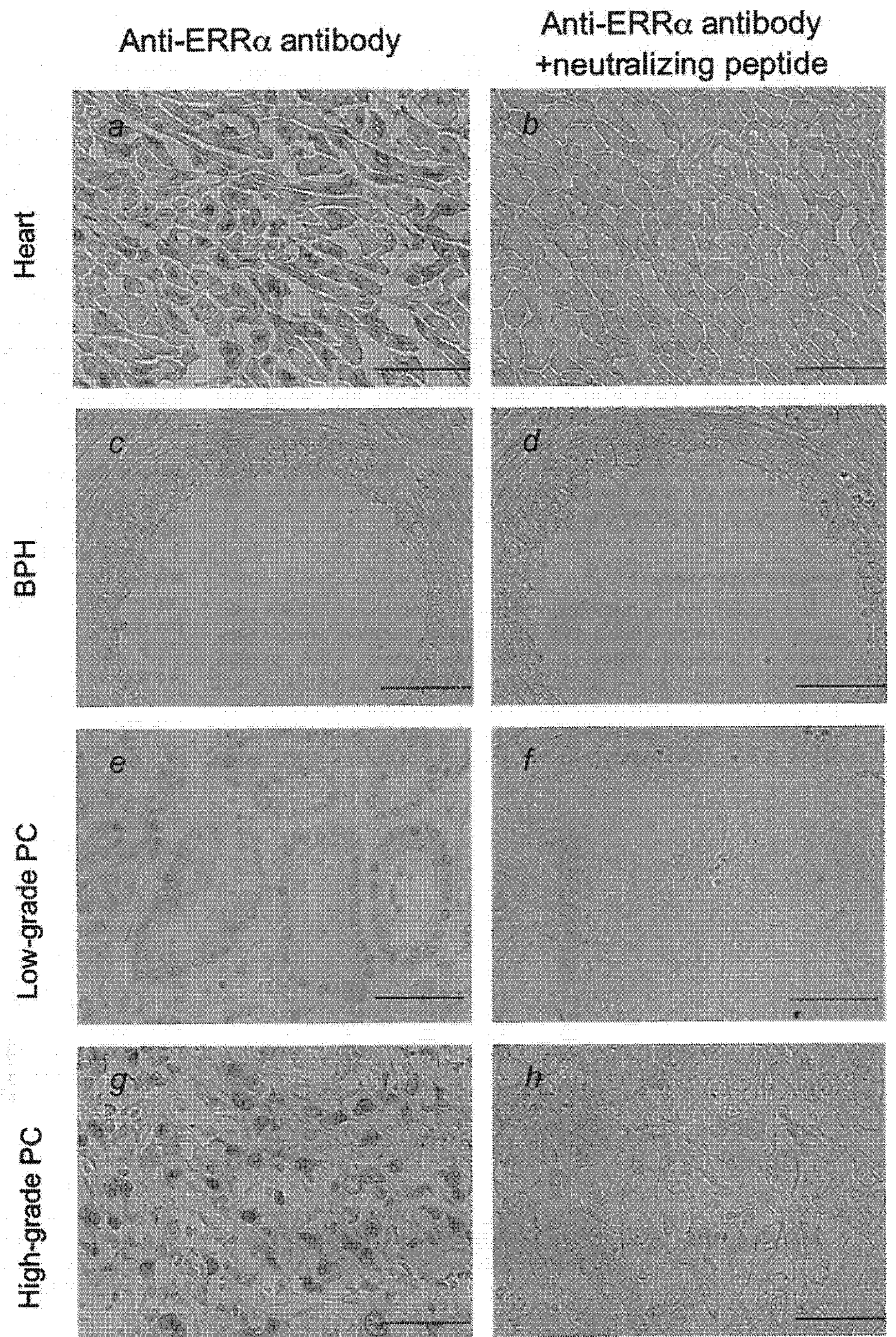

FIGURE 2 – Immunohistochemical staining (*a*, *c*, *e* and *g*) for ERRα and neutralizing peptide blocking (*b*, *d*, *f* and *h*) in human prostate and myocardium. Strong staining (IR score: 8) of ERRα was identified in the nuclei of human myocardium (*a*) Immunoreactivity was not detected in benign epithelium (*c*). Moderately increased immunoreactivity of ERRα (IR score: 6) was observed in low-grade PC (GS 6) (*e*). Extensive expression (IR score: 8) of ERRα was identified in high-grade cancer (GS 9) (*g*). Immunostaining with the ERRα antibody pre-absorbed with immunizing peptide did not identify any significant signals in all the samples examined (*b*, *d*, *f* and *h*). Original magnification; ×400, Scale bar = 50 μm.

ERRα was identified in the nuclei of human myocardium (Fig. 2*a*). Although ERRα immunoreactivity was focally detected in benign epithelium in some cases, the strong immunoreactivity (IR score: 5 or more) was rarely observed (Table I and Fig. 2*c*). No significant immunoreactivity was identified in stromal cells. In contrast, increased ERRα immunoreactivity was observed in low-grade PC (Fig. 2*e*). Extensive ERRα expression was often identified in high-grade cancer (Table I and Fig. 2*g*). Immunostaining with the ERRα antibody pre-absorbed with immunizing peptide did not identify any significant signals in all the samples examined (Figs. 2*b*,2*d*,2*f* and 2*h*). ERRα immunoreactivities were positive in 47 of 99 cases (47.5%) in benign epithelium and in 73 of 106 cancer cases (69%). The cancerous lesions showed significantly higher ERRα IR scores (3.5 ± 2.6) than 1.8 ± 2.1 of the benign foci ($p < 0.0001$). Higher-GS (8–10) cancers showed significantly higher IR score (4.3 ± 2.7), compared with the lower-GS (2–7) tumors (3.0 ± 2.6) ($p = 0.0194$)(Table I).

Clinical significance of ERRα expression in human PC

Since most of benign foci showed <5 of IR scores for ERRα, we defined IR score 5 as a cutoff for strong immunoreactivity of ERRα. Table II shows correlation of ERRα expression and clinicopathological characteristics. Cases with high serum PSA level showed a trend of strong expression of ERRα, although it did not reach statistical significance ($p = 0.07$). GS correlated with the status of ERRα expression. ERRα expression in higher-GS cancer

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(8–10) was significantly higher than that in lower GS (2–7) cancer ($p = 0.0135$).

F3 Figure 3 demonstrates a cancer-specific survival curve prepared by the Kaplan-Meier method. Eleven (10%) cases died of PC during the follow-up period. Cancer-specific survival of patients with higher ERRα expression (IR score ≥5) was significantly worse than cases with lower expression (IR score < 5) ($p = 0.0055$). Table III indicates the results of univariate and multivariate proportional analyses of cancer-specific survival with status of ERRα expression and clinicopathological parameters. ERRα expression, GS and pathological stages were significant prognostic predictors in univariable analysis ($p = 0.0141$, 0.0123 and 0.0352, respectively). Multivariate analysis showed that ERRα expression and GS are independent predictors ($p = 0.0367$ and 0.0264, respectively) among 4 parameters.

TABLE II – RELATIONSHIP OF ERRα EXPRESSION WITH CLINICOPATHOLOGICAL FINDINGS IN HUMAN PROSTATIC CANCER ($N = 106$)

	ERRα immunoreactivity[1]		*p*-value
	Weak ($n = 62$)	Strong ($n = 44$)	
Age	66.1 ± 5.8	67.4 ± 6.0	0.28
Serum PSA (ng/dl)	13.6 ± 13.0	20.5 ± 25.0	0.07
Gleason score			
2–7	43 (69.4)	20 (30.6)	0.0135
8–10	19 (44.2)	24 (55.8)	
Pathological Stage			
B, C	56 (60.9)	36 (39.1)	0.2
DI	6 (42.9)	8 (57.1)	

[1]Immunoreactivity (IR) score (0–8) was obtained as the sum of the proportion and the intensity of immunoreactivity. Proportion (0, none; 1, <1/100; 2, 1/100 to 1/10; 3, 1/10 to 1/3; 4, 1/3 to 2/3; and 5, >2/3), Intensity (0, none; 1, weak; 2, moderate; and 3, strong).IR score 0–4 and 5–8 were defined as weak and strong immunoreactivity, respectively.

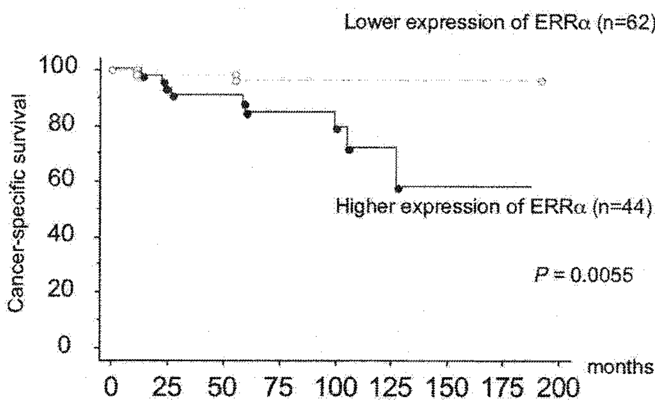

FIGURE 3 – Cancer-specific survival in 106 patients with PC according to the immunoreactivity of ERRα. Cancer-specific survival of patients with higher ERRα expression (IR score ≥ 5) was significantly worse than that of lower expression (IR score < 5) cases ($p = 0.0055$).

Discussion

Androgen deprivation and estrogen therapy have been the standard treatment for PC.[13,25,26] The growth-inhibitory effects of endocrine therapies are associated with the status of steroid receptors, such as the androgen receptor (AR) and estrogen receptor (ER).[25,26] The emergence of techniques to clone the orphan nuclear receptors in the 1980s prompted to investigate physiological functions of the orphan nuclear receptors in the targeted organs.[12,13] Among the orphan nuclear receptors, ERRα, β and γ, the three closely related members of the ERR family, all have functional links with the activities of the ERs. Two findings suggest that ERRα modulates the actions of the ERs. Firstly, ERRα shares a significant homology to ERα at the DNA-binding domain (DBD). Secondly, ERRα recognizes the ERE.[2,14,21,22,27,28] The ERRα gene is located on the long arm of chromosome 11.[29] Human ERRα was isolated from kidney and heart cDNA libraries by screening with an ERα-DBD cDNA probe.[11] ERRα mRNA was highly expressed in the heart and skeletal muscle and to a lesser degree in the kidney, pancreas, small intestine and colon.[29] Several investigations have implicated ERRα in the development in human breast cancer and colorectal cancer.[19,20,30] Further, ERRα mRNA has recently been detected in PC cell lines and human prostate tissues.[22] The present study is the first to reveal that ERRα expression is significantly higher in higher-GS cancer than in lower-GS cancer. These findings suggest that ERRα is involved in the normal and neoplastic growth of human prostate tissue.

Elevated ERRα expression has been identified as a poor prognostic factor in human breast cancer,[19,20] and has been reported to be correlated with higher histological grade and TNM stage of colorectal cancer.[30] However, there are conflicting data as to the ERRα expression in human prostate tissues. We found increased ERRα expression in PC, compared with that in benign epithelium. The previous study summarized the data and concluded that ERR proteins were detected as nuclear proteins in epithelial cells, whereas their expression became reduced in neoplastic prostate cells.[22] However, close interpretation of the results may suggest that these findings are more prominent in ERRβ expression, whereas ERRα rather expressed variably in PC on both the cell lines and human tissues. A figure of immunohistochemical analysis in the previous study demonstrated heterogeneous stain of ERRα in a low Gleason grade cancer, as the legend indicated.[22] Although the previous study did not identify number of cases examined with immunohistochemistry, we believed that our results obtained from 106 surgically resected prostate samples clearly demonstrate ERRα expression status in human prostate tissues and its potential significance in the cancer development. Of course, further investigations are warranted, and may let us gain better understanding on this interesting matter.

The present study suggests that ERRα participates in the regulation of PC besides ERs (α, β and βcx). How do these receptors correlate with the development of PC? We make two possible hypotheses. One is a functional cross-talk between ERRα and ERs in endocrine cancers in which ER exists. The binding of ERRα to several functional EREs has prompted speculation that ERRα can modify the ER function by either forming ER-ERRα heterodimer, by competing with ER for binding to ERE, or ste-

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TABLE III – UNIVARIATE AND INULTIVARIATE PROPORTIONAL HAZARD ANALYSES OF CANCER-SPECIFIC SURVIVAL ($N = 106$)

Variable	Univariate			Multivariate		
	Hazard ratio	95% index	*p*-value	Hazard ratio	95% index	*p*-value
PSA (>10 vs. ≤10)	0.79	0.23–2.8	0.73	0.47	0.13–1.76	0.26
Gleason score (High vs. Low)[1]	2.63	1.77–108.4	0.0123	11.3	1.33–95.7	0.0264
Pathological stage (DI vs. B, C)	3.77	1.01–12.9	0.0352	1.37	0.36–5.26	0.64
ERRα (Strong vs Weak)[2]	6.84	1.47–31.8	0.0141	5.24	1.11–25.7	0.0367

[1]High Gleason score: 8–10, low: 2–7.–[2]IR score 0–4 and 5–8 were defined as weak and strong immunoreactivity, respectively.

roid receptor co-activators (SRCs).[12–14] Far-Western analysis[14] and glutathione S-transferase pull-down assays have demonstrated direct interactions between ERα and ERRα.[31] Another hypothesis is that these ERs and ERRα receptors act independently. ERα and ERRα separately modulate pS2 expression in human breast cancer.[20] As we previously assessed the expression of ERs (α/ β/ βcx) in fifty patients with PC and showed their clinical significance,[2] we estimated the correlation of ERRα with ERs (α/ β/ βcx) in overlapping fifty cases. Subsequently, no significant correlations among them were identified (R^2: 0.004 for ERα vs. ERRα, R^2: 0.006 for ERβ vs. ERRα and R^2: 0.241 for ERβcx vs. ERRα). Interestingly, similar findings were reported in breast cancer,[20]and it was suggested that ERRα might modulate the activity of estrogen responsive genes, independently of ERs. Therefore, estrogen-signaling pathway via ERs (α, β and βcx), and ERRα is so complicated that we can not reach a conclusion on the basis of current findings.

Although several investigations proved that ERRα activate a variety of estrogen target genes such as pS2, aromatase and osteopontin (OPN) and play important roles in some target organs,[13] the mechanism of ERRα in PC development remains unclear at this time. For example, ERRα regulates the expression of pS2, which increase breast cancer growth.[15,20] ERRα also activates aromatase in breast, HepG2 and bone cells.[15] Aromatase, which converts androgens to estrogens, stimulates the growth of breast cancer.[32] Recent studies focus on the surveillance about anti-aromatase effects on breast cancer.[32] Aromatase is also well known to have an important role in endocrine metabolism at the prostate. Interestingly aromatase was expressed and active in LNCaP, PC-3 and DU145 cells, whereas benign prostate epithelial cells showed no expression or activity.[33] It is not demystified how over expression of aromatase in PC correlate with PC development similar to that in breast cancer[32] because estrogens are generally considered to have protective effects on PC progressions. The local synthesis of estrogens via aromatase enzyme might contribute to the estrogen-signaling pathway through ERs in PC. In addition, OPN participates in prostate biology. For instance, elevated OPN expression is found in PC both the cell lines and human tissues.[34] Increased expression of OPN is associated with Gleason score and poor cancer-specific survival.[34] These findings suggest that further investigations are needed to verify whether the ERRα-mediated effects on PC correlate with the activation of estrogen responsive genes.

Selective estrogen receptor modulators (SERMs) are synthetic estrogen ligands that can exhibit either estrogenic or anti-estrogenic effects depending on tissue types.[35] SERMs such as tamoxifene (TAM), 4-Hydroxytamoxifen (4-OHT) and raloxifene can induce apoptosis in PC cell lines such as PC-3, DU145 and LNCaP.[36–39] Toremifene treatment significantly reduces the incidence of PC in the transgenic adenocarcinoma of mouse prostate (TRAMP) mice.[40] Toremifene also reduces the incidence of PC in high-grade intraepithelial neoplasia (PIN) patients.[41] It may be that the anti-proliferate effects of SERMs on PC are mediated via ERs due to their positive expression in the prostate. Diethylstilbestrol (DES), an agent widely used for the treatment of advanced PC, represses the molecular activities of ERRα such as reporter gene trans-activation and interaction with co-activator fragments.[42] Thus, the SERMs might inhibit PC by modulating the estrogen-signaling pathway via ERRs besides the ERs. The prognostic value of enhanced ERRα expression as an independent predictor of PC suggests that an ERRα antagonist capable of specifically blocking ERRα activity may prove useful as a therapeutic agent against PC.

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ORIGINAL ARTICLE

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

1

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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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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

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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,

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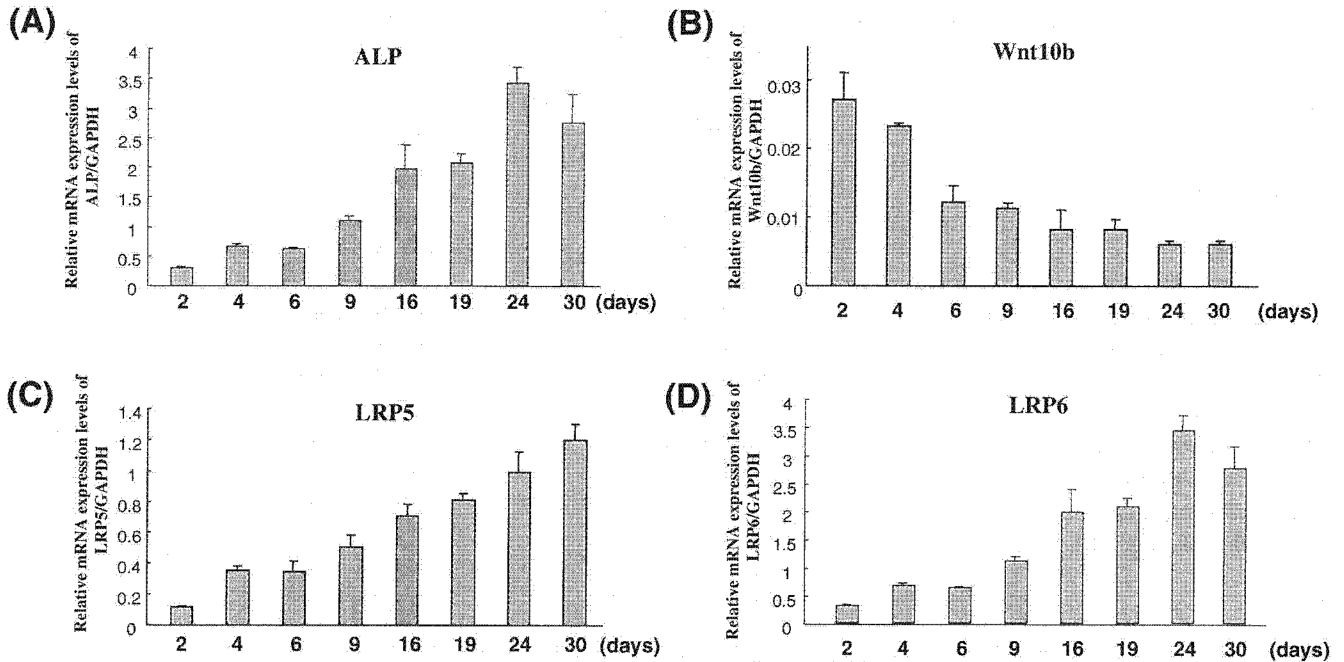

Figure 1 Expressions of the alkaline phosphatase (ALP), Wnt10b, lipoprotein receptor-related protein (LRP)5 and LRP6 mRNA during culture course of rat primary osteoblasts were analyzed by real-time reverse transcription polymerase chain reaction (RT-PCR). Rat primary osteoblasts were cultured with α-minimum 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.

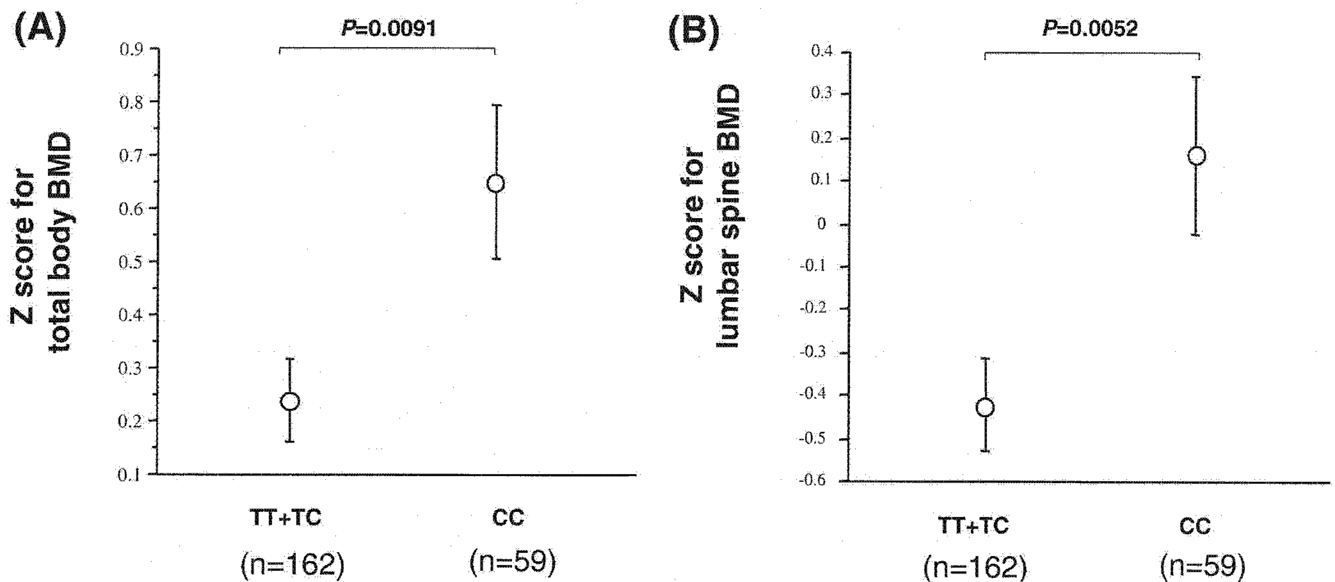

Figure 2 "Z score" values of total body and lumbar spine bone mineral density (BMD) in the groups of genotype TT + TC and genotype CC of the Wnt10b polymorphism in exon 6 (1059C/T). (A) Z score values for total BMD are shown for genotype TT + TC and for genotype CC. Values are expressed as mean ± SE.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

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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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Association of a single nucleotide polymorphism in the steroid and xenobiotic receptor (SXR) gene (IVS1-579A/G) with bone mineral density

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Running title
A SXR SNP associated with BMD

Abstract

Background: Vitamin K2 plays an important role in the bone metabolism. The SXR as a nuclear receptor activated by vitamin K2 as well as rifampicin could increase bone markers such as alkaline phosphatase in human osteoblastic cells. Thus, the SXR could mediate vitamin K2 signaling pathway in bone cells. Therefore, we analyzed expression of the SXR mRNA in human primary osteoblasts and chondrocytes. We also studied association of a single nucleotide polymorphism (SNP) in the SXR gene with bone mineral density (BMD).

Methods: Expression levels of the SXR mRNA were analyzed during the culture course of human primary osteoblasts and chondrocytes. Association of a SNP in the SXR gene in intron 1 (IVS1-579A>G) with BMD was examined in 294 healthy postmenopausal Japanese women.

Results: The SXR mRNA increased at day 5 and then decreased at day 10 in human primary osteoblasts. Its mRNA gradually increased in human primary chondrocytes until day 10. As an association study of a SNP in the SXR gene (IVS1-579A/G), the subjects without the A allele (GG; n=47) had significantly higher total BMD than the subjects bearing at least one A allele (AA + AG; n=247) (Z score $\pm$ SD; 0.635 $\pm$ 1.031 vs 0.268 $\pm$ 1.061; P=0.0298).

Conclusion: The SXR mRNA was expressed and regulated in primary human osteoblasts and chondrocytes. A genetic variation at the SXR gene locus is associated with BMD, suggesting an involvement of the SXR gene in human bone metabolism.

Key Words

Single nucleotide polymorphism (SNP), bone mineral density (BMD), osteoporosis, SXR, vitamin K2

Introduction

Osteoporosis is a skeletal disorder characterized by compromised bone strength predisposing to an increased risk of fracture (1). Twin and sibling studies have shown that bone mineral density (BMD) is under genetic control with estimates of heritability ranging from 50% to 90% (2-4). BMD is assumed to be determined by multiple genes with modest effects on bone mass and bone turnover as well as by environmental factors (5, 6). To date various polymorphisms of candidate genes have been investigated in relation to BMD (7, 8). These include vitamin D receptor (VDR) gene (9), estrogen receptor α (ERα) gene (10), collagen type Iα1 (COLIA1) gene (11) and low density lipoprotein receptor-related protein 5 (LRP5) gene (12). 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.

Vitamin K exerts an influence on the bone metabolism and is used as an anti-osteoporosis drug in Japan (13). Moreover, vitamin K intake has been found to be associated with decrease of hip fracture risk (14). In the bone homeostasis, a mechanism of vitamin K action is mediated through posttranslation modification of proteins (15). Vitamin K functions as an essential cofactor for carboxylation of glutamic acid residues to gammacarboxyglutamic acid residues. Recently, a novel mechanism was uncovered in the signaling that regulates the transcription of target genes by vitamin K through activation of a nuclear receptor, steroid and xenobiotic receptor (SXR: also known as PXR and NR1I2) (16). In the report, vitamin K2 was shown to bind to and activate the SXR that could induce bone markers such as alkaline phosphatase (ALP) and osteoprotegerin in the human osteoblastic cells (16). Therefore, the SXR could be involved in the maintenance of bone homeostasis. In the present study, we examined the expression of the SXR in human primary osteoblasts and chondrocytes and the association between a polymorphism in the SXR gene and BMD in Japanese women to investigate possible contribution of the SXR in human bone metabolism.

Materials and methods

Cell culture

Primary human osteoblasts and chondrocytes were purchased from Cambrex (MD). Primary human osteoblasts were cultured 6-cm dishes in the OGM medium (Cambrex) supplemented with SingleQuots for OGM, ascorbic acid and β-glycerophosphate for 2, 5, or 10 days according to the manufacturer's recommended protocol. Primary human chondrocytes were cultured 6-cm dishes in the CDBM medium (Cambrex) supplemented with SingleQuots for CDBM (including IGF-1, TGFβ1, insulin, transferrin and FBS) for 2, 5, or 10 days according to the manufacturer's recommended protocol.

Total RNA isolation and cDNA synthesis

Total RNAs were extracted from the cells using a ToTALLY RNA Kit (Ambion, TX). cDNA was synthesized from 1 μg of total RNA of primary osteoblasts using first strand cDNA synthesis kit (Amersham, IL).

SYBR Green real time PCR

Primers were designed using PRIMER EXPRESS 1.0 software (Applied Biosystems, CA). Definitive primers were: human GAPDH forward 5' - TGGACCTCATGGCCCACA- 3', reverse 5' -TCAAGGGGTCTACATGGCAA- 3', human alkaline phosphatase (ALP) forward 5'-TCCCACGTCTTCACATTTGGT-3', reverse 5'-AAGGGCTTCTTGTCTGTGTCACT-3', human collagen type II alpha 1 (COL2A1) forward 5'-TTGCCTATCTGGACGAAGCA-3', reverse 5'-CGTCATTGGAGCCCTGGAT-3' and human SXR forward 5'-ACTGCCTTTACTTCAGTGGGAATC-3', reverse 5'-ATTCTCTTGCTTTTCTCACTGTGAAC-3'. Quantitative PCR was carried out using a 2 x master mix composed from the SYBR Green PCR Core Reagents (Applied Biosystems) and 50 nM 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 sec at 95°C and 1 min at 60°C. ALP, COL2A1 or SXR signal was normalized to GAPDH signal.

Subjects

Genotypes were analyzed in DNA samples obtained from 294 healthy postmenopausal Japanese women (mean age ± SD; 65.5 ± 8.9). Exclusion criteria included endocrine disorders such as hyperthyroidism, hyperparathyroidism, diabetes mellitus, liver disease, renal disease, use of medications known to affect bone metabolism (e.g. corticosteroids, anticonvulsants, heparin sodium), or unusual gynecologic history. All were non-related volunteers and provided informed consent before this study. Ethical approval for the study was obtained from appropriate ethics committees.

Measurement of BMD and biochemical markers

The lumbar-spine BMD and total body BMD (in g/cm^2) of each participant were measured by dual-energy X-ray absorptiometry using fast-scan mode (DPX-L; Lunar, Madison, WI). We measured serum concentration of calcium (Ca), alkaline phosphatase (ALP), intact-osteocalcin (I-OC, ELISA; Teijin, Tokyo, Japan), intact parathyroid hormone (PTH), calcitonin (CT) and 1, 25(OH)2D3. We also measured urinary ratios of urinary deoxypyridinoline (DPD, HPLC 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 SXR gene

We extracted a polymorphic variation in the SXR gene intron 1 region from the Assays-on-Demand SNP Genotyping Products database (Applied Biosystems, Foster City, CA) and, according to its localization on the gene, denoted it IVS1-579A/G. We determined the IVS1-579A/G polymorphism of the SXR gene using the TaqMan (Applied Biosystems) polymerase chain reaction (PCR) method (17). To determine the SXR SNP we used Assays-on-Demand SNP Genotyping Products C_1834250_10 (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 A-allele and the group with only G-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

The SXR mRNA expression is regulated during the course of primary osteoblasts and chondrocytes differentiation

At the inception of this study, we measured the SXR mRNA levels during the course of differentiation in human primary osteoblasts and chondrocytes. 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 (18, 19). The continual maturation of the osteoblasts was reflected by the increase of ALP mRNA (Fig 1A). The SXR mRNA increased at day 5 and then decreased at day 10 in human primary osteoblasts (Fig 1C). In the presence of insulin and transferrin, primary chondrocytes proceed to differentiation normally (20, 21) and the continual maturation of the chondrocytes was reflected by the increase of COL2A mRNA (Fig 1B). The SXR mRNA gradually increased in human primary chondrocytes until day 10 (Fig 1C).

Association of the SXR gene polymorphism in intron 1 with BMD

We examined a SXR polymorphism at intron 1 (IVS1-579A>G) in Japanese women, using the TaqMan methods. Among 294 postmenopausal volunteers, 112 were AA homozygotes, 135 were AG heterozygotes, and 47 were GG homozygotes.

We compared Z scores for BMD of total body and lumbar spine between the subjects bearing at least one A allele (AA + AG) and subjects without the A allele (GG). Comparison of the Z scores of the lumbar BMD between those with and without A allele showed a higher average value for GG homozygote group, but its difference was not statistically significant (Z score; 0.083 ± 1.547 vs -0.224 ± 1.475; P=0.195) (Fig 2B). On the other hand, Z score of the total body BMD in GG homozygote group was significantly higher than the other group (Z score; 0.635 ± 1.031 vs 0.268 ± 1.061; P=0.0298) (Fig 2A). The background and biochemical data were not statistically different between these two groups (Table1).

Discussion

The nuclear receptor SXR (also known as PXR and NR1I2) plays a central role in the transcriptional regulation of xenobiotic detoxifying enzymes and transporters such as CYP3A4 and MDR1 (22-24). The SXR is activated by a diverse array of

pharmaceutical agents, including Taxol, rifampicin, SR12813, clotrizole, phenobarbital, hyperforin, the herbal antidepressant St. John's wort, and peptide mimetic human immunodeficiency virus protease inhibitors such as ritonavir (25-27). Recently it was shown that vitamin K2 was a novel ligand for the SXR and could induce bone marker genes through the SXR (16). The SXR is a member of nuclear receptor NR1I subfamily. The vitamin D receptor (VDR, NR1I1) is a close relative of the SXR in terms of amino acid sequence similarity and belongs to the same subfamily (22). Many reports showed that the VDR is expressed and regulated in the bone cells and VDR gene allelic variants could predict bone mineral density (28). These data prompted us that the SXR may have a role in the bone homeostasis, especially in osteoporosis, as like other NR1I subfamily members.

In the present study, during the course of primary osteoblast differentiation, the increase of ALP expression, taht is a marker of osteoblast differentiation (29) was followed by the increase of the SXR expression. Recent report also demonstrated that the SXR expression was detected in human osteosarcoma cell lines HOS, MG-63, and SaOS2 (16). Interestingly, the vitamin K2, one of the ligands for the SXR, up-regulated the steady state mRNA levels for a panel of osteoblastic bone markers including ALP in these cells. Thus, it is possible that the SXR is involved in the differentiation of osteoblasts and the regulation of ALP gene. We have shown that the SXR expression was detected in human primary chondrocytes as well as in primary osteoblasts and increased in parallel with the increased expression of COL2A, which is a marker of chondrocyte differentiation (20). It is also possible that the SXR is involved in the cartilage metabolism. Future studies should be required how vitamin K and the SXR signaling could be delivered to the regulation of skeletal differentiation.

To our knowledge, the present study is the first to investigate the influence of a polymorphism of the SXR gene on the BMD. We demonstrated that the Japanese postmenopausal women who had two alleles of an intronic change of A-G transition showed significantly higher total-body BMD. Lumbar BMD was also higher in the subjects bearing at two G alleles, although the difference was not statistically significant. Lower BMD in postmenopausal women can be considered as a result of abnormally rapid bone loss and/or lower peak bone mass. The SNP analyzed in this study would be useful as a genetic marker for low BMD and the susceptibility to osteoporosis. Although the biological meanings of this polymorphism should be revealed by functional studies, the SXR IVS1-579A/G polymorphism may modulate BMD by influencing transcription and/or expression levels of the SXR.

In conclusion, our findings suggest that the SXR gene may be a genetic determinant of BMD in postmenopausal women as is the case with its related nuclear receptor, VDR. Examining the variation in the SXR 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 SXR have been clearly implicated, including lipid and drug metabolisms (22, 24).

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Figure legend

Figure 1. Expressions of the ALP, COL2A1 and SXR mRNA during culture course of human primaty osteoblasts and chondrocytes were analyzed by real time RT-PCR. Human primary osteoblasts and chondrocytes were cultured with appropriate medium described in "Materials and methods" up to 10 days. At the indicated time, RNA was extracted and the expression levels of the ALP (A), COL2A1 (B) and SXR (C) were analyzed by real time PCR, normalized to GAPDH expression (n=4 for each group). Values are shown by means ± SD.

Figure 2. Z score values of total body and lumbar spine BMD in the groups of genotype AA+ AG and genotype GG of the SXR polymorphism in itoron 1 (IVS1-579). (A) Z score values for total BMD are shown for genotype AA+ AG and for genotype GG. Values are expressed as mean ± SE. Number of subjects is shown in parentheses. (B) Z scores for lumbar BMD as shown in the same manner as (A).

Table 1. Comparison of background, BMD and biochemical data between subjects bearing at least one A allele (AA + GA) and subjects with no A allele (GG) in the SXR gene (IVS1-579A/G).

Items	Genotype (mean ± SD)		*P* value
	AA+AG	GG	
Number of subjects	247	47	
Age (years)	65.2 ± 9.0	66.7 ± 8.7	NS
Height (cm)	150.8 ± 6.5	151.6 ± 5.4	NS
Body weight (kg)	50.5 ± 8.1	51.5 ± 7.7	NS
Lumber spine BMD (Z score)	-0.224 ± 1.475	0.083 ± 1.547	NS
Total body BMD (Z score)	0.268 ± 1.061	0.635 ± 1.031	0.0298
ALP (IU/L)	190.9 ± 62.7	177.3 ± 57.8	NS
I-OC (ng/mL)	8.3 ± 4.2	7.5 ± 3.1	NS
DPD (pmol/μmol Cr)	7.8 ± 4.4	6.8 ± 2.5	NS
Intact PTH (pg/mL)	34.7 ± 16.8	33.7 ± 8.6	NS
Calcitonin (pg/mL)	22.4 ± 10.4	20.7 ± 14.7	NS
1,25 $(OH)_2D_3$ (pg/ mL)	35.1 ± 11.3	34.5 ± 10.3	NS
% fat	31.6 ± 8.1	32.6 ± 6.2	NS
BMI	22.1 ± 3.2	22.4 ± 3.1	NS

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