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JOURNAL OF BONE AND MINERAL RESEARCH
Volume 18, Number 9, 2003

Association of Molecular Variants, Haplotypes, and Linkage Disequilibrium Within the Human *Vitamin D–Binding Protein* (*DBP*) Gene With Postmenopausal Bone Mineral Density

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ABSTRACT

Possible contribution of *vitamin D–binding protein* (*DBP*) gene for determination of BMD was tested by characterizing 13 SNPs in 384 adult Japanese women. When the effect of a specific single SNP was tested, five SNPs (−39C>T, IVS1+827C>T, IVS1+1916C>T, IVS1–1154A>G, and IVS11+1097G>C) correlated with BMD significantly at various levels. The chromosomal dosage of one haplotype (T-C-C-G-T-C in −39C>T, IVS1+827C>T, IVS1+1916C>T, IVS1–1154A>G, D432E, and IVS11+1097G>C) displayed significant correlation with adjusted radial BMD ($r = 0.15$, $p = 0.008$; $n = 331$). Multiple regression analyses revealed a most significant correlation with the combination of IVS1+827C>T and D432E ($r^2 = 0.029$, $p = 0.005$). These results indicate a complex combined effect of several SNPs within the *DBP* gene that might underlie susceptibility to low radial BMD and osteoporosis.

Introduction: Osteoporosis results from the interplay of multiple environmental and genetic determinants. The gene encoding vitamin D–binding protein (*DBP*), a key factor for regulating calcium homeostasis through the vitamin D endocrine system, is a probable candidate for conferring susceptibility to osteoporosis.
Methods: To test a possible contribution of the *DBP* gene for determination of bone mineral density (BMD) of adult women, we have characterized 13 single nucleotide polymorphisms (SNPs) within the *DBP* gene in DNA from 384 adult Japanese women and attempted to correlate specific SNPs with BMD.
Results and Conclusions: Sixteen major haplotypes accounted for 80% of the variations, indicating allelic complexity in this genomic region. Pairwise linkage disequilibrium (LD), measured by the D' and r^2 statistics, demonstrated a general pattern of decline with increasing distance, but individual LD values within small genomic segments were diverse. Regression analysis for adjusted BMD revealed significant correlation with respect to five of them (−39C>T, IVS1+827C>T, IVS1+1916C>T, IVS1–1154A>G, and IVS11+1097G>C) at various levels. An intronic SNP (IVS11+1097G>C) with the highest significance of association ($p = 0.006$) showed significant LD with four SNPs located around the first exon (r^2 values >0.18, $D' > 0.5$). A non-synonymous coding SNP, D432E, showed a comparable level of correlation, but it was in a moderate LD only with IVS11+1097G>C. The chromosomal dosage of one haplotype (T-C-C-G-T-C in −39C>T, IVS1+827C>T, IVS1+1916C>T, IVS1–1154A>G, D432E and IVS11+1097G>C) estimated in each subject displayed significant correlation with adjusted radial BMD ($r = 0.15$, $p = 0.008$; $n = 331$). Furthermore, multiple regression analyses revealed that the most significant correlation was achieved for the combination of IVS1+827C>T and D432E ($r^2 = 0.029$, $p = 0.005$).

The authors have no conflict of interest.

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These results indicate a complex combined effect of several SNPs within the *DBP* gene that might underlie susceptibility to low radial BMD and osteoporosis.

J Bone Miner Res 2003;18:1642–1649

Key words: single nucleotide polymorphism, vitamin D–binding protein, group-specific component, group-specific component of globulin, bone mineral density, association study, quantitative trait

INTRODUCTION

OSTEOPOROSIS IS A COMMON, multifactorial disease characterized by reduced bone mass, microarchitectual deterioration of bone tissue, and increased risk of fragility fractures. A number of twin studies and family studies have pointed to a strong genetic component, including factors that regulate bone mineral density (BMD) or factors that determine bone geometry.[1,2] Achievement of peak bone mass, as well as postmenopausal maintenance of skeletal homeostasis, is essential for soundness of skeletal bones in elderly individuals. A part of this developmental or homeostatic regulation is accomplished through control of systemic calcium levels, with the skeletal system serving as a calcium reservoir. Thus, a deficiency in this regulation should result in bone mineral loss and increased risk of osteoporosis.

The systemic level of calcium is regulated mainly through a feedback system involving parathyroid hormone and the vitamin D endocrine system.[3–5] Enzymatic activation/inactivation of hormones and competitive binding activities between receptors or transporters and their ligands are the major mechanisms at work.[4–6] Parathyroid hormone, calcitonin, receptors of these ligands, and calcium-sensing receptor have been analyzed in terms of contributions to predisposition to osteoporosis.[1,2,7–9] However, molecules more directly involved in the vitamin D endocrine system must be tested, because calcitriol (1,25-dihydroxyvitamin D_3), the most potent of the active metabolites of vitamin D, is the main effector of this system. For example, a nuclear variant of the vitamin D receptor (VDR) has been investigated intensely since the early 1990s.[10] Vitamin D–binding protein (DBP, also known as group-specific component of globulin [Gc]) is another example, although to date its significance has not been sufficiently defined.[11,12] DBP, a key regulator of the vitamin D endocrine system, is a member of the albumin family of gene products. In serum, DBP stores the preactivated 25-hydroxyvitamin D and other metabolites by directly binding to them[4,13] and transports them to the kidney to be metabolized through additional hydroxylation.[4,13,14] The importance of DBP for the skeletal system has been confirmed by targeted disruption of the *megalin* gene in mice, which encodes an endocytic renal receptor of multiple gene products. This receptor takes up DBP-bound vitamin D in urine for reabsorption into epithelial cells in proximal renal tubules.[15] In vitro studies have suggested that DBP may also mediate differentiation of hematopoietic cells and generate osteoclasts directly.[14,16,17] In view of these features, we considered the *DBP* gene to be a highly likely candidate for involvement in susceptibility to osteoporosis. In the work reported here, we examined the potential involvement of the *DBP* gene in the pathogenesis of osteoporosis by investigating genetic variations, constructing haplotypes, analyzing linkage disequilibrium among the variations, and carrying out multiple regression analyses to examine possible associations with BMD in 384 adult women.

MATERIALS AND METHODS

Subjects

DNA samples were obtained from peripheral blood of 384 adult Japanese women. Clinical data included questions on medical history, including medication, and a survey of the incidence of diseases. Mean ages and body mass indices (body mass index [BMI]) with SDs were, respectively, 58.4 ± 8.6 years (range, 32–69 years) and 23.7 ± 3.61 kg/m^2 (range, 14.7–38.5 kg/m^2). The BMD of radial bone (g/cm^2) of each participant was measured by DXA using a DTX-200 Osteometer (Meditech Inc., Hawthorne, CA, USA). To calculate adjusted BMD, the measured BMD was normalized for differences in age, height, and weight, using the Instat 3 software package (GraphPad Software, San Diego, CA, USA) and multiple regression analysis.[18,19] The adjustment equation for the study samples was as follows: [adjusted BMD (g/cm^2)] = [measured BMD (g/cm^2)] − 0.006375 × {58.39 − [age (years)]} + 0.008961 × {23.65 − [BMI (kg/cm^2)]}. BMD in the radius was measured according to the Guidelines for Osteoporosis Screening established for a health check-up program in Japan.[20] All subjects were nonrelated volunteers who provided written informed consent. 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 Institutional Review Board approved the study.

Selection of 13 SNPs at the DBP *locus*

Thirteen polymorphic variations in the *DBP* gene were extracted from either the JSNP database (http://snp.ims.u-tokyo.ac.jp/index.html) or the NCBI dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/). The 13 selected SNPs (Table 1) included two non-synonymous substitutions in exon 11 (rs7041 and rs4588 on dbSNP) that respectively replace nucleotide +1295 thymidine with guanosine (nt+1295T/G; D432E) and +1308 cytidine with adenosine (nt+1308C/A; T436K). Combinations of these sites are the traditionally denoted variants Gc1s, Gc1f, and Gc2 that were originally diagnosed by altered electrophoretic mobility of the protein on agarose.[21] To identify further an

TABLE 1. SUMMARY OF POLYMORPHISMS ANALYZED AT THE DBP LOCUS

No.	*Name*	*nt.*	*Location*	*JSNP-ID**	*dbSNP†*
1	−39C > T	C/T	promoter	IMS-JST087014	—
2	IVS1 + 827C > T	C/T	Intron1	IMS-JST054016	rs2298849
3	IVS1 + 1916C > T	C/T	Intron1	IMS-JST017158	rs1352845
4	IVS1 − 1155A > G	A/G	Intron1	IMS-JST017159	rs222020
5	IVS3 − 1283C > T	C/T	Intron3	IMS-JST130682	rs705124
6	IVS3 − 308C > T	C/T	Intron3	IMS-JST082799	—
7	C299C	T/C	Exon8(+896)	IMS-JST082800	rs4752
8	D432E	T/G	Exon11(+1296)	IMS-JST082801	rs7041
9	T436K	C/A	Exon11(+1307)	IMS-JST082802	rs72681
10	C445R	C/T	Exon11(+1333)	IMS-JST082803	—
11	IVS11 + 1097G > C	G/C	Intron11	IMS-JST130685	—
12	IVS11 + 1303C > T	C/T	Intron11	IMS-JST130686	—
13	IVS11 − 2344C > G	C/G	Intron11	IMS-JST054017	rs229850

* Number from Japanese SNP database (http://snp.ims.u-tokyo.ac.jp/index_ja.html).
† Number from dbSNP database of NCBI (http://www.ncbi.nlm.nih.gov/SNP/).

uncharacterized polymorphism in the promoter sequence of the *DBP* gene, 2-kb resequencing of the DNA from 12 selected subjects (24 chromosomes) were conducted, using the ABI Prism BigDye Terminator system. However, there were no uncharacterized SNPs whose minor allele frequencies are greater than 5% in our test subjects. Motifs for binding sites of transcription factors were sought by means of MatInspector V2.2 software (http://transfac.gbf.de/cgi-bin/matSearch/matsearch.pl) based on the TRANSFAC4.0 database.[22]

Genotyping

All 384 participants were genotyped for the 13 SNPs using the SNP-dependent polymerase chain reaction (Sd-PCR) method, an innovative allele-specific PCR procedure that we had previously developed for discrimination between polymorphic sequences.[19] In brief, two allele-specific primers (AS-primers) and one reverse primer are prepared for each SNP. AS-primers (long and short) each have a five-base difference between them; each has a polymorphic nucleotide of the SNP sequence at its 3′ end and an additional artificial mismatch is introduced near the 3′ end. These primer sets allow distinct discrimination of alleles. The Sd-PCR reaction was carried out using 10 ng of genomic DNA and 250 nM of each primer (two polymorphic forward and a reverse) in a 10-μl reaction mixture containing 10 mM dNTPs, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, 1 U *Taq*DNA polymerase, and 0.5 mM fluorescence-labeled dCTP (ROX-dCTP; Perkin-Elmer, Norwalk, CT, USA) on a thermal cycler (Gene-amp system 9600; Perkin-Elmer) with initial denaturation at 94°C for 4 minutes, followed by 5 cycles of stringent amplification (94°C for 20 s, 64°C for 20 s, 72°C for 20 s) and then 25 cycles of 94°C for 20 s, 62°C for 20 s, 72°C for 20 s), terminating with a 2-minute extension at 72°C. Discrimination of alleles was achieved by electrophoresis and laser scanning of the DNA fragments on the ABI Prism 377 DNA system using GeneScan Analysis Software ver2.1 (Applied Biosystems, Foster City, CA, USA). To confirm the accuracy of the Sd-PCR method, we carried out direct resequencing using the ABI Prism BigDye Terminator system (Applied Biosystems).

Determination of haplotype frequencies, tests of linkage disequilibrium, and association studies

Haplotype construction and calculation of haplotype frequencies among the 768 alleles investigated were calculated by Arlequin software (Genetics and Biometry Laboratory, Geneva, Switzerland)[23] or by SNPAlyze v2.0 (DYNACOM Co., Ltd., Chiba, Japan) based on EM algorithm and a maximum likelihood approach. The duplotype of each subject was estimated by the SNPAlyze v2.0. We tested linkage disequilibrium of all possible two-way combinations of SNPs with the absolute value of the correlation coefficient, using several widely used methods (D, D', and r^2).[24,25] Quantitative associations between genotypes and adjusted BMD values (g/cm^2) were analyzed using Instat 3 software (GraphPad Software) through ANOVA, with regression analysis as a post-hoc test. The three genotypic categories of each SNP (e.g., T/T, T/G, and G/G for rs7041, D432E = +1296T>G) were converted into incremental values 0, 1, and 2 respectively. These values correspond to the number of chromosomes possessing a minor allele of the SNP. Significant association was defined when the given p value of the ANOVA f test was less than 5% ($p < 0.05$). Dominant effects were tested by the Student's t-test ($p < 0.05$); χ^2 tests ascertained Hardy-Weinberg equilibrium among genotypes ($p > 0.05$).

RESULTS

Molecular variants, haplotypes, and LD

To characterize genetic variations and LD structure within the *DBP* locus, we genotyped 13 SNPs in a total of 768 chromosomes from 384 postmenopausal Japanese women. Distributions of genotypes and allele frequencies are given in Table 2. The observed allelic frequencies at each SNP site were in Hardy-Weinberg equilibrium. We

TABLE 2. SUMMARY OF CORRELATION ANALYSIS

No.	*Name*	*nt.*	*Allele frequency (heterozygosity)*	n*	*Correlation coefficient (r)*†	p *Value*
1	−39C > T	C/T	0.69:0.31 (38%)	381	0.102	0.047‡
2	IVS1 + 827C > T	C/T	0.69:0.31 (38%)	365	0.132	0.019‡
3	IVS1 + 1916C > T	C/T	0.69:0.31 (37%)	365	0.088	0.011‡
4	IVS1 − 1155A > G	A/G	0.70:0.30 (38%)	371	0.060	0.043‡
5	IVS3 − 1283C > T	C/T	0.82:0.18 (28%)	346	0.026	0.626
6	IVS3 − 308C > T	C/T	0.87:0.13 (24%)	329	0.080	0.145
7	C299C	T/C	0.83:0.17 (27%)	362	0.066	0.213
8	D432E	T/G	0.76:0.24 (35%)	383	0.096	0.059
9	T436K	C/A	0.75:0.25 (40%)	368	0.013	0.807
10	C445R	C/T	0.96:0.04 (6.3%)	363	0.015	0.777
11	IVS11 + 1097G > C	G/C	0.82:0.12 (30%)	379	0.140	0.006§
12	IVS11 + 1303C > T	C/T	0.59:0.41 (50%)	375	0.065	0.059
13	IVS11 − 2344C > G	C/G	0.73:0.27 (43%)	358	0.013	0.923

* Number of genotyped subjects.
† Absolute values of correlation efficient.
‡ $p < 0.05$; § $p < 0.01$; p values are calculated for regression analysis with ANOVA F-test.

constructed haplotypes and calculated their frequencies using the Arlequin algorithm and the SNPAlyze v2.0. Although rare SNPs with minor-allele frequencies of less than 1% were excluded, both programs identified 16 distinct haplotypes among the 384 Japanese subjects; the frequencies of six of them were greater than 5%. The 16 major haplotypes accounted for approximately 80% of all alleles present in our study population (Fig. 1A).

Pair-wise LD, measured according to D' and r^2 statistics, was analyzed for all possible two-way comparisons between SNPs. When the extent of LD was evaluated, we saw a general pattern of decline with increasing distance, but individual LD values varied within any given small genomic region. Strong LD was verified among four SNPs localized around the first exon (−39C>T, IVS1+827C>T, IVS1+1916C>T, and IVS1−1154A>G) (Fig. 1B). We also detected relatively strong LD between those four SNPs and three others (C299C, IVS11+1097G>C, and IVS11+2344C>G).

Association of BMD with multiple SNPs in the DBP *gene*

To test whether specific genetic variants of the *DBP* gene might be involved in the pathogenesis of postmenopausal osteoporosis, we tested correlation between adjusted radial BMD and each of the 13 SNPs by ANOVA and linear regression. Five SNPs (39C>T, IVS1+827C>T, IVS1+1916C>T, IVS1−1154A>G, and IVS11+1097G>C) displayed significant associations with BMD (Table 2). Among them, IVS11+1097G>C in intron 11 showed the most significant correlation with radial BMD ($p = 0.006$; Fig. 2B). It also showed significant LD (r^2 values >0.18, $D' > 0.5$) with the four significantly correlated SNPs located around the first exon, as well as moderate LD with a non-synonymous coding SNP (D432E) that had shown a comparable level of correlation. No evidence was found for LD between D432E and the four SNPs around the first exon, indicating an independent association of D432E with BMD. Evidence for a dominant effect of the minor E allele was found with Student's t-test (E/E; 0.42 ± 0.003, $n = 26$, D/E and D/D; 0.40 ± 0.003, $n = 357$, $p = 0.02$). The correlation observed between IVS11+1097G>C and adjusted BMD may represent a combined effect on postmenopausal bone mass of multiple SNPs within the DBP locus. Multiple regression analyses were conducted to detect interactive effects among the SNPs. For every combination that was tested, each panel of two to four SNPs (e.g., −39C>T, IVS1+827 and D432E ($r^2 = 0.033$, $p = 0.008$) or IVS1+827, D432E, and IVS11+1097 ($r^2 = 0.035$, $p = 0.006$) displayed significant correlation with radial BMD in multiple regression analysis. However, the best combination for the lowest p value was obtained with the combination of IVS1+827 and D432E ($r^2 = 0.028$, $p = 0.005$). To evaluate the combined effects, haplotypes consisting of the five significantly correlated SNPs and one marginally correlated SNP were estimated by means of the SNPAlyze. Although we estimated 36 distinctive haplotypes in our subjects, 11 major haplotypes accounted for about 94% of the total (Fig. 2A). To analyze correlation between chromosomal dosage of a distinctive haplotype and adjusted radial BMD, a "duplotype" in each individual was estimated with the SNPAlyze v2.0. This analysis revealed a significant correlation between the genetic dose of one haplotype, T-C-C-G-T-C, and the adjusted BMD ($r = 0.15$, $p = 0.008$, $n = 331$; Fig. 2C).

DISCUSSION

In the study reported here, we investigated haplotypes and linkage disequilibrium among molecular variants of the *DBP* gene and looked for associations of these features with BMD among 384 postmenopausal Japanese women. We

A.

Haplotype No.	-30C>T	IVS1+827C>T	IVS1+1916C>T	IVS1-1155A>G	IVS3-1230C>T	IVS3-388C>T	C299C (T>C)	D432E (T>G)	T436K (C>A)	C445R	IVS11+1997G>C	IVS11+1360C>T	IVS11-2344C>G	Frequency
1	C	T	T	A	C	C	T	T	A	C	G	C	G	22.0%
2	C	T	T	A	G	C	T	G	C	C	G	T	C	15.4%
3	C	T	T	A	T	C	C	T	C	C	G	T	C	9.5%
4	T	C	C	G	C	C	T	T	C	C	G	C	C	6.1%
5	T	C	C	G	C	T	T	T	C	C	C	C	C	5.2%
6	T	C	C	G	C	C	T	T	C	C	C	C	C	5.0%
7	C	T	T	A	C	C	T	T	C	C	G	C	C	3.9%
8	T	C	C	G	C	C	T	G	C	C	G	T	C	2.7%
9	C	T	T	A	C	C	T	T	C	C	G	T	C	2.1%
10	C	T	T	A	T	C	C	T	C	3	G	T	C	2.0%
11	C	T	T	A	C	C	T	T	C	C	C	C	C	1.9%
12	C	T	T	A	C	C	T	T	C	C	G	C	G	1.4%
13	C	T	T	G	C	C	T	T	C	C	G	C	C	1.3%
14	T	C	C	G	C	T	T	T	A	C	G	C	G	1.1%
15	C	T	T	A	T	C	T	T	A	C	G	C	G	1.1%
16	T	C	T	A	C	T	T	T	C	C	C	C	C	1.0%

B.

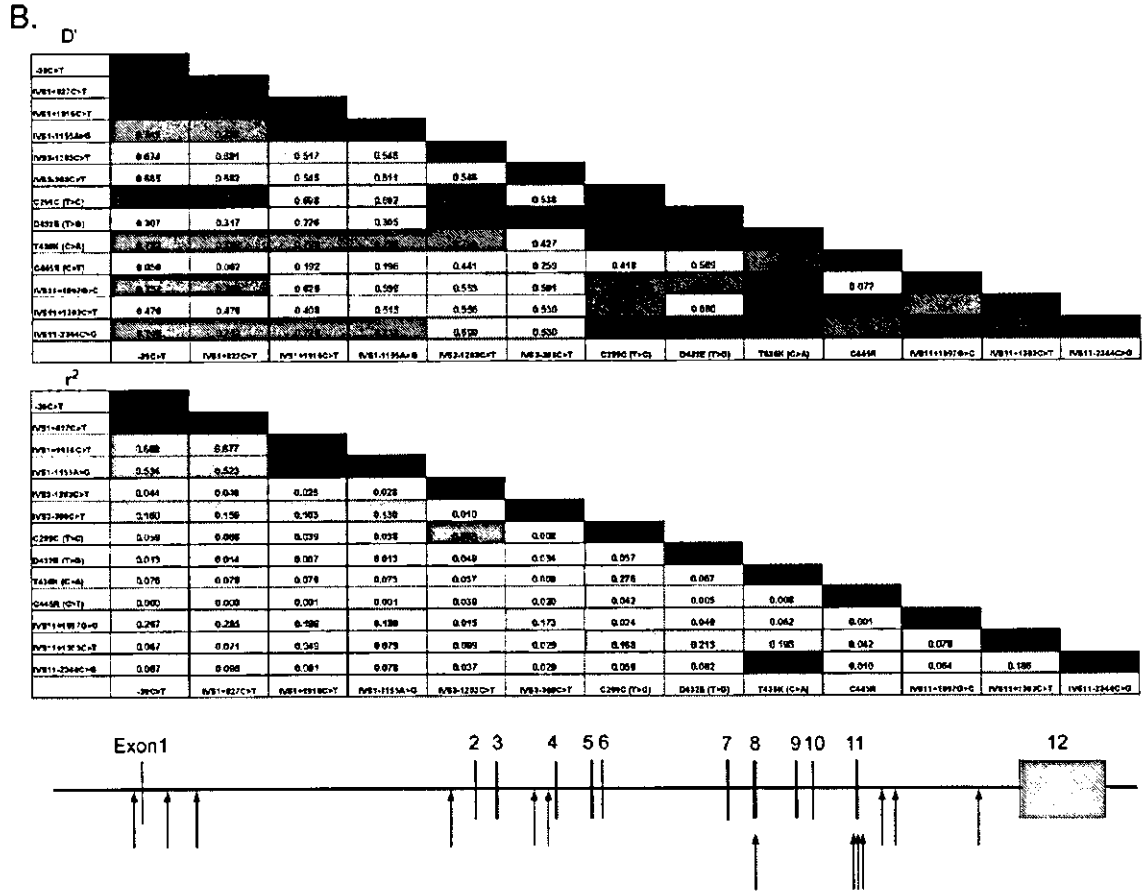

FIG. 1. Analysis of haplotypes and linkage disequilibrium among 13 SNPs at the *DBP* locus. (A) The 16 major predicted haplotypes, ordered according to predicted frequencies indicated in percentages in the second column from the right. The far right column indicates summed occupancy ratios; the sum of predicted frequencies of the 16 major haplotypes accounts for 79.4% of the entire sample. (B) Indices of linkage disequilibrium, D' and r^2, presented in separate tables. D' values greater than 0.4 and r^2 values greater than 0.1 are highlighted with gray halftone. The schematic diagram of the *DBP* gene structure below indicates locations of the 13 tested SNPs by upward arrows.

considered DBP to be a likely candidate for susceptibility to osteoporosis because it is a key regulator of calcium by way of vitamin D endocrine system. It binds to 25-hydroxyvitamin D and other vitamin D metabolites to maintain vitamin D storage in the body and transports these molecules to the kidney for further hydroxylation.[4,13,14] A quantitative-trait locus (QTL) analysis in mice had indicated possible involvement of the chromosomal region around a

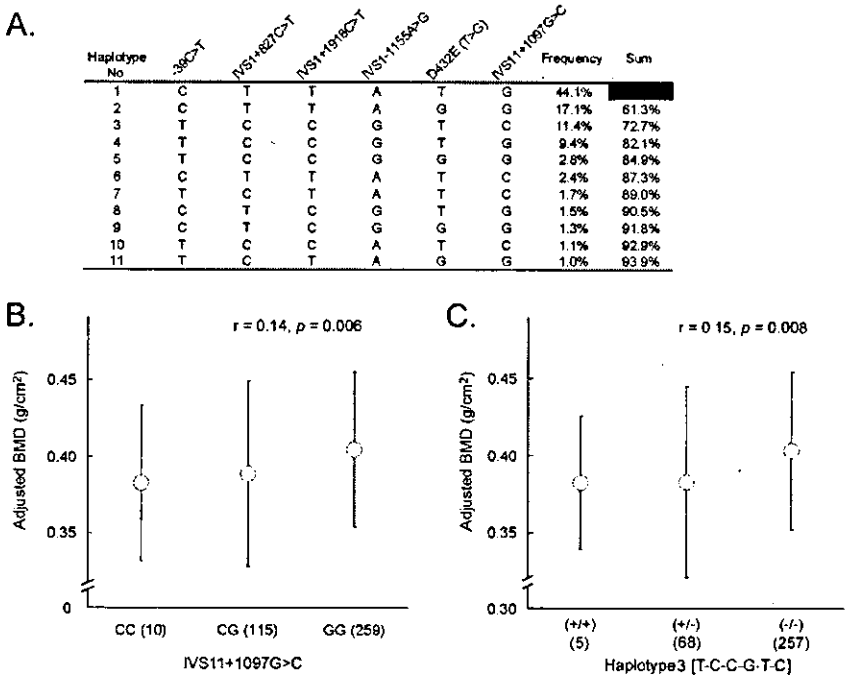

FIG. 2. Haplotype analysis of six SNPs that showed association with adjusted radial BMD. (A) The 11 major predicted haplotypes are presented in the order of their frequencies, as in Fig. 1A. (B) Adjusted BMD levels among 384 subjects plotted against three genotypically classified subgroups for the most significantly associated SNP (IVS11+1097G>C). Open circles indicate mean values; error bars indicate SDs. Correlation between the number of major alleles possessed and the adjusted BMD was tested by linear regression analysis ($r = 0.14$, $p = 0.006$). (C) Adjusted BMD of three duplotypically classified subgroups plotted against the significantly associated haplotype (T-C-C-G-T-C) among our 384 subjects. Open circles indicate mean values and error bars indicate SDs. Correlation between the number of haplotypes possessed (2,1,0) and adjusted BMD was tested by linear regression analysis ($r = 0.14$, $p = 0.006$).

microsatellite marker (Mit112) on murine chromosome 5, suggesting that osteoporosis-susceptibility genes might exist within the corresponding human chromosomal region (4q11–13). This region happens to contain the *DBP* gene.[24]

To determine whether genetic variations in DBP were associated with radial BMD in humans, our correlation studies used age- and BMI-adjusted BMD as a reliable quantitative trait. A hierarchical strategy was adopted to detect association by studying each genetic variant separately, then haplotypes, and finally LD among the SNPs. Previous studies had defined three isoforms of DBP in plasma, Gc1s, Gc1f, and Gc2, and analyzed them for association with common diseases.[14,25–29] Diagnosis was based on altered electrophoretic mobility in agar of isoforms that combined two amino acid variations, D432E and T436K. In addition to those variations, a TAAA-repeat polymorphism in the 8th intron of *DBP* was investigated previously.[12] However, rather than concentrating on this limited number of indirect marker variations, we chose to combine analyses of multiple SNPs spanning the entire 42.4-kb sequence of the *DBP* gene. Using this strategy we selected 13 SNPs, including an additional non-synonymous coding SNP, C445R (nt+1333C/T; IMS-JST082803) for examination in our cohort. Another known coding SNP, R445H (+1334G/A) was not detected in this population sample. Resequencing of the 2-kb 5′ flanking region of 32 chromosomes indicated that no other SNPs with minor-allele frequencies exceeding 3% were present. Combined analyses of the 13 selected SNPs revealed associations of multiple SNPs within the *DBP* gene with radial BMD levels. In general, the alleles affecting radial BMD were common, with frequencies ranging from 12% to 31% (Table 2).

Because multiple SNPs might act in combination to affect BMD, we constructed haplotypes to examine relationships to variations in radial BMD. This procedure increased the significance of any correlation previously found (Fig. 2). In addition, multiple regression analysis was effective to show that several combinations of three or four SNPs from among the "correlation group" consisting of −39C>T, IVS1+827C>T, D432E, and IVS11+1097G>C were significantly associated with radial BMD at the level of $p < 0.01$. Although D432E showed only a marginal tendency for correlation to the adjusted BMD by a separated analysis, we added this SNP to the analysis, because of its implicated functional importance and significant LD with IVS11+1097G>C. Interestingly, this was true when another non-synonymous coding SNP in the exon11, T436K, was included in the analysis; there still remained a significant level of correlation (data not shown).

Of interest, the functional importance of these polymorphic nucleotides of the SNPs that provided better p values was more predictable than the other SNPs. T436K and D432E are non-synonymous coding SNPs that alter protein structure, as evidenced by shifts in electrophoretic mobility of the protein on agarose gels. Although the affected amino acids do not lie within known structural domains,[30] they could affect the affinity of DBP for binding to vitamin D or other proteins. The SNPs in the 5′-flanking region (−39C>T) and in the first intron of *DBP* (IVS1+827C>T) may be in part of enhancer elements. Motif analysis using the MatInspector program revealed that −39C>T, in the

promoter region, lies within a consensus binding site for δ-EF1 (δ-crystallin/E2-box factor 1) [ggtcaccta(*C/T*)a].[31] δ-EF1 is an important transcription factor for differentiation of various types of cells. It is active in many organs, not only in eyes, and may regulate tissue-specific expression of target genes including the crystalline gene in lens and collagen and osteocalcin genes in osteoblasts.[32–34] Of course, in addition to the above-mentioned promoter SNP and coding SNPs, the significantly associated SNPs in noncoding regions (within introns and the 3′ untranslated region) might still influence alternative splicing, splicing efficiency, or mRNA turnover; such mechanisms have been reported for other disease-causing genes. Those possibilities should be tested. Nevertheless, the genetic data reported here, as well as the obvious physiological role of DBP in calcium homeostasis, suggest that alterations in DBP activity or expression level may underlie abnormalities in bone mineral metabolism.

In summary, our genetic analysis of *DBP* variations, haplotypes, and LD, and correlation of those features with radial BMD, suggests that multiple SNPs at the *DBP* locus might act in combination to increase the risk of osteoporosis in postmenopausal Japanese women. The possible involvement of genetic variations in the *DBP* gene may explain, at least in part, the pathogenesis of postmenopausal osteoporosis and may contribute to the establishment of suitable treatment designs and plans for prevention of the disease.

ACKNOWLEDGMENTS

We thank Dr Hironori Iwasaki at Ryukyu University for his advice and discussions, and Mina Kodaira, Kyoko Shimizu, Mayumi Tanaka, and Naoko Tsuruta for their technical assistance. This work was supported in part by a special grant for Strategic Advanced Research on "Cancer" and "Genome Science" from the Ministry of Education, Science, Sports and Culture of Japan, a Research Grant for Research from the Ministry of Health and Welfare of Japan, and a Research for the Future Program Grant of The Japan Society for the Promotion of Science.

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Received in original form January 13, 2003; in revised form February 11, 2003; accepted March 19, 2003.

J Hum Genet (2003) 48:342–345
DOI 10.1007/s10038-003-0035-1

ORIGINAL ARTICLE

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Association of genetic variation of the *RIL* gene, encoding a PDZ-LIM domain protein and localized in 5q31.1, with low bone mineral density in adult Japanese women

Received: 27 January 2003 / Accepted: 9 April 2003 / Published online: 6 June 2003

Abstract Twin and family studies had shown that genetic factors are important determinants of bone mass. Multiple genes might be involved. One candidate gene, the reversion-induced LIM gene (*RIL*), is a PDZ and LIM-domain-containing protein and has been localized within the cytokine cluster of chromosome 5 (5q31.1). In a genetic study of 370 adult Japanese women, we investigated the correlation between radial bone mineral density (BMD) and a genetic variation (−3333T → C) of the 5'-flanking region of *RIL* gene. A significant association was identified between the *RIL* variation −3333T → C and radial BMD ($r = 0.15$, $P = 0.003$). The variation of the *RIL* locus may be an important determinant of osteoporosis.

Keywords Single-nucleotide polymorphism · *RIL* · Bone mineral density · Association study

Introduction

Osteoporosis is a multi-factorial common disease that is characterized by reduced bone mass and increasing risk of fracture. Genetic and environmental factors play important roles in the determination of bone mineral density (BMD) (Hirota et al. 1992; Suleiman et al. 1997; Pocock et al. 1987; Krall and Dawson-Hughes 1993).

Previous studies have examined associations of candidate gene polymorphisms with BMD. Examples are the genes encoding the vitamin D receptor, the estrogen receptor, the apolipoprotein E and type I collagen, combinations of which may determine individual BMD levels (Morrison et al. 1994; Melhus et al. 1994; Greenfield and Goldberg 1997; Kobayashi et al. 1996; Shiraki et al. 1997; Uitterlinden et al. 1998). In addition to these makers, numbers of unidentified polymorphic genes may participate in determining the bone mass of an individual. Candidates might be genes involved in cytokine-signaling pathways, the hormonal regulation of calcium balance and bone mineral, or the cellular function of bone cells.

One possible candidate gene localized within the cytokine cluster of chromosome 5 (5q31.1) is the reversion-induced LIM gene (*RIL*), whose mRNA expression in human bone marrow stromal cells has been strongly detected in a previous study (Bashirova et al. 1998). Although its exact function is not defined as yet, an involvement of osteoblast development/function is implicated.

In this study, we have carried out a correlation study of genetic variations in *RIL* gene for radial BMD levels. Involvement of an *RIL* gene polymorphism was tested with respect to the regulation of BMD.

Subjects and methods

Subjects

DNA samples were obtained from peripheral blood of 370 adult Japanese women (Shinohara et al. 2001). BMD was measured in all of these subjects. Mean ages and body mass indices (BMI) with standard deviations (SD) were 58.4 ± 8.6 years (range:

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32-69 years) and 23.7 ± 3.61 kg/m^2 (range: 14.7–38.5 kg/m^2), respectively. All subjects were non-related volunteers and gave their informed consent prior to 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 was receiving estrogen-replacement therapy.

BMD measurement

The BMD of radial bone (expressed in g/m^2) in each participant was measured by dual energy X-ray absorptiometry (DEXA) with DTX-200 (Osteometer Meditech, Hawthorne, Calif., USA). To calculate the adjusted BMD, the measured BMD was normalized for differences in age and BMI by multiple regression analysis with the Instat 3 software package (GraphPad Software, San Diego, Calif.). The adjustment equation for the study samples was as follows: {adjusted-BMD (g/m^2)} = {measured-BMD (g/m^2)} − 0.006375 × [58.39 − {age (years)}] + 0.008961 × [23.65 − {BMI (kg/m^2)}]. BMD in the distal radius was measured according to the Guidelines for Osteoporosis Screening in a health check-up program in Japan (Orimo et al. 2000). Although radial bone is not a first-choice measurement site for BMD in this program, we took advantages of radial BMD that represented the quality of both cortical bone thickness and cancellous bone volume when the distal part near the wrist joint was measured. This is suitable for dealing with large number of study participants and is rarely affected by old fractures, degenerative changes, or deformities.

Genotyping procedure

Genotypes of single-nucleotide polymorphisms (SNPs) were determined by using the SNP-dependent polymerase chain reaction (Sd-PCR) method, a refined allele-specific PCR to discriminate polymorphic sequences as described previously (Iwasaki et al. 2003). In brief, Sd-PCR transforms nucleotide differences (G, A, T, or C) between two alleles at a single site into size-differences between the respective alleles. The procedure incorporates double-nucleotide mismatches at the 3' end of polymorphic (forward) primers representing each allele, one mismatch corresponding to the natural SNP to be tested and the other being designed to allow distinct allelic discrimination through the almost exclusive amplification of one allele over the other. Two allele-specific primers (AS-primers) and one reverse primer were prepared per SNP as follows: RIL-FL: 5'-TTTTTGGGGCCTGGATCTGGCTCTTCGGAC-3', RIL-FS: 5'-CCGGCCTGGATCTGGCTCTTCCCAT-3', RIL-RS: 5'-GTTTAGCTGTGGCCACTGGGTAT-3'. The AS-primers (long and short) had a 5-base difference between them; each had the polymorphic nucleotide of the SNP sequence at the 3' ends and an additional artificial mismatch introduced near the 3' end. These primer sets allowed the distinct discrimination of alleles. Each genomic DNA sample (10 ng) was amplified with 250 nM each primer (two polymorphic forward primers and one reverse primer) in a 10-μl reaction mixture containing 10 mM dNTPs, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, 1 U *Taq* DNA polymerase, and 0.5 mM fluorescence-labeled dCTP (ROX-dCTP; Perkin-Elmer, Norwalk, Conn., USA; Nakazawa et al. 2001). The Sd-PCR reaction was carried out on a thermal cycler (Gene-amp system 9600, Perkin-Elmer) with initial denaturation at 94°C for 4 min, followed by five cycles of stringent amplification (94°C for 20 s, 64°C for 20 s, 72°C for 20 s) and then 25 cycles of 94°C for 20 s, 62°C for 20 s, 72°C for 20 s), terminating with a 2-min extension at 72°C (Harada et al. 2001). Allele discrimination was carried out by electrophoresis and laser scanning of the DNA fragments on an ABI Prism 377 DNA system with GeneScan Analysis Software ver2.1 (Applied Biosystems, Foster City, Calif.; Hattori et al. 2002). To confirm the accuracy of the Sd-PCR method, direct re-sequencing was carried out by using the ABI Prism BigDye Terminator system (Applied Biosystems).

Statistical analysis

BMD data of each subject were normalized with age and BMI, by means of Instat 3 software package (GraphPad Software, San Diego, Calif.) via multiple regression analysis. Quantitative association between genotypes and adjusted BMD values (g/m^2) was analyzed via one-way analysis of variance (ANOVA) with regression analysis as a post hoc test. Three genotypic categories of each SNP were converted into incremental values (0, 1, and 2) corresponding to the number of chromosomes possessing the minor allele. Significant association was defined when the given P-value of the ANOVA F-test was less than 5% ($P < 0.05$; Ota et al. 2001). To ascertain the Hardy-Weinberg equilibrium among genotypes of the subjects, the chi-squared test was used ($P > 0.05$).

Results

BMD association of the *RIL* gene locus was examined with *RIL* as one of the likely candidates for osteoporosis susceptibility genes. We first examined the polymorphic nature of a promoter SNP, −3333T → C, localized −3333 bp upstream from the translation initiation site of *RIL* and archived in the Japanese SNP (JSNP) database (referenced contig; NT_007072.10 from GenBank, Hs 79691 from Unigene) in 32 chromosomes of Japanese subjects. The SNP from the JSNP database turned out to be moderately polymorphic in the test population. Genotypes of the SNP were determined for all the 370 subjects, and an ANOVA with a linear regression test was performed. The distribution of SNP genotypes of the *RIL* gene in our subjects fitted the expectations of the Hardy-Weinberg equilibrium based on allele frequencies (chi-squared test, $P = 0.99$).

The 5' flanking SNP −3333T → C revealed a significant correlation with a variation in radial adjusted BMD ($r = 0.15$, $P = 0.003$, $n = 370$). The homozygous T-allele carriers had the lowest adjusted BMD (0.370 ± 0.053 g/cm^2, $n = 14$), heterozygous individuals had an intermediate adjusted BMD (0.391 ± 0.054 g/cm^2, $n = 116$), and homozygous C-allele carriers had the highest adjusted BMD (0.404 ± 0.055 g/cm^2, $n = 240$), implying an allelic dose effect of this variation on influences on BMD (Fig. 1). The results suggest that genetic variation in *RIL* contribute to the development of osteoporosis. We hypothesized that the T-allele homozygote for *RIL* −3333T → C SNP is an important risk factor for decreased BMD in adult women.

Discussion

In the work reported here, we showed an association of the −3333 T/C SNP variation in the promoter region of the *RIL* gene with radial BMD in a population of adult Japanese women. Adjusted BMD was lowest in T/T homozygotes, intermediate among heterozygotes, and highest among C/C homozygotes in the test population. The data implied that variation in the 5' flanking region of the *RIL* gene might have affected bone metabolism in these women, eventually introducing a variation

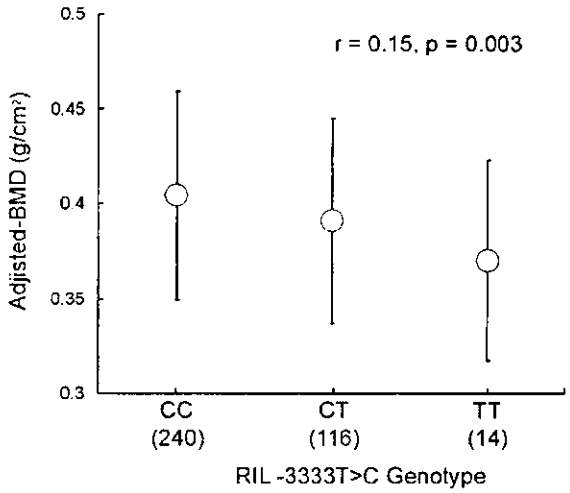

Fig. 1 Adjusted BMD among three genotypic groups of −3333T → C SNP of the *RIL* gene correlated with the variation ($r = 0.15$, $P = 0.003$). Values are expressed as means ± SD

in BMD. A lowered BMD in adult women could be a result of the reduced acquisition of bone mass before maturation and/or accelerated bone loss.

Originally, the *RIL* gene was identified, in rat fibroblasts, as a candidate tumor suppressor gene that encodes a PDZ and LIM-domain-containing protein (Kiess et al. 1995). PDZ and LIM-domain-containing proteins (PDZ-LIM proteins) are a class of LIM-domain proteins. The proposed function of PDZ-LIM proteins is related to the organization of the plasma membrane of polarized cells, based on the indicated function of both domains in protein-protein interactions and the subcellular localization of these proteins in a specific domains of plasma membrane by binding to F-actin and related cytoskeleton structures (Boden et al. 1998; Cuppen et al. 1998).

Structural similarity (identity: 27%) of the RIL protein to the other member of the PDZ-LIM protein family, LIM mineralization protein-1 (*LMP-1*, also known as *ENIGMA*), implies a functional resemblance of these gene products. LMP-1 is induced by bone morphogenetic protein-6 and may mediate the regulation of the osteoblast differentiation program (Boden et al. 1998). Similarly, *RIL* might be involved in the regulation of osteoblast differentiation and bone formation. Functional studies of genetic variation(s) in the *RIL* gene will be required to elucidate these problems. Of course, one possibility that cannot be ruled out is that this variation may itself be in linkage disequilibrium with other unmeasured functional variants that lie at or near the *RIL* locus and that represent the true mechanistic basis for the associations. The *RIL* gene is localized within the cytokine cluster of chromosome 5. Genetic variations of this region may have to be evaluated thoroughly. In addition, functional studies may also be required. Other genes in the same signaling cascade also need to be scrutinized to clarify the molecular mechanism for PDZ-LIM domain family regulation in osteoblast differentiation.

In summary, we have shown a significant association of the −3333T → C variant in the 5' flanking region of the *RIL* gene with radial BMD in adult Japanese women.

Acknowledgements This work was supported in part by a special grant for Strategic Advanced Research on "Cancer" and "Genome Science" from the Ministry of Education, Science, Sports and Culture of Japan, by a Research Grant for Research from the Ministry of Health and Welfare of Japan, and by a Research for the Future Program Grant of The Japan Society for the Promotion of Science.

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Bone 33 (2003) 237–241

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Association of a promoter haplotype (−1542G/−525C) in the tumor necrosis factor receptor associated factor-interacting protein gene with low bone mineral density in Japanese women

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Received 24 November 2002; revised 16 April 2003; accepted 21 April 2003

Abstract

Osteoporosis, a multifactorial common disease, is believed to result from the interplay of multiple environmental and genetic factors that regulate bone mineral density (BMD). Tumor necrosis factor receptor associated factor-interacting protein (I-TRAF) is an essential effecter of the tumor necrosis factor receptor-signaling cascade, one of the most potent bone-resorbing systems. In genetic studies of 382 Japanese adult women, we found that genotypes of two promoter variations of *I-TRAF* gene, −1542T/G and −525G/C, were similarly associated with radial BMD levels. Two variations were in almost complete linkage disequilibrium ($D' = 0.978$, $r^2 = 0.917$, $\chi^2 = 695.2$, $P = 3.4 \times 10^{-153}$), and there were two exclusive haplotypes (−1542T/−525C, frequency 0.74, and −1542G/−525G, frequency 0.24) among our test subjects. When BMD values were compared among the three haplotypic categories (−1542G/−525G homozygotes, heterozygotes, and −1542T/−525C homozygotes), BMD was lowest among −1542G/−525C homozygotes (mean ± SD = 0.382 ± 0.060 g/cm^2), highest among −1542T/−525G homozygotes (0.405 ± 0.051 g/cm^2), and intermediate among heterozygotes (0.395 ± 0.056 g/cm^2) ($r = 0.11$, $P = 0.030$). The observed trend supported a codominant effect of the relevant haplotype of *I-TRAF* gene in determination of radial BMD. These results suggested that variation of *I-TRAF* might be an important determinant for postmenopausal osteoporosis.

Keywords: Single nucleotide polymorphism; I-TRAF; Bone mineral density; Association study; Quantitative trait; Linkage disequilibrium

Introduction

Cytokine pathways including tumor necrosis factor α (TNF α) and interleukin-1 (IL 1) are considered among the most potent of all bone-resorbing mechanisms [1–4]. TNF receptor associated factor (TRAF) proteins bind to members of the TNF receptor superfamily to transduce signals from them. Some of these signaling cascades are known to participate in osteoclast differentiation and may affect bone mineral density (BMD). TRAF-interacting protein (I-TRAF), which binds to the TRAF-C domains of TRAF1, TRAF2, and TRAF3, is a regulator of TRAF-mediated signal transduction, which is likely to play an important role in bone metabolism [5].

Osteoporosis is characterized by low bone mineral density and by deterioration of the microarchitecture of bone tissue. The consequent increase in fragility and susceptibility to fracture is a social problem as well as an individual concern [6]. An important predictor of fracture, BMD is

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8756-3282/$ – see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/S8756-3282(03)00226-6

thought to be determined both by genetic factors and by environmental factors [6–10]. For adequate diagnosis, prevention, and early treatment of osteoporosis, genetic elements must be clarified that are supposed to be more consistent determinants of BMD than environmental factors. If the relevant genes were identified, the pathogenesis of osteoporosis presumably is explained by a combination of variations in those genes or in loci adjacent to them.

Several genes have already been investigated as potential risk factors for osteoporosis [2,11–16]. However, an extended panel of genes must be examined in detail, considering the polygenic nature of BMD distribution and the multiplicity of endocrine and local factors known to regulate bone mass and bone turnover. For the present study, we chose I-TRAF as a most likely candidate molecule that affects BMD levels of human. We investigated a possible association of nucleotide variations in the promoter region of the *I-TRAF* gene with the adjusted value of radial BMD in adult women.

Materials and methods

Subjects

DNA samples were obtained from peripheral blood of 384 adult Japanese women. Mean ages and body mass indices (BMI) with standard deviations (SD) were 58.4 ± 8.6 (range 32–69) years and 23.7 ± 3.61 (range 14.7–38.5) kg/m^2. The BMD of radial bone (expressed in grams per square centimeter) of each participant was measured by dual energy X-ray absorptiometry using DTX-200 (Osteometer Meditech, Inc., Hawthorne, CA, USA). To calculate the adjusted BMD, the measured BMD was normalized for differences in age, height, and weight by multiple regression analysis using the Instat 3 software package (GraphPad Software, San Diego, CA, USA).[7, 17]. The adjustment equation for the study samples was as follows: {adjusted-BMD (g/cm^2)} = {measured-BMD (g/cm^2)} − 0.006375 × [58.39 − {age (years)}] + 0.008961 × [23.65 − {BMI (kg/cm^2)}]. BMD in the radius was measured according to the Guidelines for Osteoporosis Screening in a health checkup program in Japan [18]. All were nonrelated volunteers and gave their informed consent prior to 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 was receiving estrogen replacement therapy.

Genotyping for molecular variants in the I-TRAF gene

Of the archived five SNPs in the promoter region of the *I-TRAF* gene in the JSNP database (Table 1), only two SNPs, −1542T/G and −525G/C, were examined for genotypes and haplotypes in this study. One SNP, −224G/A, that localized at the most proximal site from the transcription initiation site was excluded from the analysis, because of low minor allele (A-allele) frequency less than 1%. Two SNPs, −1808G/A and −2141G/C, were excluded because of their greater distances from the transcription initiation site.

Table 1
Summary of polymorphisms localized in the *I-TRAF* locus

No.	SNP name	JSNP-ID[a]	dbSNP[b]	Allele frequency (heterozygosity)
1	−224G/A	IMS-JST108009	rs3754975	0.99:0.01[c] —
2	−525G/C	IMS-JST108010	rs3754976	0.76:0.24[d] (38%)
3	−1542T/G	IMS-JST108011	rs979414	0.75:0.25[d] (38%)
4	−1808G/A	IMS-JST108012	rs3754978	0.80:0.20[c] —
5	−2141G/C	IMS-JST108013	rs3754979	0.79:0.21[c] —

[a] Number from Japanese SNP database (http://snp.ims.u-tokyo.ac.jp/index_ja.html).
[b] Number from dbSNP database of NCBI (http://www.ncbi.nlm.nih.gov/SNP/).
[c] Frequency and heterozygosity data is from database.
[d] Frequency and heterozygosity data is from the present study.

Genotypes of SNPs were determined using the Sd-PCR method, a modified allele-specific PCR of polymorphic sequence as previously described [11,19]. In brief, the Sd-PCR transforms nucleotide differences (G, A, T, or C) between two alleles at a single site into size differences between the respective alleles. The procedure incorporates double-nucleotide mismatches at the 3′ end of polymorphic (forward) primers representing each allele, one mismatch corresponding to the natural SNP to be tested and the other designed to allow distinct allelic discrimination through almost exclusive amplification of one allele over the other.

Two allele-specific primers (AS-primers) and one reverse primer were prepared per SNP. AS-primers (long and short) have a five-base difference between them; each has a polymorphic nucleotide of the SNP sequence at the 3′ ends and an additional artificial mismatch introduced near the 3′ end.

For SNP −1542T>G, long AS-primer; 5′-TTTTTGGTTGAGCTTGGCTGACACCCCGGA-3′, short AS-primer; 5′-CCTTGAGCTTGGCTGACACCCACGC-3′, reverse primer; 5′-GTTTAAACAATGCCCCACCAGAT-3′.

For SNP −525G>C, long AS-primer; 5′-TTTTTGGTTAAGTTCTCAAGGCAGAGAAG-3′, short AS-primer; 5′-CCTTAAGTTCTCAAGGCAGAAGAC-3′, reverse primer; 5′-GTTTTTTATAAACAGGAGGTAAAAACCAAA-3′.

These primer sets allowed distinct discrimination of alleles. Each genomic DNA sample (10 ng) was amplified with 250 nM of each primer (two polymorphic forward and a reverse) in a 10-μl reaction mixture containing 10 mM dNTPs, 10 mM Tris–HCl, 1.5 mM MgCl^2, 50 mM KCl, 1 U Taq DNA polymerase, and 0.5 mM fluorescence-tabeled dCTP (ROX-dCTP; Perkin-Elmer, Norwalk, CT, USA). The Sd-PCR reaction was carried out on a thermal cycler

(Gene-amp System 9600, Perkin-Elmer) with initial denaturation at 94°C for 4 min, followed by 5 cycles of stringent amplification (94°C for 20 s, 64°C for 20 s, 72°C for 20 s) and then 25 cycles of 94°C for 20 s, 62°C for 20 s, 72°C for 20 s), terminating with a 2-min extension at 72°C. Allele discrimination was carried out by laser scanning of the DNA fragments electrophoresed on ABI Prism 377 DNA system, using GeneScan Analysis software ver2.1 (Applied Biosystems, Foster City, CA, USA). To confirm the accuracy of the Sd-PCR method, direct resequencing was carried out using the ABI Prism BigDye Terminator system (Applied Biosystems).

Statistical analysis of linkage disequilibrium and tests for association with BMD

Among 384 individuals investigated, genotyping data for both of the SNPs were defined in 379 individuals. Haplotype frequencies among the representative 758 alleles investigated were calculated by Arlequin software (http://lgb.unige.ch/arlequin/software/). Maximum-likelihood haplotype frequencies are computed using an expectation–maximization algorithm [20]. This procedure is an iterative process aiming at obtaining maximum-likelihood estimates of haplotype frequencies from multilocus genotype data when the gametic phase is unknown. This program infers haplotypes probabilistically.

Linkage disequilibrium was investigated for the comparison of the polymorphisms according to Thompson's method (D, D,' and r^2) [14,21]. The coefficient of disequilibrium (D) is the difference between the observed haplotype frequency and the frequency expected under statistical independence, which is given by the following formula: $D = (p\mathrm{AB} \times p\mathrm{ab}) - (p\mathrm{Ab} \times p\mathrm{aB})$. (In the formula, "$p$AB" represents the probability that a chromosome have both "A" and "B" alleles. Similarly, "$p$ab", "paB," and "pAb" represent the probabilities that a chromosome have respective two alleles.) The normalized disequilibrium coefficient (D') is given by the formula $D' = D/|D|\mathrm{max}$, in which $|D|\mathrm{max}$ represents a maximum possible value of D ($|D|\mathrm{max} = \min(p\mathrm{A} \times p\mathrm{B}, p\mathrm{a} \times p\mathrm{b})$ if $D < 0$, and $|D|\mathrm{max} = \min(p\mathrm{A} \times p\mathrm{b}, p\mathrm{a} \times p\mathrm{B})$ if $D > 0$). The correlation coefficient (r^2) is given by the formula $r^2 = D^2/(p\mathrm{A} \times p\mathrm{B} \times p\mathrm{a} \times p\mathrm{b})$. Significance levels were determined by χ^2 statistics for the corresponding 2 × 2 table.

Differences in the adjusted-BMD (grams per square centimeter) among the three genotypic/haplotypic categories were analyzed by analysis of variance (ANOVA) and linear regression test as a post hoc test. Based on the almost complete LD of the two SNPs (−1542T/G and −542G/C) in our study subjects, we included 376 individuals (752 alleles) who definitely had one of the two major haplotypes homozygously (−1542T/−542G or −1542G/−525C), or was supposedly carrying both of the two major haplotypes heterozygously. One individual predicted to have a rare haplotype was excluded. Statistical significance was considered when the P values were less than 5% (ANOVA, F test $P < 0.05$).

Results

Almost complete linkage disequilibrium between −1542T/G and −525G/C SNPs

A panel of 384 Japanese adult women was genotyped for two SNPs in the 5′ flanking region of the *I-TRAF* gene −1542T/G and −525G/C, using a refined method for allele-specific amplification for SNP discrimination (Sd-PCR). Genotypes were determined in 383 individuals for −1542T/G and in 380 individuals for −525G/C. Individuals with ambiguous results were excluded. There was no deviation of genotype frequencies from Hardy–Weinberg equilibrium in any of the polymorphisms among our subjects. However, we found an obvious consistency of genotypes observed between the two SNPs, −1542T/G and −525G/C. Thus, we analyzed linkage disequilibrium between the two variations among 379 subjects, using Thompson's method (D, D', and r^2), confirming almost complete linkage disequilibrium of these SNPs ($D' = 0.978$, $r^2 = 0.917$, $\chi^2 = 695.2$, $P = 3.4 \times 10^{-153}$). On this basis, we calculated the frequencies of the constructed haplotypes using the Arlequin algorithm, which predicted two major haplotypes among the test population. The most frequently observed haplotype, −1542T/−525G, constituted 74.3% of the haplotypes predicted. The other haplotype, −1542G/−525C, represented 24.1% of the haplotypes. Because the predicted frequencies of two other haplotypes, −1542T/−525C and −1542G/−525G, were rare, only about 1% level, we regarded them as negligible in the present analysis.

Association of −1542G/−525C haplotype with low BMD

Correlation of the determined genotypes and haplotypes to the adjusted BMD was analyzed among 384 adult women. A significant correlation was identified by ANOVA and linear regression test for both of the SNPs, −1542T/G ($r = 0.13$, $P = 0.012$; $n = 383$) and −525G/C ($r = 0.11$, $P = 0.027$; $n = 380$). Similar analysis for the number of the most frequent haplotype and the adjusted BMD levels in individuals revealed a significant correlation ($r = 0.11$, $P = 0.030$; $n = 376$) (Fig. 1). When the adjusted BMD values among the three haplotypic categories, −1542G/−525C homozygotes, heterozygotes, and −1542T/−525G homozygotes, were compared, BMD was lowest among −1542G/−525C homozygotes (mean SD = 0.382 ± 0.060 g/cm^2), highest among −1542T/−525G homozygotes (0.404 ± 0.051 g/cm^2), and intermediate among heterozygotes (0.396 ± 0.056 g/cm^2). A trend of resistance for lowering BMD by possession of the −1542T/−525G haplotype was suspected, which indicated a codominant effect

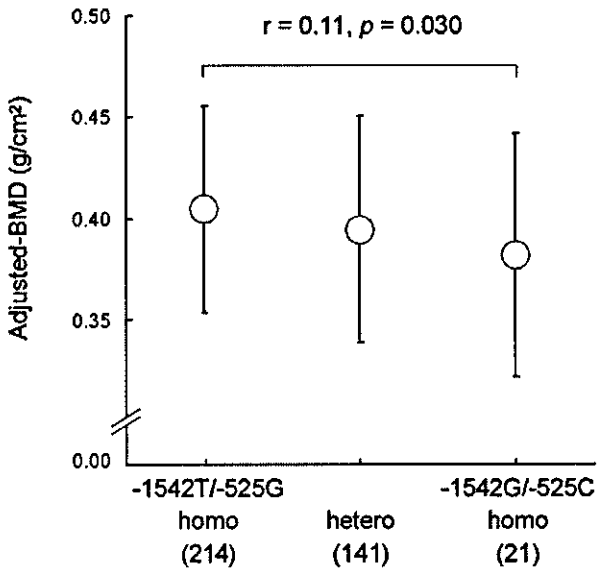

Fig. 1. Effect of I-TRAF variations on adjusted BMD. Haplotypic dose effect of the −1542G/−525C and the −1542T/−525G was analyzed by ANOVA with a linear regression analysis as a post hoc test. Open circles indicate mean values; error bars indicate standard deviation. *P* values were calculated by ANOVA *F* test.

of the relevant haplotype of the *I-TRAF* gene in the determination of radial BMD.

Discussion

In the work reported here we studied two 5′ flanking SNPs in the potential promoter region of the *I-TRAF* gene among adult Japanese women. By analyzing haplotypes and correlation of forearm cancellous BMD, we demonstrated a significant association of lower BMD to one of the major haplotypes of this gene, −1542T/−525G, that was constructed by two SNPs almost in complete linkage disequilibrium. The adjusted BMD was lowest in −1542G/−525C homozygotes, intermediate among heterozygotes, and highest among −1542T/−525G homozygotes. The data implied that variation in the potential promoter region of the I-TRAF gene might have affected bone metabolism in these women, eventually introducing variation in BMD. Lowered BMD could be a result of accelerated bone loss after menopause and/or lesser acquisition of bone mass before maturation. Future longitudinal analysis of bone loss rate and *I-TRAF* variation over the years would be required to prove either hypothesis.

There are several interpretations for the observed associations for the two SNPs that are in strong linkage disequilibrium. First, one site or the other may play a predominant mechanistic role that explains the associations. Second, both nucleotide changes together are required to impart the functional change that underlies the observed association (codominant effect of the two polymorphisms). Third, these markers may themselves be in linkage disequilibrium with other unmeasured and functional variants that are the true mechanistic basis for the associations. Although these possibilities would be distinguished by functional studies, we assume SNP−1542T/G may be more important on the theoretical grounds that it is located in a consensus-binding site for a transcription factor GATA-2 (CAGGATCCTG; underlined "T" is a variant nucleotide). This binding site is intact on the chromosome carrying the "−1542T" allele, whereas it is lost on the "−1542G" allele carriers. GATA-2 is required for generation of osteoclast progenitors [22]. Although target genes of GATA-2 on this action are not defined yet, I-TRAF might be one of them. The exact function of I-TRAF is still in controversial. However, in mammalian cells, excessively expressed I-TRAF inhibits the TRAF-mediated NF-κB activation that is known to be essential for osteoclastgenesis [13]. Therefore, if the *I-TRAF* gene is one of the target genes of GATA-2, its expression might be negatively regulated. Experimental examinations of promoter activity would be required. Loss of efficient control of I-TRAF expression in the homozygous G-allele carriers by GATA-2 may result in disorganized osteoclast differentiation, and altered bone resorption/turnover would be the result. The mechanisms of how the altered GATA-2 regulation of TRAF system results in lowered BMD should be investigated in the future study.

The effects of molecules acting on inflammatory cytokine pathways such as IL1 and TNF α have been investigated on bone mineral status [5,8,10,23–25]. In addition to these molecules and I-TRAF, there must be many other effectors and inhibitors acting on this signaling cascade. Clarification of their contributions is awaited for further investigation. Such investigations may help to clarify mechanisms of postmenopausal bone loss as well as general bone loss associated with bone-resorbing cytokine-exerted pathways.

In summary, we have identified a novel candidate osteoporosis-susceptibility gene, *I-TRAF*, whose two promoter variations were significantly associated with bone mineral density among adult Japanese women. Structural inspection indicated a possible contribution of −1542T/G variation in I-TRAF, which is located in the GATA-2 binding site. The possible involvement of genetic variations in I-TRAF may explain, at least in part, the pathogenesis of postmenopausal osteoporosis and may contribute to the establishment of designs for suitable treatments and preventive strategies for this disease.

Acknowledgments

This work was supported in part by a special grant for Strategic Advanced Research on "Cancer" and "Genome Science" from the Ministry of Education, Science, Sports, and Culture of Japan; by a Research Grant for Research from the Ministry of Health and Welfare of Japan; and by a

Research for the Future Program Grant of the Japan Society for the Promotion of Science.

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0013-7227/03/$15.00/0
Printed in U.S.A.

The Journal of Clinical Endocrinology & Metabolism 88(3):1333–1340

doi: 10.1210/jc.2002-021015

Zone-Dependent Expression of Estrogen Receptors α and β in Human Benign Prostatic Hyperplasia

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Estrogen, which acts through estrogen receptors (ERs) α and β, has been implicated in the pathogenesis of benign and malignant human prostatic tumors, *i.e.* benign prostatic hyperplasia and prostate cancer, thought to originate from different zones of the prostate [the transition zone (TZ) and peripheral zone (PZ), respectively]. Here, we examined the cellular distribution of ERα and ERβ in human normal and hyperplastic prostate tissues, using *in situ* hybridization and immunohistochemistry. ERα expression was restricted to stromal cells of PZ. In contrast, ERβ was expressed in the stromal cells of PZ as well as TZ. ERβ-positive epithelial cells were evenly distributed in PZ and TZ of the prostate. Our results suggest that estrogen may play a crucial role in the pathogenesis of benign prostatic hyperplasia through ERβ. (*J Clin Endocrinol Metab* 88: 1333–1340, 2003)

THE HUMAN PROSTATE is a sex hormone target organ, and both benign prostatic hyperplasia (BPH) and prostate cancer (PC) could develop in this organ. In fact, about 50% of men 51–60 yr old and 90% of men over 80 yr of age show histological evidence of BPH (1), and suffer from various urinary symptoms. Because these diseases are sex-hormone-sensitive, patients are often treated with antiandrogens or estrogens (2, 3). Although a number of studies have reported that certain sex hormones, such as androgens and estrogens, stimulate prostatic cell proliferation in experimental animal models, the role of cell proliferation in the progression of human BPH has not been established yet, because only a few proliferating cells are usually detected in the tissue (4). Thus, our understanding of the involvement of sex hormone in the pathogenesis of BPH is still only limited.

The prostate is absolutely dependent on androgens to maintain its size and secretory function (5). Although the androgen-dependency of the prostate gland has long been known, the role of estrogen in the prostate has been only recently recognized. In canine studies, castrated dogs showed regrowth and normal function after administration of exogenous androgens. When estrogens were added to the treatment protocol, the dogs developed glandular hyperplastic prostate, including an increase in total cell number (6). In other studies, pharmacological doses of estrogens induced a marked proliferative response of prostatic glandular epithelium (termed: squamous metaplasia) in a variety of mammals, including human (7).

Whereas serum estrogen levels are low in healthy men [approximately half the levels in nonpregnant women (8)], serum and intraprostatic estradiol levels (both absolute levels and those relative to testosterone) increase in men with age, accompanied by an increase in the prostate volume (9–11). In addition, patients with larger volumes of BPH tend to have high levels of serum estradiol (12). Therefore, the estrogen-dominant status in men after middle age has been implicated in the induction and progression of BPH (10). However, the molecular mechanism of estrogen action in the development of human BPH is largely unknown.

Estrogens require the presence of estrogen receptors (ERs) for their actions. ERs belong to a nuclear receptor superfamily of ligand-activated transcription factors; and, at present, two types of ERs (ERα and ERβ) have been characterized (13–16). ERα was known as ER before the discovery of ERβ, and its distribution has been thoroughly investigated in various human organs, including prostate and endometrium, breast cancer cells, and ovarian interstitial cells, which contain mostly ERα (17–20). ERα has been detected in stromal cells of human prostate, although the cellular localization of ERα remains controversial. At present, estrogen is thought to act indirectly on glandular cell proliferation, through ERα-positive stromal cells, in a paracrine manner (21).

ERβ has been recently cloned in the rat prostate and human testis (15, 16). ERα and ERβ share very high amino acid homology in the DNA binding domain, at 96%, but only show 53% homology in the ligand binding domain, as reported previously (22). The distribution of ERα and ERβ is variable, depending on tissues. In human, the testis contains mostly ERβ, and this subtype is also present in a variety of tissues, including kidney, interstitial mucosa, lung parenchyma, bone marrow, bone, brain, endothelial cells, and prostate gland (23). Although the biological action of ERβ in human prostate remains to be clarified, ERβ knockout mice develop prostatic hyperplasia with aging, suggesting that ERβ may play an important role in the development of BPH

Abbreviations: AR, Androgen receptor; BPH, benign prostatic hyperplasia; DAB, 3,3′-diaminobenzidine/4HCl; ER, estrogen receptor; HRP, horseradish peroxidase; IHC, immunohistochemistry; ISH, *in situ* hybridization; PC, prostate cancer; PZ, peripheral zone; SSC, standard saline citrate; TZ, transition zone.

(24, 25). At present, however, there are conflicting reports on the cellular localization of ERβ in human prostate. Enmark *et al.* (26) and Bonkhoff *et al.* (27) demonstrated that ERβ expression was only marginal or absent in human prostate. On the other hand, using immunohistochemistry (IHC), Royuela *et al.* (19) reported that ERβ was significantly expressed and localized only in the nuclei of the basal cells, whereas others detected a similar expression in the nuclei of stromal cells as well as the basal cells and also the cytoplasm of glandular epithelial cells by IHC (20, 28). Considering that the results of IHC are often influenced by the stability of the epitope and fixation procedure, analysis of ERβ transcript by *in situ* hybridization (ISH) would be appropriate to resolve the controversy.

According to zonal anatomy described by McNeal *et al.* (29, 30), all BPH nodules and most PC foci arise in the transition zone (TZ) and the peripheral zone (PZ). To our knowledge, there are no studies that have examined the zonal differences, if any, in the expression of ERα and ERβ and whether such difference may reflect the pathogenesis of these prostatic diseases. In the present study, we first examined the cellular distribution of ERβ mRNA and protein in normal human prostate tissues, using nonradioactive ISH and IHC, respectively. The antibody for ERβ was raised by immunizing rabbits with a synthetic peptide of a part of the C-terminal domain of human ERβ (22), and the specificity of this antibody was confirmed by Western blot analysis. We then investigated the expression of ERα and ERβ in human BPH tissues, by IHC, focusing on the difference between PZ and TZ. Our results revealed a discrete cellular distribution of ERs and that such distribution varied between the TZ and PZ, suggesting that the differential expression of ERα and ERβ may be involved in the pathogenesis of prostatic diseases, especially BPH. Our results also indicated that the zone-dependent staining for ERα and ERβ may explain, at least partly, the discrepancy for the distribution of ERs.

Materials and Methods

Chemicals and biochemicals

DAB (3,3′-diaminobenzidine/4HCl) was purchased from Dojin Chemical Co. (Kumamoto, Japan). SDS-PAGE was purchased from Daiichi Pure Chemicals Company Ltd. (Tokyo, Japan). Immobilon, polyvinylidene difluoride membranes, was purchased from Millipore Corp. (Bedford, MA). The enhanced chemiluminescence system was purchased from Amersham International (Buckinghamshire, UK). Yeast tRNA, salmon testicular DNA, BSA, Brij 35, Tween 20, and 3-aminopropyltriethoxysilane were purchased from Sigma (St. Louis, MO). Formamide was purchased from Nacalai Tesque (Kyoto, Japan).

Antibodies

Antiserum against human ERβ was obtained from a rabbit immunized with this peptide conjugated to keyhole limpet hemocyanin, as described previously (31). The synthetic oligopeptide sequence (511-CSPAEDSKSKEGSQNPQSQ-530) corresponding to the C-terminal amino acid residues of human ERβ (22) was selected. When the titer of antibody reached a plateau, the entire peripheral blood was drawn, and the serum was separated. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Nagasaki University School of Medicine.

Mouse monoclonal antihuman ERα IgG1 (1D5), normal goat serum, and normal rabbit serum were purchased from DAKO Corp. (Glostrup, Denmark). Mouse monoclonal antihuman androgen receptor (AR) IgG1 (NCL-AR-318) was purchased from Novocastra Laboratories (Newcastle, UK). Horseradish peroxidase (HRP)-conjugated goat antirabbit IgG F(ab′)$_2$ was purchased from MBL (Nagoya, Japan). HRP-conjugated goat antimouse IgG F(ab′)$_2$ was purchased from Chemicon International (Temecula, CA). Normal goat IgG and normal mouse IgG were purchased from Sigma. HRP-conjugated mouse monoclonal anti-T-T IgG was from Kyowa Medex (Tokyo, Japan).

All other reagents used in this study were from Wako Pure Chemical Industries Ltd. (Osaka, Japan) and were of analytical grade.

Tissue collection and preparation

Prostate tissues were obtained from 16 patients: 10 prostatic needle biopsy specimens (age, 55–75 yr) and 6 radical cystoprostatectomized prostates (age, 56–67 yr) from patients with primary muscle-invasive bladder cancer. Eleven of these tissues were diagnosed clinically and histopathologically as BPH, whereas the remaining 5 were normal. All specimens were obtained from patients at the Department of Urology, Nagasaki Atomic Bomb Hospital, in 2001. None of the patients had received any treatment before tissue sampling. The specimens of needle prostatic biopsies were obtained by the transperineal approach, under the guidance of transrectal ultrasonography. On the other hand, the samples of PZ or TZ were also taken from radical cystoprostatectomy, under direct vision. Some sections from all specimens were stained with hematoxylin and eosin for pathological examination to confirm the absence of PC and prostatic intraepithelial neoplasia.

For Western blot analysis, prostate tissue specimens (obtained from radical cystoprostatectomy) and human testis (obtained from patients with orchiectomy) for PC treatment were cut into several small pieces and rapidly frozen in liquid nitrogen and later used for the extraction of protein. For IHC, all prostatic samples were fixed with 10% neutral buffered formalin and embedded in paraffin using a standard procedure. The specimens were cut serially into 5-μm-thick sections and mounted on 3-aminopropyltriethoxysilane-coated glass slides.

IHC for ERα, ERβ, and AR

We used indirect enzyme-IHC for detection of ERα, ERβ, and AR. For IHC of ERβ, paraffin sections were dewaxed with toluene and rehydrated by serial graded ethanol solutions. Then the sections were microwaved at 95 C for 20 min in 10 mM citrate buffer (pH 6.0). The following protocol was essentially similar to that previously established for rabbit polyclonal antibody (31). Briefly, after washing the sections with PBS, endogenous peroxidase activity was inhibited by immersing the slides in 0.3% H_2O_2 in methanol for 30 min. After the slides were washed with PBS again, they were preincubated with 10% normal goat serum and 1% BSA in PBS for 1 h to reduce nonspecific binding of antibodies. Then, the sections were reacted overnight with anti-ERβ antiserum at a dilution of 1:400. After washing with 0.075% Brij 35 in PBS and rinsing with PBS, the sections were incubated with HRP-labeled goat antirabbit IgG F(ab′)$_2$ at a 1:200 dilution for 1 h. After washing with 0.075% Brij 35 in PBS and rinsing with PBS, the sites of HRP were visualized by DAB and H_2O_2. The sections were counterstained with Mayer's hematoxylin. As a control, some sections were reacted with normal rabbit serum instead of the specific antiserum, at the same concentration.

For IHC of ERα and AR, paraffin sections were processed in a manner similar to that described above. After preincubation with 500 μg/ml normal goat IgG and 1% BSA in PBS for 1 h, the sections were reacted with mouse monoclonal anti-ERα IgG1 at a dilution of 1:200 and anti-AR IgG1 at a dilution of 1:50 overnight. After washing with 0.075% Brij 35 in PBS and rinsing with PBS, sections were incubated with HRP-labeled goat antimouse IgG F(ab′)$_2$ at a dilution 1:100 for 1 h. After washing in 0.075% Brij 35 in PBS and rinsing in PBS, the sites of HRP were visualized with DAB and H_2O_2 and then counterstained with Mayer's hematoxylin. As a negative control, some sections were reacted with normal mouse IgG instead of the specific primary antibody, at the same concentration.

The frequency of positive cells for ERβ, ERα, and AR was graded as negative (−), occasionally positive (+), often positive (++), and abundantly positive (+++), relative to the background staining with normal rabbit serum or normal mouse IgG.

To confirm the zonal difference of ERβ expression in the stroma of BPH specimens, the proportion of ERβ-positive cells in the stroma of both PZ and TZ was calculated by counting at least 1000 cells at ×400