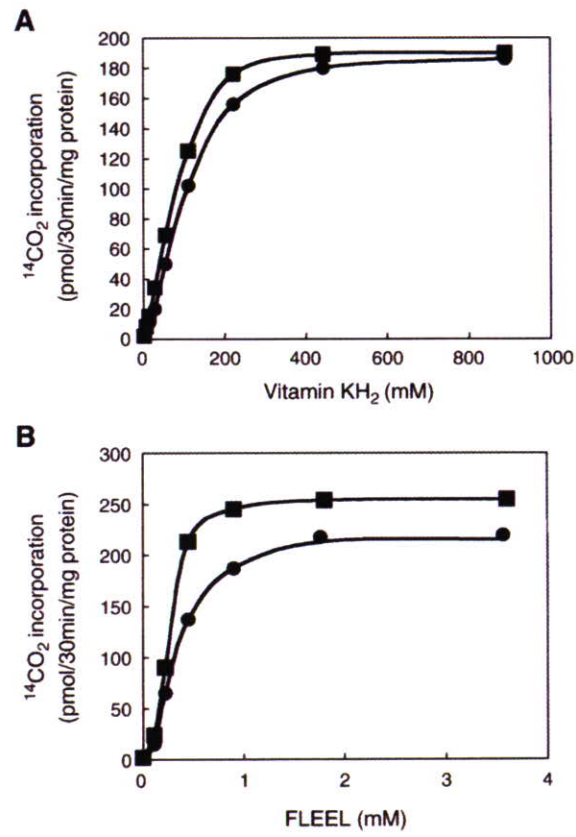

Fig. 1. Influence of vitamin KH_2 or FLEEL on the carboxylase activity of GGCX 325Gln or 325Arg. (A) Carboxylation of 3.6 mM FLEEL at vitamin KH_2 concentrations between 0 and 888 mM was measured by $^{14}CO_2$ incorporation for both 325Gln (filled squares) and 325Arg (filled circles). (B) Carboxylation in 222 mM vitamin KH_2 at FLEEL concentrations between 0 and 3.6 mM was measured by $^{14}CO_2$ incorporation for both 325Gln (filled squares) and 325Arg (filled circles).

as a 130 kDa band in Western blot analysis using antibodies to V5 and His (data not shown).

GGCX 325-Gln (c.8762=A) was determined to have a Km for vitamin KH_2 of 71.34±6.20 μM (mean±SD). The Km of GGCX 325-Arg (c.8762=G) for vitamin KH_2 was 90.31±4.63 μM, about 1.3-fold higher than that of 325-Gln (p=0.029). The Vmax determined for 325-Arg (186±7.88 pmol/30 min/mg) was lower than that of 325Gln (191±9.45 pmol/30 min/mg) (p=0.033). The Vmax/Km for vitamin KH_2 with 325-Arg (2.06±0.12 pmol/30 min/mg/μM) was thus reduced by 24% compared with 325Gln (2.68±0.20 pmol/30 min/mg/μM) (p=0.032) (Fig. 1 and Table 3a).

Kinetic constants for FLEEL carboxylation were determined in the presence of saturating concentrations of ProFIX19. The Km of 325-Gln for FLEEL was 0.27±0.02 mM. The Km of 325-Arg for FLEEL was 0.32±0.03 mM, about 1.3-fold higher than that of 325-Gln (p=0.016). Comparison of Vmax for FLEEL carboxylation showed that the reaction rate of 325-Arg (215±5.28 pmol/30 min/mg) was about 1.2-fold slower than that of 325-Gln (255±6.33 pmol/30 min/mg) (p=0.011). The Vmax/Km for FLEEL with 325-Arg (671.9±10.79 pmol/30 min/mg/mM) was thus reduced about 30% compared with 325Gln (944.4±9.21 pmol/30 min/mg/mM) (p=0.018) (Fig. 1 and Table 3b). These results indicate that 325-Gln has higher carboxylase activity than 325-Arg.

Association study of BMD

The subjects with 325-Gln had the highest adjusted Z score but the difference was not statistically significant in all subjects; 0.297±0.866 (mean±SD), 0.030±0.959 and 0.057±0.882 for 325-Gln, 325-Arg/Gln, or 325-Arg, respectively. From this result, we assumed that the effect of GGCX polymorphism c.8762 G>A is allele G dominant, and compared 325-Gln vs. 325-Gln/Arg+325-Arg.

Table 3
Comparison of the kinetic parameters of GGCX 325-Gln and 325-Arg

a. Using vitamin KH_2 as a substrate

Substrate	Enzyme	Km (μM)	Vmax (pmol/30 min/mg)	Vmax/Km (pmol/30 min/mg/μM)
Vitamin KH_2 [a]	325Gln	71.34±6.20	191±9.45	2.68±0.20
	325Arg	90.31±4.63	186±7.88	2.06±0.12
p value		0.029	0.033	0.032

b. Using FLEEL as a substrate

Substrate	Enzyme	Km (μM)	Vmax (pmol/30 min/mg)	Vmax/Km (pmol/30 min/mg/mM)
FLEEL [b]	325Gln	0.27±0.02	255±6.33	944.4±9.21
	325Arg	0.32±0.03	215±5.28	671.9±10.79
p value		0.016	0.011	0.018

All assays were performed in quadruplicate.
Data were shown as mean±SD. The p values were calculated by the unpaired t-test.
[a] Determined at 3.6 mM FLEEL.
[b] Determined at 222 mM vitamin KH_2.

Table 4
Comparison of BMI-adjusted Z score of BMD among the genotypes of GGCX Arg325Gln

Genotype	325-Gln	325-Arg or 325-Arg/Gln	p value
All	0.297±0.866 (32)	0.045±0.915 (468)	0.132
<75	0.022±0.926 (18)	−0.017±0926 (275)	0.861
>75	0.650±0.883 (14)	0.133±0.650 (193)	0.038

The data are expressed as mean±SD.
The p values were calculated by the Mann–Whitney's U test.

The adjusted Z score seemed higher in the subjects with 325-Gln than those with other genotypes. However, the difference was not statistically significant (Table 4). Then we divided the subjects into two groups according to the age; above and below 75 years. As a result, the adjusted Z score in the subpopulation older than 75 years (n=207) was higher in those with 325-Gln (0.650±0.883, mean±SD) than those with 325-Gln/Arg or 325-Arg (0.133±0.064) (p=0.0383). On the other hand, this association was not found in the subpopulation younger than 75 years.

Discussion

This is the first report to demonstrate the different activities of GGCX between the common genotypes. Although the association study of these genotypes with BMD provided a statistically significant results, the limited size of the samples should make us cautious and modest and requires further studies. Because the site of the BMD measurement in this study was limited to the radius, the association of GGCX genotype with BMD of spine and proximal femur would be another important issue. In addition, low BMD does not explain all of the pathophysiology of bone fragility in osteoporotic patients. Our results would explain a part of BMD determinants not the osteoporosis of itself, and suggest that the GGCX gene polymorphism may be involved in the pathogenesis of osteoporosis.

The physiological roles of vitamin-K-dependent Gla proteins in bone metabolism have not been elucidated yet, but it was reported that under-carboxylated osteocalcin (ucOC) was negatively correlated with BMD [22] and that administration of vitamin K decreased ucOC and worked to increase BMD [23]. Another vitamin-K-dependent protein, MGP (matrix Gla protein) has been reported to be a regulator of calcification in several studies. For example, MGP-deficient mice exhibited inappropriate calcification of various cartilages including the growth plate, which eventually led to short stature [24]. In addition, it was suggested that under-carboxylated MGP was biologically inactive and may increase the risk for vascular calcification [25]. Therefore, properly carboxylated MGP is assumed to be necessary to protect vascular system from calcification and skeletal system from abnormal ossification [26]. People with 325-Gln might have a higher efficiency of carboxylation of these proteins with the given status of vitamin K, considering that carboxylase activity of 325-Gln was higher than that of 325-Arg *in vitro*. Because this variation of carboxylase activity between the genotypes was examined

using only a standard substrate, FLEEL, in this study, functional significance of the polymorphisms in physiological target molecules and tissues should be investigated further. For example, *in vitro* studies using other substrates as well as measurements of the serum levels and carboxylation status of vitamin-K-dependent proteins would be informative.

In order to be carboxylated, vitamin-K-dependent proteins are assumed to be bound specifically to 343–355 residues of GGCX with high affinity. Among these residues, 343 (Cys) and 345 (Tyr) were suggested to be located near the catalytic center [27]. Moreover, it was also reported that chemical modification of 323-Cys and 343-Cys decreased its carboxylase activity [28]. Considering a study of human GGCX membrane topology, human GGCX probably may span the endoplasmic reticulum membrane 5 times and the interval of fourth and fifth transmembrane region may be composed of amino acids 313–361 [29]. Since amino acids 323-Cys, 325-Arg/Gln, 343-Cys, 345-Tyr and 343–355 are involved in the interval of fourth and fifth transmembrane regions (313–361), the amino acid substitution of 325 residue (Arg/Gln) may affect enzymatic activity directly or indirectly through influencing the function of these residues.

The higher Km value of GGCX 325-Arg would mean that higher intake of vitamin K may cancel the effects of this genotype. Therefore, the influence of GGCX polymorphisms should be studied further from the viewpoints of gene–environment interactions as well as ethnic/race differences. When the association study with the GGCX gene polymorphism is designed among different ethnicities, the intake of vitamin Ks should be handled carefully, because there are large ethnic differences in vitamin K status as we reported previously [30]. On the other hand, the difference in Vmax between the 325-Gln and 325-Arg suggests that the GGCX polymorphism might affect the carboxylase activity at the pharmacological level of vitamin K and might contribute to the difference of individual sensitivity to vitamin K treatment for osteoporosis.

The reason for age-dependent effect of GGCX polymorphism is not obvious, but we reported the similar age-dependent effects of functional SNP in tissue non-specific alkaline phosphatase gene [31]. It is reported that dietary vitamin K intake decreases with age, and elevated levels of ucOC may result from subclinical vitamin K deficiency and are frequently observed in the elderly [32]. The administration of vitamin K increased osteocalcin's hydroxyapatite binding capacity, decreased urinary calcium and hydroxyproline excretion in postmenopausal women but no effect was observed in premenopausal women [33]. These evidences may explain partly that the effects of different carboxylase activity between 325-Gln and 325-Arg might become obvious in advancing aging.

In conclusion, we reported here for the first time the different activities of GGCX between the common genotypes which were associated with BMD in elderly Japanese women. There are two major limitations in this study as discussed above; the limited sample size and the lack of extensive functional studies. Further studies are absolutely necessary to delineate any conclusion regarding the GGCX polymorphism and osteoporosis.

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ONCOGENOMICS

Identification of novel androgen response genes in prostate cancer cells by coupling chromatin immunoprecipitation and genomic microarray analysis

K Takayama[1,3], K Kaneshiro[2], S Tsutsumi[2], K Horie-Inoue[3], K Ikeda[3], T Urano[1,3], N Ijichi[3], Y Ouchi[1], K Shirahige[4], H Aburatani[2,5] and S Inoue[1,3]

[1]Department of Geriatric Medicine, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan; [2]Genome Science Division, Research Center for Advanced Science and Technology, The University of Tokyo, Tokyo, Japan; [3]Division of Gene Regulation and Signal Transduction, Research Center for Genomic Medicine, Saitama Medical University, Saitama, Japan; [4]Division for Gene Research, Center for Biological Resources and Informatics, Tokyo Institute of Technology, Tokyo, Japan and [5]CREST, Japan Science and Technology Agency (JST), Tokyo, Japan

The androgen receptor (AR) plays a key role as a transcriptional factor in prostate development and carcinogenesis. Identification of androgen-regulated genes is essential to elucidate the AR pathophysiology in prostate cancer. Here, we identified androgen target genes that are directly regulated by AR in LNCaP cells, by combining chromatin immunoprecipitation (ChIP) with tiling microarrays (ChIP-chip). ChIP-enriched or control DNAs from the cells treated with R1881 were hybridized with the ENCODE array, in which a set of regions representing approximately 1% of the whole genome. We chose 10 bona fide AR-binding sites (ARBSs) ($P<1e-5$) and validated their significant AR recruitment ligand dependently. Eight upregulated genes by R1881 were identified in the vicinity of the ARBSs. Among the upregulated genes, we focused on UGT1A and CDH2 as AR target genes, because the ARBSs close to these genes (in UGT1A distal promoter and CDH2 intron 1) were most significantly associated with acetylated histone H3/H4, RNA polymerase II and p160 family co-activators. Luciferase reporter constructs including those two ARBSs exhibited ligand-dependent transcriptional regulator/enhancer activities. The present study would be powerful to extend our knowledge of the diversity of androgen genetic network and steroid action in prostate cancer cells.
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Correspondence: Dr S Inoue, Department of Geriatric Medicine, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan.
E-mail: INOUE-GER@h.u-tokyo.ac.jp
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Introduction

Androgen is a key regulator of male sexual differentiation as well as prostate development and carcinogenesis. Androgen-regulated gene expression is mediated by the action of androgen receptor (AR), which is a member of nuclear receptor superfamily that functions as a ligand-dependent transcription factor. Prostate cancer is originally an androgen-dependent tumor, whose growth and survival are under the control of AR signaling. Thus, androgen deprivation is the most common option of the cancer treatment. The therapy, however, eventually fails and most patients will relapse owing to adaptive progression of the surviving prostate cancer cells. The recurrent cancer is usually referred to as 'androgen independent' (Grossmann *et al.*, 2001). Nevertheless, advanced prostate cancer often continues to express AR and androgen-regulated genes, suggesting a functional role of AR in the recurrent stage. Alterations of the AR gene including mutation and amplification are also shown in some recurrent tumors, but these mechanisms will not explain the hormone-refractory responses in the majority of patients after androgen deprivation. Indeed, a modest increase in AR mRNA has been shown to be associated with the resistance to anti-androgen therapy in isogenic prostate cancer xenograft models (Chen *et al.*, 2004). Therefore, understanding the global aspects of AR signaling network and the distinct roles of AR target genes are essential for the development of new diagnostic procedures and therapeutic options for prostate cancer in various disease states.

AR regulates the expression of target genes by binding to androgen response elements (AREs) in the genome, or by interacting with other transcription factors bound to their specific recognition sites. AR-mediated gene transcription has been studied using prostate-specific antigen (PSA) as a prototypic model, and AREs in PSA promoter and enhancer have been shown to recruit various co-activators and general transcription factors including histone acetyltransferases,

p160 family, mediator and RNA polymerase II (PolII) (Wang *et al.*, 2005). Efforts have been paid to search various androgen target genes by using microarray techniques since last decade, identifying hundreds of genes with altered expression by hormone stimulation in cells. The gene expression profiling is powerful to depict the global function of androgen in a specified model; however, the technique will not be suitable to determine whether the alteration of gene expression is owing to direct or indirect action of AR transcription. Recent advance of human genome project enables to search putative AREs bioinformatically in the transcription regulatory regions of androgen target genes; yet, few AREs are identified as physiological elements in AR signaling (Horie-Inoue *et al.*, 2004, 2006). Thus, the development of a new high-throughput method that identifies bona fide AR-binding sites (ARBSs) in the genome is a prerequisite for the elucidation of AR gene network.

Recently, a combined technique of chromatin immunoprecipitation (ChIP) analysis with DNA microarray has been established to identify chromatin-interacting domains of transcription factors in a genome-wide manner (Cawley *et al.*, 2004; Bernstein *et al.*, 2005). Regarding nuclear receptors, ligand-dependent estrogen receptor (ER)-binding sites have been recently shown by this ChIP-chip technique using Affymetrix-tiling oligonucleotide microarrays of chromosomes 21 and 22 (Carroll *et al.*, 2005), or a custom-made promoter microarrays (Laganière *et al.*, 2005). In this study, we have performed ChIP-chip using a sampler DNA microarray of the human genome, the so-called ENCODE chip. In this microarray, a set of regions representing approximately 1% (30 Mb) of the whole genome are included as the target for the pilot project that has been selected by the research consortium of the ENCyclopedia of DNA Elements (ENCODE Project Consortium, 2004). Fifty percent of the 30-Mb genomic regions, consisting of 14 regions (ENm001–ENm014), were manually selected, and the remaining 50% were composed of 30, 500 kb regions (ENr111–ENr334) selected according to a stratified random-sampling strategy based on gene density and level of non-exonic conservation.

Here, we find a discrete number of ARBSs in the selected regions of the ENCODE regions. Intriguingly, most of the AR-interacting regions have been shown to locate in non-promoter proximal regions; yet, they contained ARE sequences and were validated to recruit AR ligand dependently. In the vicinity of the functional ARBSs, we found several genes with upregulated transcript levels by hormone stimulation. Some of the AR target genes that have been identified in this study are previously known to be associated with AR expression, whereas some are novel targets. Our ChIP-chip analysis and transcriptional study indicate that non-promoter ARBSs play roles in the AR-dependent transcriptional regulation, potentially dissecting a series of AR-regulated mechanisms in a genome-wide manner.

Results

Screen of ARBSs on ENCODE DNA microarray

To perform a screen of ARBSs in AR-positive cells on tiling oligonucleotide microarrays, we first investigated the time course of ligand-dependent AR recruitment in human prostate cancer LNCaP cells. After 3-day hormone depletion, cells were stimulated with vehicle or a synthetic androgen R1881 (10 nM) for 2, 6 or 24 h. Cross-linked protein–DNA complexes extracted from the cells were immunoprecipitated with anti-AR antibody, and quantitative polymerase chain reaction (qPCR) for ARE regions in the proximal promoter and enhancer of PSA was performed using the purified precipitated DNAs as templates. AR binding in response to ligand stimulation exhibited maximal levels at 24 h (data not shown).

We next performed ChIP-chip analyses using the ENCODE tiling microarrays comprised of the total 30-Mb human genomic DNA, which corresponds to 1% of the genome. The chromatin DNAs immunoprecipitated by anti-AR or without ChIP (input control) were amplified unbiasedly by *in vitro* transcription (IVT), and the amplified DNAs were fragmented and biotin-labeled, then hybridized with the ENCODE chips for duplication. Using the Affymetrix Tiling Analysis Software, raw intensity data of duplicate arrays for each experimental group were transformed and signal and *P*-values for each genomic position interrogated were determined after quantile normalization. The results were mapped to genomic positions that could be visualized in the Affymetrix Integrated Genome Browser or the UCSC Genome Browser (NCBI Build 35). Applying a *P*-value cutoff of 1e-5 for a significant AR binding, we identified 10 ARBSs (Table 1) in the ENCODE genomic regions. Among them, five ARBSs were involved in the manually defined regions of the 30-Mb ENCODE regions (ARBSs no. 3–no. 7), whereas the remaining five binding sites were derived from the randomly selected regions.

Notably, most of the ARBSs were located within intronic regions or gene upstream regions at least 10 kb apart from the transcriptional start sites (TSSs) of their closest genes. One of the ARBSs included in the ENCODE chips was ARBS no. 1, which was located adjacent to UGT1A locus, in the 5′ upstream region > 17 kb upstream of UGT1A1 gene TSS or in intron 1 of UGT1A3 on chromosome 2q37. As another example, ARBS no. 10 was situated in intron 1 of CDH2 on chromosome 18q11.2 (Figure 1).

We next investigated whether the 10 ARBSs included sequences highly similar to the previously established consensus AREs. Using a weighted matrix-based finder TRANSFAC (Matys *et al.*, 2003) with the matrix conservation > 75% or a sequence analysis utility of JASPER with the relative profile score threshold > 70% (Sandelin *et al.*, 2004), we identified canonical ARE sequences in all of the ARBSs (Table 2).

To verify whether the identified ARBSs in ChIP-chip were authentic ARBSs in the genome, we performed new independent ChIP experiments in LNCaP cells. We