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Original Article

Association between single nucleotide polymorphisms of the estrogen receptor 1 and receptor activator of nuclear factor kappa B ligand genes and bone mineral density in postmenopausal Taiwanese

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Abstract

Objective: To investigate the relationship between single nucleotide polymorphisms (SNPs) of the genes encoding the estrogen receptor 1 (*ESR1*) and the receptor activator of nuclear factor kappa B ligand (*RANKL*) and bone mineral density (BMD) in postmenopausal Taiwanese.
Materials and Methods: Five *ESR1* SNPs and three *RANKL* SNPs in 467 women were genotyped. Results of genotyping were correlated with BMD that had been adjusted for body mass index (BMI), age, and years after menopause.
Results: Those with the *ESR1* $C_{rs1884054}$ allele were found to have a lower BMD at LS_{2-4}/Lateral view ($p = 0.005$ and permutated $p = 0.046$), and those with the *ESR1* haplotype $T_{rs2234693}$-$A_{rs922996}$ had a higher risk for low BMD also at LS_{2-4}/Lat (OR = 1.8, 95% CI = 1.1-2.9). In addition, women without the *RANKL* haplotype $G_{rs2148072}$-$C_{rs2200287}$-$G_{rs922996}$ had a higher risk for low BMD at LS_{1-4}/AP (OR = 2.09, 95% CI = 1.21 ~ 3.64). Stratification analyses revealed that those with *ESR1* $AA_{rs1884054}$ and *RANKL* $A_{rs2148072}$ ($p = 0.032$) or *RANKL* $T_{rs2200287}$ ($p = 0.007$) had a lower BMD at LS_{1-4}/AP.
Conclusion: Genotypes of these SNPs of *ESR1* and *RANKL* may help us predict the osteoporosis risk in menopausal women.

Keywords: bone mineral density; *ESR1*; single nucleotide polymorphism; *RANKL*; *TNFSF11*

Introduction

Osteoporosis is a condition of decreased bone mass. The chance of developing osteoporosis increases with age, and the prevalence of osteoporosis is higher in females. Bone mineral density (BMD) is a useful indicator for assessment of osteoporosis. In twin and family studies, 50-80% of variations in BMD are attributed to genetic factors [1–3]. More than 100 candidate genes related to BMD have been found, including those of the sex steroid and the OPG-RANK-RANKL pathways [2,3]. Estrogen plays a critical role in bone homeostasis as shown in the observation that women experience an accelerated loss of bone mass after menopause when their serum estrogen levels start to decrease [4]. Low estrogen levels lead to imbalanced bone metabolism and increased production of pro-inflammatory cytokines such as IL-1, IL-6, and TNF-α [5]. IL-6 facilitates the proliferation of osteoclast

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precursors, while IL-1 and TNF-α enhance the function of osteoclasts, thus increasing bone resorption.

Another important factor that promotes bone resorption is the signal cascades activated by the receptor activator of nuclear factor kappa B ligand (RANKL). RANKL binds to its receptor RANK on osteoclast precursor cells and activates osteoclastogenesis leading to an increase in the number of osteoclasts and a decrease in BMD [6]. RANKL also enhances the function and survival of osteoclasts and exacerbates bone resorption. Estrogen prevents bone loss by decreasing the production of RANKL in bone marrow cells [7], thus inhibiting the ability of hematopoietic cells to form osteoclasts in response to RANKL. Therefore, the interaction between estrogen and RANKL is one of most important factors in speedy bone loss after menopause.

The functions of estrogen are mainly mediated through its receptor, estrogen receptor 1 (*ESR1*). Studies have shown that *ESR1* knockout mice exhibit a severe bone loss [8], whereas *RANKL* knockout mice display a decreased number of osteoclasts and severe osteopetrosis [9]. Since both *ESR1* and *RANKL* have major effects on BMD, a number of studies on *ESR1* and *RANKL* (also known as *TNFSF11*) genes have been conducted. Both *ESR1* [10,11] and *RANKL* [12,13] genes have been found to be significantly associated with BMD. However, the results on the association of SNPs of these two genes with spine or hip BMD in postmenopausal women remain controversial, probably owing to the differences in race. Therefore, we decided to determine the association between the polymorphisms of *ESR1* and *RANKL* and BMD in a Taiwanese postmenopausal population. The effects of gene-gene interaction between *ESR1* and *RANKL* on BMD were also investigated in this study.

Materials and methods

Study participants

Participants, aged 45-63 years, were selected from the volunteers who visited the Menopause Health Education Clinic at the Chang Gung Memorial Hospital-Kaohsiung Medical Center, Taiwan, between August 2002 and December 2007. None of the women had any history of major surgeries, such as bilateral oophorectomy or hip or joint replacement. Patients with conditions that may affect bone mass were excluded, including chronic disorders of vital organs, metabolic diseases (diabetes, hypo- or hyperparathyroidism, and hyper- or hypothyroidism), skeletal diseases (Paget's disease, osteogenesis imperfecta, and rheumatoid arthritis), and malnutrition conditions, such as those resulting from chronic diarrhea or ulcerative colitis. Chronic users of drugs that may affect bone metabolism such as corticosteroid, anticonvulsions, anti schizophrenia, antibone resorption, and immunosuppressive drugs were also excluded. A total of 500 healthy postmenopausal women who met the criteria were selected. Informed consents were obtained from each woman. The study protocol was approved by the Institutional Review Board of Chang Gung Memorial Hospital.

Measurements of BMD and other covariates

BMD (g/cm^2) of the lumbar spine LS_1-LS_4 AP (LS_{1-4}/AP) view and LS_2-LS_4 lateral (LS_{2-4}/Lat) view, total hip, and femur neck was measured using a dual energy X-ray absorptiometer (DXA, Hologic Delphi A, MA, USA). The T-scores that indicate the quality of bone mass were calculated automatically by built-in software using the data of healthy American-Japanese women as the reference. Body height was measured using a fixed stadiometer, and body weight without shoes was measured on a standard clinical scale. BMI was calculated as kg/m^2. The weekly intake of calcium of each woman was estimated on the basis of the consumption of calcium and multivitamin supplements and high-calcium content foods such as dried tofu, dried small fish or shrimps, seaweeds, and green beans.

SNP genotyping

Genomic DNA was extracted from 300 μL of peripheral blood from each woman using the Puregene DNA purification kit (Gentra system, USA), dissolved in 100 μL of the DNA hydration solution in the kit, and stored in a -20 °C freezer until used. The concentration of each DNA sample was approximately 100 μg/ml. The entire gene and 2 kb on both 5′ and 3′ sides of the gene of both *ESR1* and *RANKL* were searched for tag SNPs (tSNPs) that are representative SNPs of a certain haploblock. Only the tSNPs with a pair-wise linkage disequilibrium value $r^2 > 0.8$ and a minor allele frequency >0.2 in the Han Chinese reference panel (CHB) of the International HapMAP Project were selected [14]. The *ESR1* tSNPs selected for this study included rs9340954, rs1884054, rs3020314, rs9340799, rs2234693, and rs3798577. For *RANKL* tSNPs, rs922996, rs2200287, and rs2148072 were selected. All of these SNPs were located in introns (Fig. 1) [11]. A small fragment of 200-650 bp containing a certain SNP in the genome of each woman was amplified by PCR. The PCR products were then used to perform allele-specific extensions, and the extended products were analyzed by matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry as described previously [15–17]. Oligonucleotide primers used for SNP genotyping are shown in Tables 1 and 2.

Statistical analyses

The Hardy-Weinberg equilibrium (HWE) test was performed for each SNP to determine whether the distribution of its allele frequency deviates from expectation. The association between SNP and adjusted BMD and the effects of gene-gene interaction on BMD were assessed by the additive linear regression analysis. BMD was adjusted for age, BMI, and years after menopause. Haplotype and haploblock were constructed by using the Haploview. All other statistical analyses were performed using the software SPSS (version 15.0; SPSS Inc., Chicago, IL). A p value <0.05 was considered significant after being corrected for multiple tests by the permutation scan

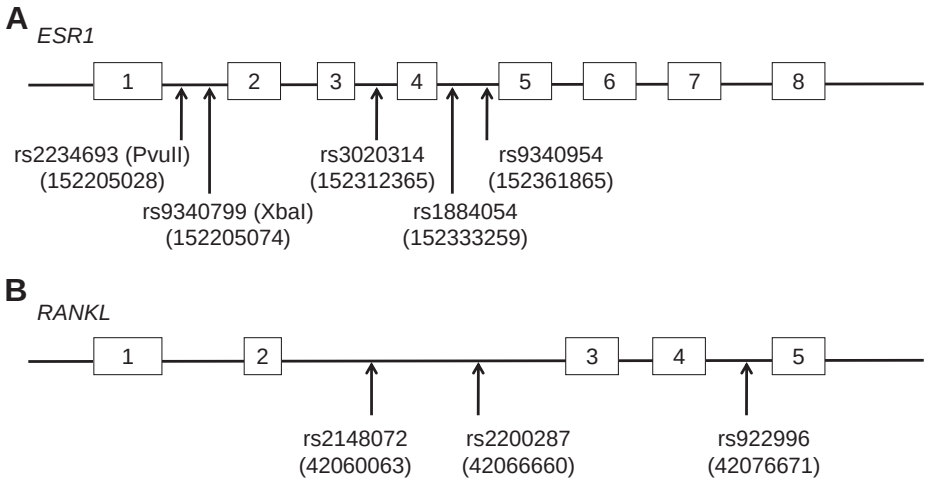

Fig. 1. Locations of various SNPs examined in this study. (A) *ESR1*. (B) *RANKL*. Boxed regions are exons. Numbers in parentheses are nucleotide numbers. *ESR1* is located at human chromosome 6q25.1, and *RANKL* is located at 13q14. *ESR1* rs2234693 and rs9340799 were previously referred to as PvuII and XbaI RFLP sites, respectively [11].

statistics. Odds ratios were determined by logistic regression analysis and used to assess the strength of association of various genotypes with normal or low BMD.

Results

Baseline demographics of participants

The mean year after menopause of all participants was 2.64 ± 2.3, and 85.4% of the women had menopause for less than or equal to 5 years. Demographics and relevant clinical information of the participants, including age, years after menopause, body weight and height, BMI, BMD at various anatomic regions, serum levels of FSH and E2, and average weekly calcium intake, are summarized in Table 3.

Allele frequencies of selected RANKL and ESR1 tSNPs and their associations with BMD

Six *ESR1* and three *RANKL* tSNPs of each woman were genotyped, and 467 of the 500 participants were successfully typed for all the tSNPs. For *ESR1*, the minor allele frequencies (MAFs) of tSNPs rs9340954 (A > G), rs1884054 (C > A), rs3020314 (C > T), rs9340799 (T > G), and rs2234693 (T > C) were 43.3%, 31.2%, 19.7%, 20.6%, and 40.9%, respectively (Table 4). For *RANKL*, the MAFs for tSNPs of rs922996 (A > G), rs2200287 (T > C), and rs2148072 (A > G) were 48.4, 19.2, and 19.3%, respectively (Table 4). These MAFs were very similar to those of the Beijing Han population in the International HapMap project (HapMap Build 35). Genotype distribution of *ESR1* rs3798577 was deviated from HWE with a $p < 0.0001$; therefore, this tSNP was excluded for further analysis. With the genetic additive dominant modeling, only the *ESR1* rs1884054 (C > A) polymorphisms were found to have a significant correlation with BMD at LS_{2-4}/Lat ($p = 0.004$; $p_c = 0.046$) after correction for multiple tests by full scan permutation analysis (Table 4). Participants with the *ESR1* Crs1884054 allele were found to have a lower BMD at LS_{2-4}/Lat than those with the AA genotype.

BMD T-scores at four different bone sites and their relationship with ESR1 rs1884054

Based on the built-in reference BMD values in the DXA system, the individuals were divided into two categories, normal T-score (≥−1.0 SD) and low T-score (<−1.0 SD), according to the WHO criteria. Those with the *ESR1*

Table 1
PCR primers for amplification of regions containing SNP.

SNP	Forward primer sequence	Reverse primer sequence	Length (bp)
RANKL rs2148072	ATGGGAAACAGATCCCCTTG	CAAAGGGAAAGAGGCAATGA	647
RANKL rs2200287	TCAGAGCTGGCTCAATCTCA	GTTTGGGGCAGTTATTCAGC	501
RANKL rs922996	TCCTTCTCTAGAGGCCCACA	TTTCTGGACAGAGGGATTGG	225
ESR1 rs2234693	CATGAACCACCATGCTCAGT	ACCAATGCTCATCCCAACTC	269
ESR1 rs9340799	CATGAACCACCATGCTCAGT	AGACCAATGCTCATCCCAAC	300
ESR1 rs3020314	TGGACCAGTAAACCCTGCTC	TGCATATTGCCAGTCCAGAG	401
ESR1 rs1884054	CACAGGTTCCTCTCCTCCAG	CAAAGGGCCAAGTTCCATAA	494
ESR1 rs9340954	TTGCCATGGATTCCTAGTCC	GCTTTCTCTGGTGCCTGAAC	511
ESR1 rs3798577	TGCATGATGAGGGTAAATGG	CCTAGGTAGCTGCAGCCTGT	205

Table 2
Primers used for allele-specific extension and mass of extended products.

Allele	Primer for extension reaction	Mass of allele 1[a]	Mass of allele 2[b]
RANKL rs2148072 G/A	GCCACTATTTCTCATTT	G = P + G, 5399.5	A = P + AC, 5672.6
RANKL rs2200287 C/T	CATTTACAGCAAAGGATACG	C = P + C, 6407.1	T = P + TC, 6711.3
RANKL rs922996 C/T	GCCATCCAACGGTGGGGCAA	C = P + C, 6425.1	T = P + TC, 6729.3
ESR1 rs2234693 C/T	AGTTCCAAATGTCCCAGC	C = P + C, 5716.6	T = P + TG, 6060.9
ESR1 rs9340799 G/A	GACCCTGAGTGTGGTCT	G = P + G, 5530.6	A = P + AG, 5843.8
ESR1 rs3020314 C/T	TCCTGGAGAGATGACAGAAG	C = P + C, 6488.1	T = P + TC, 6792.3
ESR1 rs1884054 C/A	TTTGTAGGGAAGCAAAT	C = P + C, 5546.5	A = P + AC, 5859.7
ESR1 rs9340954 G/T	TGTATCAGCGTCAATGTCTGAGT	G = P + G, 7382.8	T = P + TG, 7687.0
ESR1 rs3798577 T/C	TGGGGCATGGAGCTGAACAGTAC	T = P + T, 7441.7	C = P + CT, 7730.9

[a] Unextended product; [b] Extended product.

Crs1884054 allele had a higher risk for low bone mass than those with the AA genotype at all bone sites examined, including LS_{2-4}/Lat (OR = 2.54, $p = 0.006$), LS_{1-4}AP (OR = 1.84, $p = 0.03$), total hip (OR = 3.26, $p = 0.008$), and femur neck (OR = 1.93, $p = 0.02$) (Table 5).

Linkage disequilibrium between SNPs in RANKL and ESR1 genes and haplotype frequencies in relation to BMD T-scores

Values (r^2) of pair-wise linkage disequilibrium of various *RANKL* SNPs were determined and only rs2148072-rs2200287 and rs3742257-rs922996 pairs were found to have significant linkage disequilibrium with r^2 values of 0.92 and 0.94, respectively (Table 6). Among a total of 11 *RANKL* tSNP haplotypes, the haplotype Grs2148072-Crs2200287-Grs922996 was predominant with a population frequency of 51.1% (Table 7). Women without this haplotype were found to have a higher risk for low BMD at LS_{1-4}/AP with an odds ratio of 2.09 (95% CI: 1.21–3.64) as determined by the logistic regression analysis, followed by adjustment for multiple tests. For *ESR1* haplotypes, the only significant linkage disequilibrium was found between the Trs2234693 and Ars9340799 alleles (D′ = 95, $r^2 = 0.34$) (Table 6), and those with this haplotype had a higher risk for low BMD at LS_{2-4}/Lat (OR = 1.80, 95% CI = 1.1-2.9) (Table 8).

Table 3
Demographics of the 467 menopausal women.

	Mean	SD	Min	Max	95% CI
Age (y)	51.7	4.2	45.0	63.0	47.0–60.0
Years after menopause	2.8	2.4	1.5	14.0	2.35–8.0
Weight (kg)	57.2	8.2	40.0	88.8	43.1–74.0
Height (cm)	156.1	5.0	143.0	173.0	146.0–166.0
BMI (kg/m^2)	23.3	3.1	18.3	30.2	18.4–30.4
BMD at LS_{1-4}/AP (g/cm^2)	0.921	0.131	0.581	1.345	0.7–1.2
BMD at LS_{2-4}/Lat (g/cm^2)	0.676	0.117	0.586	1.086	0.65–0.9
BMD at total hip (g/cm^2)	0.844	0.107	0.465	1.151	0.647–1.068
BMD at femur neck (g/cm^2)	0.719	0.103	0.416	1.127	0.527–0.943
FSH (IU/dL)	62.1	27.3	34.0	159.0	39.7–122.2
E2 (pg/dL)	27.5	33.4	5.0	35.6	24.3–30.7
Calcium intake per wk (mg)	4283.2	2254.7	220	15100	909–9579

BMD = bone mineral density; E2 = 17-estradiol; FSH = follicular stimulating hormone.

Gene-gene interactions in relation to BMD

Gene-gene interaction analyses revealed that participants with combinations of *ESR1* rs1884054 AA and *RANKL* rs2148072 A allele had a lower BMD than those with *ESR1* rs1884054 AA and *RANKL* rs2148072 GG genotypes (0.832 ± 0.080 vs. 1.029 ± 0.047 g/cm^2, $p = 0.04$) (Fig. 2A). Similarly, those with combinations of *ESR1* rs1884054 AA and *RANKL* rs2200287 T allele had a lower BMD than those with *ESR1* rs1884054 AA and *RANKL* rs2200287 CC genotypes (0.810 ± 0.076 vs. 1.061 ± 0.046 g/cm^2, $p = 0.008$) (Fig. 2B).

Table 4
Multiple linear regression analyses of additive genetic modeling for associations between various SNPs and BMD at different bone sites.

SNPs	MAF (%)	LS_{1-4}/AP	Total hip	LS_{2-4}/Lat	Femur neck
		p[a]	p[b]	p[c]	p[d]
ESR1 rs9340954 (T > G)	43.3	1	1	1	1
ESR1 rs1884054 (C > A)	31.2	0.326	0.244	**0.046***	1
ESR1 rs3020314 (C > T)	19.7	0.221	0.06	0.655	0.291
ESR1 rs9340799 (A > G)	20.6	0.999	1	1	1
ESR1 rs2234693 (T > C)	40.9	1	0.998	0.791	1
RANKL rs922996 (A > G)	48.4	0.181	1	0.996	1
RANKL rs2200287 (C > T)	19.2	0.693	1	1	0.901
RANKL rs2148072 (G > A)	19.3	0.505	0.996	1	0.663

Equation for multiple linear regression: $Y = \beta \times SNP + \alpha_1 \times age + \alpha_2 \times BMI + \alpha_3 \times$ years from menopause + ρ.
MAF = minor allele frequency.
*Statistically significant $p < 0.05$.
[a] Corrected p value for multiple tests by full scan permutation for BMD at lumbar spine AP view; [b] Corrected p value for multiple tests by full scan permutation for BMD at total hip; [c] Corrected p value for multiple tests by full scan permutation for BMD at lumbar spine lateral view; [d] Corrected p value for multiple tests by full scan permutation for BMD at femur neck.

Table 5
ESR1 rs1884054 genotypes and BMD T-scores.

ESR1 rs1884054	AC and CC (No. of individuals)	AA (No. of individuals)	OR[a]	*p*
LS_{2-4}Lat T-score <−1.0	268	16	2.54	0.006
T-score ≥−1.0	158	24		
LS_{1-4}AP T-score <−1.0	175	11	1.84	0.03
T-score ≥−1.0	251	29		
Total hip T-score <−1.0	61	1	3.26	0.008
T-score ≥−1.0	365	39		
Femur neck T-score <−1.0	167	10	1.93	0.02
T-score ≥−1.0	259	30		

OR = odds ratio.
[a] OR adjusted for years after menopause.

Discussion

In this study, we investigated five *ESR1* and three *RANKL* SNPs for their potential association with BMD in 467 postmenopausal Taiwanese and provided evidence for a statistically significant association between genetic variations in *ESR1* and *RANKL* genes and BMD for the first time in Taiwanese population. We found that participants with the *ESR1* Crs1884054 allele had a significant lower BMD at LS_{2-4}/Lat than those with the *ESR1* $AA_{rs1884054}$ genotype (Table 5 and Fig. 2). In addition, individuals with the *ESR1* $T_{rs2234693}$-$A_{rs922996}$ haplotype had a higher risk for low BMD at LS_{2-4}/Lat (OR = 1.8, 95% C.I. = 1.1-2.9) (Table 6). Those without the *RANKL* $G_{rs2148072}$-$C_{rs2200287}$-$G_{rs922996}$ haplotype also had a higher risk for low BMD at LS_{1-4}/AP (OR = 2.09, 95% CI = 1.21-3.64) (Table 8). Stratification analyses revealed that the interaction between the *ESR1* $AA_{rs1884054}$ genotype and the *RANKL* $A_{rs2148072}$ allele ($p = 0.032$) or $T_{rs2200287}$ allele ($p = 0.007$) might result in a lower BMD at SL_{1-4}/AP (Fig. 2). Successful linking of these two genes to BMD might be attributed to the homogeneity of the study participants as the great majority of them were in the early postmenopausal stage. Unlike several other studies in which the mean year after menopause was over 10 years, ours was 2.6 years; therefore, the chance for bias in population stratification was minimal.

The *ESR1* rs1884054 is located only 200 bp away from *ESR1* rs1884052 that was investigated by the Framingham 100 k genome-wide association study (GWAS) [18]. This GWAS examined 241 families, including 159 original and 487 offspring women of predominantly Caucasians and found that *ESR1* rs1884052 and rs3778099 are two of the top 40 SNPs significantly associated with BMD at femur neck. The *ESR1* $C_{rs1884054}$ allele wassignificantly related to low BMD at all bone sites examined in this study. Therefore, it is conceivable that the *ESR1* rs1884054 C > A polymorphisms are associated with low BMD.

The relationship between *ESR1* polymorphisms and BMD was first described in a Japanese population (45–91 years old) by Kobayashi et al [19]. They found that *ESR1* exhibits PvuII and XbaI restriction fragment length polymorphisms and that the Px haplotype is associated with low BMD in menopausal women, where P represents those without the PvuII restriction site (C allele), and x indicates those with the XbaI site (A allele) in the *ESR1* gene. The PvuII recognition sequence CAGCTG is now known as *ESR1* rs2234693 (the 5th *ESR1* SNP listed in Table 4) in which the fifth base of the sequence CAGCTG is polymorphic and may be T or C, with C being the minor allele [20]. The XbaI recognition sequence TCTAGA is now known as *ESR1* rs9340799 (the 4th *ESR1* SNP listed in Table 4) in which the fourth base of the sequence TCTAGA may be G or A. The G allele is minor and is denoted by X (XX = GG, xx = AA) [20]. However, another study, also in a Japanese population, showed no significant association of the PvuII polymorphism with BMD, but participants with the XbaI GG genotype were found to

Table 6
Linkage between various SNP pairs.

SNP Pair	D′	r^2
RANKL rs2148072-rs2200287	0.97	0.92
RANKL rs2200287-rs922996	1.00	0.24
RANKL rs2148072-rs922996	0.98	0.23
RANKL rs3742257-rs922996	0.96	0.94
ESR1 rs2234693-rs9340799	0.95	0.34

D′ = pair-wise linkage disequilibrium; r^2 = correlation coefficient.

Table 7
RANKL and *ESR1* haplotype frequencies.

Haplotype	Frequency (%)
RANKL G-C-G	51.1
RANKL G-C-C	28.8
RANKL A-T-A	18.6
RANKL G-T-A	0.6
RANKL A-C-G	0.4
RANKL A-C-A	0.3
RANKL G-T-G	0.1
RANKL others	0.1
ESR1 T-A	58.6
ESR1 C-A	20.9
ESR1 C-G	20.5

RANKL haplotype: rs2148072 (A > G), rs2200287 (T > C), and rs922996 (A > G). , *ESR1* haplotype: rs2234693 (T > C) and rs9340799 (G > A).

Table 8
Association between haplotype and normal (≥−1.0) and low (<−1.0) BMD T-scores at lumbar spine.

Haplotype copy number	T-score ≥−1.0	T-score <−1.0	Odds ratio[a]	95% CI
RANKL G-C-G[a]	*N* (%)	*N* (%)		
2	79 (28.2)	34 (18.2)	1.00	
1	138 (49.3)	94 (50.3)	1.61	0.99–2.65
0	63 (22.5)	59 (31.6)	2.09	1.21–3.64
ESR1 T-A[b]	*N* (%)	*N* (%)		
0	43 (23.6)	41 (14.8)	1.00	
2 or 1	139 (76.4)	237 (85.2)	1.80	1.1–2.9

[a] *RANKL* haplotype: rs2148072 (A > G), rs2200287 (T > C), and rs922996 (A > G); odds ratio significant at bone sites LS_{2-4}Lat and LS_{1-4}AP; [b] *ESR1* haplotype: rs2234693 (T > C) and rs9340799 (G > A); odds ratio significant at bone site LS_{2-4}Lat.

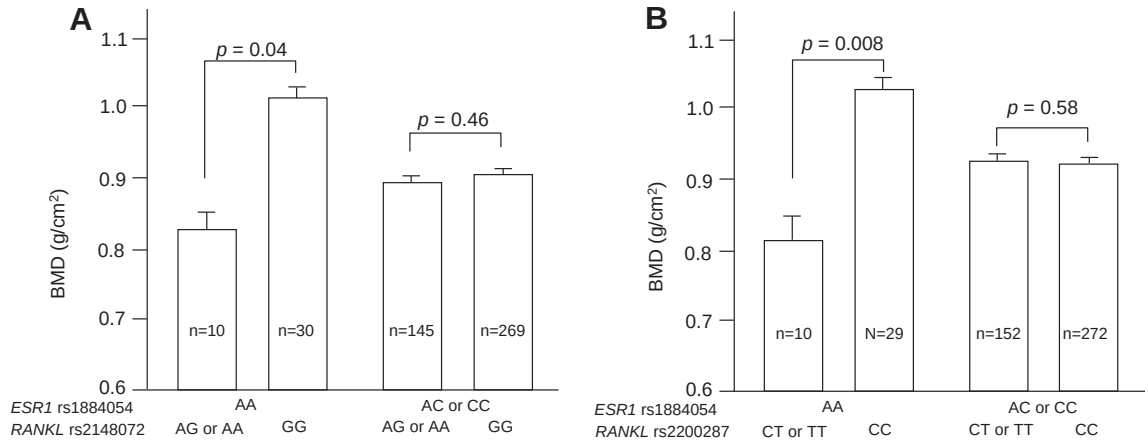

Fig. 2. Stratification analyses for correlations between *ESR1-RANKL* interactions and BMD LS_{1-4}/AP. (A) Participants with the *ESR1* $AA_{rs1884054}$ genotype and the *RANKL* $A_{rs2148072}$ allele had significantly lower BMD than those with *ESR1* $AA_{rs1884054}$ and *RANKL* $GG_{rs2148072}$ genotype ($p = 0.04$). (B) Participants with the *ESR1* $AA_{rs1884054}$ genotype and the *RANKL* $T_{rs2200287}$ allele had significantly lower BMD than those with *ESR1* $AA_{rs1884054}$ and *RANKL* $CC_{rs2200287}$ genotype ($p = 0.007$).

have a lower BMD than those with the A allele [21]. In a third study of a Japanese-American population [22], individuals with the PvuII CC or TC genotype had a lower lumbar spine BMD than those with the TT genotype. These results were opposite to that of Kobayashi et al [19,22]. In our study, the *ESR1* px (Trs2234693-Ars922996) haplotype was found to be significantly related to a higher risk for low BMD at LS_{2-4}/Lat (OR = 1.8, 95% CI = 1.1-2.9) (Table 4). Our results were consistent with those of Binh et al, where they found that postmenopausal Vietnamese with the px haplotype had increased osteoporosis risk [23]. Our results also agree with those of the meta-study of 20,000 Caucasians by Ioanni et al, where they found that participants with XbaI XX (rs9340799GG) and PvuII PP (rs2234693CC) (PX) genotypes had a slightly better BMD than those with other XbaI and PvuII genotypes [11]. By contrast, in another meta-analysis study of results published between 1994 and 2006 involving approximately 4,000 Chinese, the PvuII PP (rs2234693CC) genotype was found significantly associated with low BMD at femur neck (difference: −0.011, 95% CI = 0.022-0.000, $p = 0.047$) but not at lumbar spine. These discrepancies are likely owing to the different frequencies of *ESR1* rs2234693 and rs9340799 polymorphisms in different ethnic populations.

The three *RANKL* tSNPs, rs2148072, rs2200287, and rs922996 investigated in this study are located in the same haploblock at chromosome locations 42060063, 42066660, and 42076671, respectively (Fig. 1). All of these tSNPs have a minor allele frequency greater than 19% in our study population. A recent meta-analysis found that *RANKL* is one of the nine osteoporosis candidate genes [12,13], and many *RANKL* SNPs have been found to be associated with BMD. For example, rs9594766 and rs2062305 are associated with lumbar spine BMD [12]; rs9594782 is related to hip bone BMD in men [24]; and rs9594738 and polymorphism are associated with BMD at lumbar spine in Korean women [25]. In this study, we did not find significant associations between any of the individual *RANKL* SNPs and BMD at the hip bone. It is possible that most of the participants were in the early postmenopausal stage in which trabecular bone (the main component of lumbar spine) loss was more prevalent [26]. The *RANKL* gene encodes two different mRNAs and thus two different proteins of 244 (variant 1) [27] and 317 (variant 2) [28] amino acids. Variant 2 has 73 more amino acids than variant 1 at the N-terminus. We found that women without the *RANKL* $G_{rs2148072}$-$C_{rs2200287}$-$G_{rs922996}$ haplotype had a higher risk for low BMD at lumbar spine. Since all three tSNPs of this haplotype are located in introns, it is unlikely that these tSNPs affect coding and thus the function of the RANKL protein. However, it is possible that they are miRNA binding sites and that these SNPs result in differences in miRNA binding and hence altered the production of the RANKL protein.

Stratification analyses revealed that participants with the *ESR1* $AA_{rs1884054}$ and *RANKL* $A_{rs2148072}$ or $T_{rs2200287}$ had lower BMD at lumbar spine (Fig. 2), suggesting that these two genes together have effects on BMD. One example of this possibility is that *ESR1* may increase the production of RUNX or OPG to inhibit the activity of RANKL [29]. *ESR1* may also inhibit NF-κB activation, leading to a decrease in the production of RANKL and subsequently reduction in bone resorption [30].

In summary, we found that Taiwanese postmenopausal women with the *ESR1* Crs1884054 allele had a significant lower BMD at LS_{2-4}/Lat, and those with the *ESR1* Trs2234693-Ars922996 haplotype had a higher risk for low BMD at LS_{2-4}/Lat. Participants without the *RANKL* $G_{rs2148072}$-$C_{rs2200287}$-$G_{rs922996}$ haplotype also had a higher risk for low BMD at LS_{1-4}/AP. We also found that the interaction between the *ESR1* $AA_{rs1884054}$ genotype and the *RANKL* $A_{rs2148072}$ allele or $T_{rs2200287}$ allele could result in a lower BMD at SL_{1-4}/AP. These findings may extend our understanding on the effect of genetic factors on BMD and establish a foundation for the development of genotyping *ESR1* and *RANKL* to predict the osteoporosis risk in menopausal women.

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