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Takeshi Mizuguchi · Itsuko Furuta · Yukio Watanabe
Kazuhiro Tsukamoto · Hiroshi Tomita
Mitsuhiro Tsujihata · Tohru Ohta · Tatsuya Kishino
Naomichi Matsumoto · Hisanori Minakami
Norio Niikawa · Koh-ichiro Yoshiura

LRP5, low-density-lipoprotein-receptor-related protein 5, is a determinant for bone mineral density

Abstract Osteoporosis is a multifactorial trait with low bone mineral density (BMD). We report results of an association study between BMD and nine candidate genes (*TGFB1, TGFBR2, SMAD2, SMAD3, SMAD4, IFNB1, IFNAR1, FOS* and *LRP5*), as well as of a case-control study of osteoporosis. Samples for the former association study included 481 general Japanese women. Among the nine candidate genes examined, only *LRP5* showed a significant association with BMD. We identified a strong linkage disequilibrium (LD) block within *LRP5*. Of five *LPR5* single nucleotide polymorphisms (SNPs) that are located in the LD block, three gave relatively significant results: Women with the C/C genotype at the c.2220C > T SNP site had higher adjusted BMD (AdjBMD) value compared to those with C/T and T/T ($p = 0.022$); and likewise, G/G at IVS17–30G > A and C/C women at c.3989C > T showed higher AdjBMD than those with G/A or A/A ($p = 0.039$) and with C/T or T/T ($p = 0.053$), respectively. The case-control study in another series of samples consisting of 126 osteoporotic patients and 131 normal controls also gave a significant difference in allele frequency at c.2220C > T ($x^2 = 6.737$, $p = 0.009$). These results suggest that *LRP5* is a BMD determinant and also contributes to a risk of osteoporosis.

Keywords Bone mineral density · Osteoporosis · Association study · Haplotype analysis · LRP5

T. Mizuguchi · Y. Watanabe · N. Matsumoto
N. Niikawa · K. Yoshiura (✉)
Department of Human Genetics,
Graduate School of Biomedical Sciences,
Nagasaki University, Sakamoto 1-12-4,
Nagasaki 852-8523, Japan
E-mail: kyoshi@net.nagasaki-u.ac.jp
Tel.: +81-95-8497120
Fax: +81-95-8497121

T. Mizuguchi · K. Tsukamoto · T. Ohta · T. Kishino
N. Matsumoto · N. Niikawa · K. Yoshiura
Core Research for Evolutional Science and Technology (CREST),
Japan Science and Technology Corporation (JST),
Kawaguchi, Japan

Y. Watanabe
Department of Obstetrics and Gynecology,
Asahikawa Medical College, Asahikawa, Japan

I. Furuta · H. Minakami
Department of Obstetrics and Gynecology,
Graduate School of Medicine,
Hokkaido University, Sapporo, Japan

K. Tsukamoto
Department of Clinical Pharmacy,
Graduate School of Biomedical Sciences,
Nagasaki University, Nagasaki, Japan

H. Tomita
Nagasaki Prefectural Medical Health Center,
Nagasaki, Japan

M. Tsujihata
Nagasaki Kita Hospital, Nagasaki, Japan

T. Ohta · T. Kishino
Division of Functional Genomics,
Center for Frontier Life Sciences,
Nagasaki University, Nagasaki, Japan

Introduction

Osteoporosis, a multifactorial trait with low mineral density (BMD), is common among postmenopausal women who could have bone fractures in spite of minimal traumas. The BMD, a good scale to predict the risk of subsequent fractures, is regulated by both polygenes and environmental factors. Although both twin and family studies have shown that 50–80% of age-related variance of BMD are genetically determined (Eisman 1999; Stewart and Ralston 2000), genetic susceptibility to osteoporosis has not fully been understood. Previous studies demonstrated that BMD is associated with several genes/proteins. They included genes for parathyroid hormone (*PTH*) (Hosoi et al. 1999), alpha 1 chain of type I collagen (*COL1A1*) (Grant et al. 1996), vitamin D receptor (*VDR*) (Morrison et al. 1994), transforming growth factor beta 1 (*TGFB1*) (Yamada et al. 1998;

Bertoldo et al. 2000), interleukin-6 (*IL6*) (Ota et al. 2001), osteoprotegerin (*OPG*) (Ohmori et al. 2002), and tumor necrosis factor receptor superfamily, member 1B (*TNFRSF1B*) (Spotila et al. 2000; Albagha et al. 2002). However, conflicting results have been reported in some of them.

Analysis of a monogenic disorder, Camurati-Engelmann disease (MIM #131300), uncovered TGF-β1 as one regulatory factor of bone metabolism (Kinoshita et al. 2000; Saito et al. 2001). A T-to-C transition at the nucleotide position $+29$ in the signal sequence region of the TGF-β1 gene (*TGFB1*) showed a significant association with bone mass at the lumbar spine in Japanese postmenopausal women (Yamada et al. 1998). Both *Ifnb1*-/- and *Ifnar1*-/- mice exhibited characteristic features of osteoporosis (Takayanagi et al. 2002). Nonsense or missense mutations of *LRP5*, which encodes the transmembrane-spanning protein involving in the Wnt signaling pathway, caused autosomal recessive osteoporosis-pseudoglioma syndrome (OPPG, MIM #259770) (Hey et al. 1998; Gong et al. 2001), while heterozygotes for truncation type mutations showed lower BMD than age- and gender-matched controls (Gong et al. 2001). Heterozygous glycine-to-valine substitution at codon 171 in *LRP5*-exon 3 was found in autosomal dominant high-bone-mass trait (HBM, MIM #601884) (Boyden et al. 2002; Little et al.2002). Therefore, all these genes may contribute to the BMD phenotype.

Here we report on the results of two association studies: a study with a series of samples between BMD and either of the candidate genes *TGFB1*, *TGFBR2*, *SMAD2*, *SMAD3*, *SMAD4*, *IFNB1*, *IFNAR1*, *FOS* and *LRP5*; and a case-control study with the other series between osteoporosis and *LRP5*.

Materials and methods

Association study between BMD and TGFB1, TGFBR2, SMAD2, SMAD3, SMAD4, IFNB1, IFNAR1, FOS or LRP5

Subjects and their BMD values

The subjects studied included 481 healthy Japanese women aged 54.2 ± 12.4 years who had a health checkup provided by the Nagasaki Prefectural Government as a population-based system. They underwent a radiological examination to be evaluated for BMD values. BMD measurements were performed at the left radial bone by dual-energy X-ray absorptiometry (DEXA) using DTX-200 DexaCare (Osteometer MediTech Inc., Hawthorne, CA, USA). Because BMD is affected by many factors, differences by age, height and weight were calculated with multiple regression analysis using Statview software (SAS Institute Inc. Cary, NC, USA) and adjusted with the following correcting formulas: body mass index (BMI)=body weight (kg)/body height^2 (m^2); and adjusted BMD (AdjBMD) value=BMD$-0.00447 \times (54.23-$age$) + 0.00802 \times (22.65-$BMI). The AdjBMD values among the samples were statistically analyzed by Student's t test. Study protocols were approved by the Committee for the Ethical Issues on Human Genome and Gene Analysis at Nagasaki University. Under written, informed consent, genomic DNA was extracted from participants using the standard method.

Single nucleotide polymorphism (SNP) identification and genotyping

For *TGFB1*, *TGFBR2*, *SMAD2*, *SMAD3*, and *SMAD4*, we used 106 SNPs and 11 other types of variations together with their primers, reported previously (Watanabe et al. 2002). Four primers were generated to cover the *IFNB1* sequence between 1 kb upstream from the translation initiation site and 800 bp downstream from the termination codon. Two and five primer sets were made for *IFNAR1* and *FOS*, from the JSNP database (Hirakawa et al. 2002). To identify highly informative SNPs in these genes, we recruited 16 healthy Japanese volunteers other than the samples above and confirmed six SNPs in *IFNB1*, five in *IFNAR1*, and seven in *FOS*. SNP genotyping for *TGFB1* and the four other related genes was performed by the TaqMan PCR procedure (Applied Biosystems, Foster City, CA, USA). Genotype for other genes was determined by the PCR sequencing method. In short, after the amplified products were purified with ExoSAP-IT (Amersham-Pharmacia, Cleveland, OH, USA), they were subjected to sequencing using BigDye terminator kit, version 3 (Applied Biosystems) on an ABI 3100 DNA automated sequencer (Applied Biosystems). Electropherograms were aligned by Auto-Assembler software (Applied Biosystems) to detect SNPs and genotype.

A total of 23 primers were made for *LRP5* to amplify all exons and exon-intron boundaries. Two other primers were also generated to verify intron 1 SNPs deposited in the GenBank and in the JSNP database. Seventy-seven samples were sequenced to identify *LRP5* SNPs and genotyped by either PCR-direct sequencing or the TaqMan PCR procedure.

Statistical analysis

The 481 samples were divided into three groups according to their BMD values. Group 1 (low BMD group) consisted of 60 women with BMD < 0.33 g/cm^2 and age > 50 years, group 2 (high BMD group) included 46 women with BMD > 0.50 g/cm^2 and age > 50 years, and group 3 was comprised of the other 375 women. As for genes involving the TGF-β1 and interferon β signaling pathways, we chose SNPs referring both to allele frequencies between groups 1 and 2 and to linkage disequilibrium (LD) data. When allele frequency distortion was observed between the two groups, we expanded genotyping to all 481 samples and then compared the average AdjBMD value in each genotype by Student's t test. For *LRP5*, we quantified LD, expressed as "D" and "r-square", between all pairs of biallelic loci using SNPAlyze program (Dynacom, Yokohama, Japan). The haplotype frequencies and the estimated diplotype of each individual were also calculated by SNPAlyze using genotyping data. Quantitative associations between genotypes/haplotypes and AdjBMD were analyzed by Statview software. Comparison of the average AdjBMD value in each genotype/haplotype was done by Student's t test. We categorized genotypes C/C, C/T, and T/T at LRP5–9 in each of the 481 women into three incremental values, 0, 1, and 2, to calculate the Spearman's correlation coefficient.

Case-control study on osteoporosis and *LRP5*

The samples used consisted of 126 women with osteoporosis and 131 normal control women, all of whom were admitted to the Obstetrics and Gynecology Department of Hokkaido University Hospital for cancer screening, osteoporotic risk evaluation, hormone replacement therapy, or treatment for a mild-to-moderate climacteric disorder. Medical history was obtained from all these women, and those with disorders known to affect BMD were excluded. BMD at the lumbar spine (L2–L4) was measured by dual-energy X-ray absorptiometry (DEXA) with QDR2000 instrumentation (Hologic Inc., Waltham, MA, USA). The Z-score values for lumbar BMD showing a deviation from the weight-adjusted average BMD of each age were calculated based on the previously reported data for the general Japanese women (Soda et al. 1993).

Diagnosis was made under the World Health Organization (WHO) criteria established in 1994. All women were genotyped for five SNPs (LRP5–1, LRP5–8, LRP5–9, LRP5–20, and LRP5–21) in *LRP5*, and allele frequencies were statistically analyzed by χ^2 test.

Results

By the χ^2 test for allele frequencies in group 1 and 2 samples in the study on BMD, two SNPs in *TGFB1* (rs1800469 and rs1982073), one each in *TGFBR2* (IVS3 + 21274T > A), *SMAD2* (rs1787186) and *SMAD4* (rs2276163), and two each in *IFNB1* (rs1424855 and rs1364612) and *IFNAR1* (rs2253413 and rs2243599) were found to show relatively low *p* values. Comparison among the 481 samples revealed no significant association between their AdjBMD values and genotypes and between the AdjBMD values and combinations of genotypes for these SNPs ($p > 0.05$).

With 21 (14 known and 7 novel) SNPs in *LRP5* (Table 1), we genotyped 77 of the 481 samples to analyze LD within the gene region. We chose nine of the 21 SNPs showing minor allele frequency > 0.2 to identify LD block and defined a strong LD region between two SNPs, LRP5–7 (IVS7–118T > G) and LRP5–21 (c.3989C > T) (Fig. 1, Table 2). Using 15 SNPs located in the LD block, we identified four common haplotypes with $> 5\%$ frequency, accounting for 89% of all chromosomes. We then determined three haplotype-tagging SNPs (HT-SNPs) to distinguish these four common haplotypes from each other and to capture the majority

of haplotype diversity (Johnson et al. 2001) (Fig. 1). Regarding these three HT-SNPs and their neighboring SNPs, LRP5–10 (IVS10 + 6T > C), LRP5–11 (IVS10 + 100A > G), LRP5−21 (c.3989C > T), we expanded genotyping to all 481 samples. We estimated haplotype frequencies and diplotypes among the 481 samples with SNPAlyze. Association study on *LRP5* revealed that women with one copy (heterozygotes) or two copies (homozygotes) of haplotype 3 had significantly lower AdjBMD than those without it ($p = 0.023$) (Table 3).

Of five SNPs located within the LD block, three, LRP5–9 (c.2220C > T), LRP5–20 (IVS17–30G > A), and LRP5–21 (c.3989C > T), were identified to show a statistically significant association with AdjBMD values (Table 3). In LRP5–9, women with C/C genotype had a higher AdjBMD value, compared to those with C/T and T/T ($p = 0.022$). Furthermore, the Spearman's correlation coefficient calculated for the three genotypes revealed that C/C women had the highest AdjBMD value, C/T women an intermediate value, and T/T women the lowest value ($r = -0.118$, $p = 0.009$), suggesting a dosage effect of alleles on BMD values (Fig. 2). Likewise, women with the G/G genotype for LRP5–20 showed higher AdjBMD than those with G/A or A/A genotypes ($p = 0.039$) and women with C/C for LRP5–21 revealed higher values than those with C/T or T/T genotypes ($p = 0.053$). In other words, since minor alleles of these three SNPs almost represent haplotype 3, heterozygotes or homozygotes for the minor alleles had significantly lower AdjBMD values than those without

Table 1 Single nucleotide polymorphisms (SNPs) in *LRP5* used in the present study. Adenine of the ATG initiation codon is denoted as nucleotide +1 in exon. Intronic SNPs are designated by the intron number. Plus number is counted from guanine of GT splice donor site, and minus number is from guanine of AG splice acceptor site. Allele frequencies were estimated among 77 samples selected from 481 Japanese women

| SNPs	Position	Base alteration	Amino acid substitution	Allele frequency (minor allele)	dbSNP in NCBI and/or JSNP
LRP5–1	Intron 1	IVS1 + 4689G > C		0.453 (G)	rs312014
LRP5–2	Intron 1	IVS1−4340G > A		0.060 (A)	
LRP5–3	Intron 1	IVS1−4226T > C		0.099 (C)	rs74744, JST-IMS137909
LRP5–4	Intron 1	IVS1−4106G > A		0.039 (A)	rs74743
LRP5–5	Exon 2	c.266A > G	Q89R	0.087 (G)	
LRP5–6	Intron 6	IVS6 + 8G > A		0.061 (A)	
LRP5–7	Intron 7	IVS7−118T > G		0.283 (G)	rs686921
LRP5–8	Exon 8	c.1647C > T	F549F	0.072 (T)	rs545382
LRP5–9	Exon 10	c.2220C > T	N740 N	0.211 (T)	ds2306862, JST-IMS064599
LRP5–10	Intron 10	IVS10 + 6T > C		0.039 (C)	rs4988322
LRP5–11	Intron 10	IVS10 + 100A > G		0.293 (G)	rs576118
LRP5–12	Intron 10	IVS10 + 120T > C		0.283 (C)	rs667126
LRP5–13	Intron 10	IVS10−173A > G		0.041 (G)	
LRP5–14	Intron 11	IVS11 + 37C > T		0.039 (T)	rs2242339, JST-IMS014541
LRP5–15	Intron 11	IVS11 + 78G > A		0.276 (A)	rs689179, JST-IMS014542
LRP5–16	Intron 16	IVS16−213T > C		0.342 (C)	rs608343
LRP5–17	Intron 16	IVS16−82C > T		0.336 (T)	rs607887, JST-IMS064603
LRP5–18	Intron 17	IVS17−125G > A		0.046 (A)	
LRP5–19	Intron 17	IVS17−63C > T		0.046 (T)	
LRP5–20	Intron 17	IVS17−30G > A		0.092 (A)	
LRP5–21	Exon 18	c.3989C > T	A1330V	0.217 (T)	JST-IMS080311
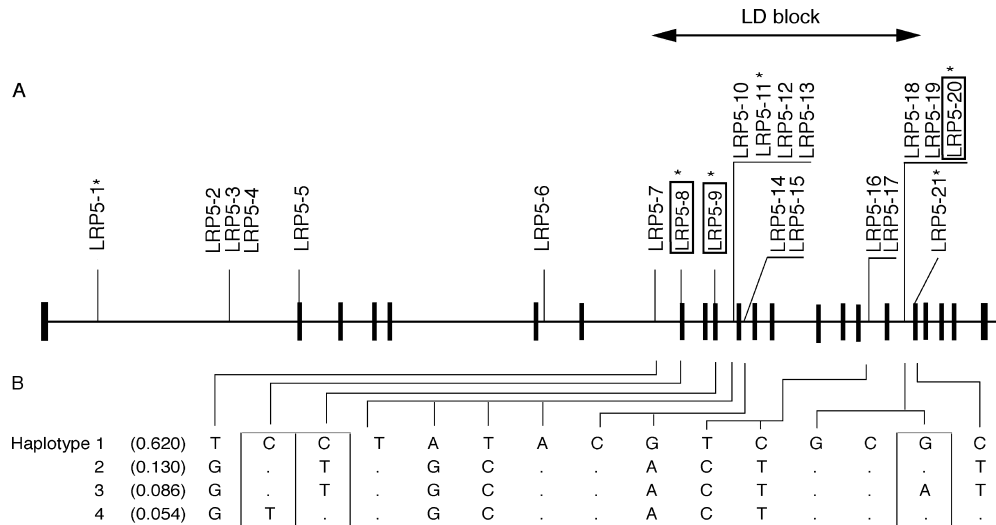
Fig. 1A, B Single nucleotide polymorphisms (SNPs) identified in *LRP5*, and four common haplotypes constructed based on the SNPs. **A** Gene structure of *LRP5* and SNP sites. *Black vertical lines* indicate exons. *Asterisks* and *open boxes* indicate SNPs used for association study and HT-SNPs, respectively. **B** Haplotype diversity in the region screened. The four haplotypes account for 89% of all chromosomes. *Dots* show the same SNP alleles as those found in haplotype 1, and SNPs in *boxes*, corresponding to the HT-SNPs above, are those used to distinguish the four common haplotypes. Haplotype frequencies are shown in *brackets*

them. There was no association between AdjBMD and LRP5–1 (IVS1 + 4689G > C) located outside the LD block (data not shown).

The case-control study on *LRP5* and osteoporosis revealed that the allele "T" at LRP5–9 SNP was more frequently deposited in patients (*n* = 126) than in normal control women (*n* = 131) (*p* = 0.009) (Table 4). A similar association was also observed when sampling postmenopausal women with osteoporosis (*n* = 99) and their controls (*n* = 88) (*p* < 0.01). Significant difference (*p* = 0.001) in allele frequency was also observed at a rare SNP, LRP5–8 (c.1647C > T) (Table 4).

Discussion

Prior to the present candidate gene-oriented association studies between AdjBMD and osteoporosis, we searched and identified SNPs for *TGFB1, TGFBR2, SMAD2, SMAD3, SMAD4, IFNB1, IFNAR1, FOS,* and *LRP5* in the normal Japanese population of women. A total of 481 women having a health checkup were subjects for this study, their BMD values were adjusted by age and BMI, and they were classified by genotype for SNP alleles. There was no significant difference in AdjBMD value among any SNP genotypes of the eight former genes. Although a C- > T SNP (P10L) at + 29 in *TGFB1* exon 1 was reported to be associated with BMD in postmenopausal Japanese women (Yamada et al. 1998), no such relationship was reproduced in our series of samples. The discrepancy between the two studies may be due to the small sample size of our series, different populations used, and/or different skeletal sites examined for BMD. Alternatively, the C- > T substitution may not generally affect BMD values in Japanese

Table 2 Pairwise linkage disequilibrium (LD) coefficients for nine *LRP5* single nucleotide polymorphisms (SNPs) with the allele frequency > 0.20. LD, expressed as |D'| and *r* square, is calculated with genotype data of 77 samples. Pairs in LD, |D'| > 0.9 and *r* square > 0.5 are shown by *italics* and **bold** respectively

SNPs	|D'| and r^2	SNPs							
		LRP5–7	LRP5–9	LRP5–11	LRP5–12	LRP5–15	LRP5–16	LRP5–17	LRP5–21
LRP5–1	|D'|	0.001	0.219	0.162	0.062	0.066	0.100	0.152	0.219
	r^2	0.000	0.011	0.009	0.001	0.001	0.006	0.014	0.011
LRP5–7	|D'|		*1.000*	*0.966*	*0.967*	*0.966*	*0.962*	*0.925*	*0.954*
	r^2		**0.676**	**0.876**	**0.936**	**0.904**	**0.702**	**0.668**	**0.639**
LRP5–9	|D'|			*1.000*	*1.000*	*1.000*	*1.000*	*1.000*	*1.000*
	r^2			**0.634**	**0.676**	**0.698**	**0.513**	**0.528**	**0.962**
LRP5–11	|D'|				*1.000*	*1.000*	*0.928*	*0.892*	*0.952*
	r^2				**0.938**	**0.908**	**0.696**	**0.663**	**0.598**
LRP5–12	|D'|					*1.000*	*1.000*	*0.963*	*0.954*
	r^2					**0.968**	**0.759**	**0.724**	**0.639**
LRP5–15	|D'|						*1.000*	*0.961*	*0.955*
	r^2						**0.734**	**0.699**	**0.662**
LRP5–16	|D'|							*1.000*	*1.000*
	r^2							**0.971**	**0.533**
LRP5–17	|D'|								*1.000*
	r^2								**0.549**

Table 3 Comparison of adjusted BMD (AdjBMD) values with diplotypes for haplotype 3 (H3)/other haplotypes, and with genotypes for single nucleotide polymorphisms (SNPs) in *LPR5* among 481 women

Locus or SNP site	Diplotype or genotype	No. of samples	AdjBMD value (g/cm^2)	p value
Haplotype	others/others	377	0.442 ± 0.064*	
	H3/others	94	0.424 ± 0.063	
	H3/H3	10	0.435 ± 0.046	
	H3/H3 + H3/others	104	0.425 ± 0.062*	0.023
LRP5–9	C/C	275	0.444 ± 0.065**	
	C/T	166	0.432 ± 0.064	
	T/T	40	0.425 ± 0.051	
	C/T + T/T	206	0.430 ± 0.062**	0.022
LRP5–20	G/G	375	0.441 ± 0.064***	
	G/A	93	0.426 ± 0.064	
	A/A	13	0.434 ± 0.045	
	G/A + A/A	106	0.427 ± 0.062***	0.039
LRP5–21	C/C	271	0.443 ± 0.065****	
	C/T	165	0.434 ± 0.065	
	T/T	45	0.424 ± 0.049	
	C/T + T/T	210	0.432 ± 0.062****	0.053

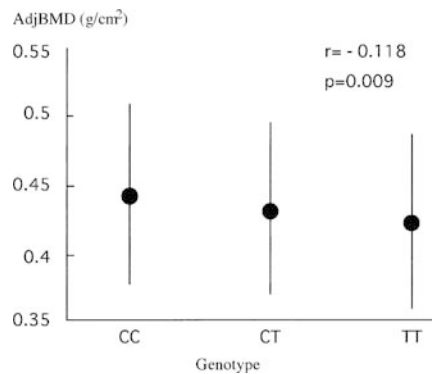Comparisons were done between values with asterisks by Student's *t* test

Fig. 2 Correlation between adjusted bone mineral density (AdjBMD) values (mean ± SD) and three genotypes at LRP5–9 (c.2220C > T) among 481 women. Spearman's correlation coefficient (*r*) was calculated after categorizing the genotypes CC, TC, and TT among the women into three incremental values, 0, 1, and, 2 respectively

Table 4 Allele frequency distribution between patients with osteoporosis and normal controls

SNP	Allele frequency (minor allele)		x^2	p value
	Patients	Controls		
LRP5–1	0.472 (C)	0.511 (C)	0.791	0.374
LRP5–8	0.008 (T)	0.061 (T)	10.731	0.001*
LRP5–9	0.302 (T)	0.202 (T)	6.737	0.009*
LRP5–20	0.123 (A)	0.084 (A)	2.118	0.146
LRP5–21	0.302 (T)	0.237 (T)	2.759	0.097

* Significant difference

esis (Takayanagi et al. 2002). Under a hypothesis that the two genes as well as *FOS* might be candidates for osteoporosis, we examined whether a certain genotype is associated with BMD in our series of samples. We studied four SNPs, i.e., rs1424855 and rs1364612 in *IFNB1*, and rs2253413 and rs2243599 in *IFNAR1*. However, no significant association was observed. Thus, it is less likely that all the eight genes are major genetic determinants for BMD. Since all 481 women in the present study were from a small district in Nagasaki Prefecture, the samples may appear homogeneous with a similar genetic background.

By a survey of LD patterns in *LRP5* and its flanking region in the European population, Twells et al. (2003) identified three seeming LD blocks, such as a block between intron 1 and the 5' flanking region, another block between introns 1 and 3, and the other encompassing from exon 17 to the 3' flanking region. The LD block we identified between LRP5–7 and LRP5–21 (or between intron 7 to exon 18) overlaps with the last LD block found by Twells et al. (2003). Among the four common haplotypes which we constructed within the LD block, haplotype 3 appears significantly associated with AdjBMD, as heterozygotes or homozygotes for it had lower AdjBMD than those without it (*p* < 0.023). Furthermore, association study on three SNPs in the block gave significant results with low *p* values (< 0.022–0.053). Among them, c.2220C > T at exon 10 is a synonymous substitution, and IVS17–30G > A at intron 17 does not seem to affect the splicing process. Although c.3989C > T at exon 18—an alanine-to-valine substitution at codon 1330—is located at the low-density lipoprotein receptor domain class A that contains seven cysteine-rich repeats and highly conserved, negatively-charged amino acids, these two amino acids involved in the substitution are chemically similar. Therefore, it is less likely that any of these SNPs directly alter the LRP5 gene function, suggesting the existence of another significant SNP within the LD block.

The case-control study on *LRP5* also gave a positive result, i.e., the T allele at LRP5–9 may predispose to osteoporosis. The finding is compatible with that of the present association study in the general population that homozygotes for the major allele (C) at the SNP site had

women. By a genome-wide screening, Koller et al. (2000) provided evidence for a linkage of polymorphic markers to BMD and obtained various LOD scores (θmax = 1.87–3.5) at four different skeletal regions. In their report, the radial bone we used was not included. Albagha et al. (2002) showed an association between allelic variations in *TNFRSF1B* and BMD at the femoral neck, while no association was found with lumbar spine BMD. Thus, a specific gene, even if it is associated with BMD, may have different effects on BMD at different skeletal sites. It remains to be seen whether an association is present at other bone regions in our series of samples.

Mice deficient in either *Ifnb1* or *Ifnar1* exhibited reduced BMD, resulting from enhanced osteoclast gen-

significantly higher AdjBMD than those without it. However, there was no statistical significance between case and control at LRP5–20 or LRP5–21. This may be explained by the small sample size in the case-control study. A recently performed whole genome linkage study supported our results by showing an association of BMD with markers at 11q12–13 (Koller et al. 1998), although other studies never gave any association (Deng et al. 2001; Wilson et al. 2003). In conclusion, both the present association and case-control studies suggest that *LRP5* is a BMD determinant and also contributes to a risk of osteoporosis.

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