

Comprehensive Association Analysis of 10 Single Nucleotide Polymorphisms Associated With Osteoporosis among a Taiwanese Population

Chiao-Wen Hwang¹, Ching-Hsiang Lu², Shu-Fen Sun¹, Tzu-Ying Sung¹, Hsin-Yeh Chung¹, Shi-Ying Huang³, Han-Chun Hung³, Chun-Hong Chen^{3,4}, Yu-Min Sun^{3,4}, Yen-You Lin³, Wen-Sheng Liu⁵ and Zhi-Hong Wen^{3*}

¹*Department of Physical Rehabilitation & Medicine, Kaohsiung Veterans General Hospital, Kaohsiung 813, Taiwan*

²*Department of Neurosurgery, Kaohsiung Armed Forces General Hospital, Kaohsiung 802, Taiwan*

³*Department of Marine Biotechnology and Resources, National Sun Yat-sen University, Kaohsiung 804, Taiwan*

⁴*Doctoral Degree Program in Marine Biotechnology, National Sun Yat-sen University, Kaohsiung 804, Taiwan*

⁵*Asia-Pacific Biotech Developing, Inc.*

KEYWORDS Osteoporosis. Bone Mineral Density. Single Nucleotide Polymorphisms. Taiwanese

ABSTRACT In the present study, we tested for an association between single nucleotide polymorphisms (SNPs) and bone mineral density (BMD) of the hip and lumbar spine in a Taiwanese population by analyzing 252 healthy persons (non-osteoporosis) and 193 persons with osteoporosis. We found that age; body mass index; family history; and consumption of coffee or vitamin D were associated with osteoporosis in our Taiwanese population. Our results also indicated that osteoprotegerin (OPG) SNP (rs6993813 T→C) and receptor activator of nuclear factor kappa-B ligand (RANKL) SNP (rs9594738 C→T) were significantly associated with BMD in our Taiwanese population. Additionally, we propose that the mean threshold value of integration of 7 wild-type SNPs (rs7524102 and rs6696981 of 1p36, rs11898505 of 2p16, rs9479055 of 6q25, rs326340 and rs1289759 of 3q13, and rs9594738 of 13q14) can be used for both a Taiwanese reference for bone density testing and to achieve the effect of grading.

INTRODUCTION

Osteoporosis, which literally means “porous bones,” is a multifactorial skeletal bone disease characterized by microarchitectural deterioration and low bone mineral density (BMD) of bone tissue. In the United States alone, the direct medical costs for osteoporosis were estimated to be about \$13.8 billion in 1995, of which \$11 billion (80.4 %) were attributed to the treatment of women (Ray et al. 1997). To date, the expenditure of this disease in the United States is \$17 billion every year (Richards et al. 2008), and the annual cost of fractures is expected to increase another 50% by 2025 (Burge et al. 2007). However, osteoporosis is a worldwide public health issue that is present not only in

Western countries but also in Asia (Delmas 2002). According to the World Health Organization (WHO), osteoporosis is defined in those whose BMD value is more than 2.5 SD below the mean BMD of the ethnicity-matched and young adult population (Murphy et al. 2003). Based on this WHO definition, many women over the age of 80 years suffer from osteoporosis (Boonen 2010). Osteoporosis can cause symptoms such as fracture of the hip, spine, and wrist (Huang et al. 2003), with hip fracture often resulting in high mortality.

Recently, some studies have examined the effectiveness of available treatments for osteoporosis, including calcium, vitamin D, hormone replacement therapy, selective estrogen receptor modulators (SERMs), bisphosphonates, parathyroid hormone (PTH), and calcitonin (Altkorn & Vokes 2001; Delmas 2002). However, these medicines are not omnipotent for every patient. In clinical practice, treatments of osteoporosis need to be evaluated on a case-by-case basis. Therefore, the identification of effective diagnostic markers in advance of os-

*Address Correspondence to:

Dr. Zhi-Hong Wen
Department of Marine Biotechnology and Resources,
National Sun Yat-sen University,
#70 Lien-Hai Rd, Kaohsiung 804, Taiwan
Telephone: +(886)-7-5252021; Fax: +(886)-7-5255020;
E-mail: wzh@mail.nsysu.edu.tw

teoporosis merits further investigation. Osteoporosis is associated with environmental factors and with many genes (Rivadeneira et al. 2009). Some studies have indicated that genetic factors could be used for evaluating population variation of BMD (Brown et al. 2005; Crawford et al. 2010). There is abundant evidence for a genetic contribution to variation in BMD, with heritability estimates between 0.6 and 0.8 (Peacock et al. 2002). Moreover, BMD may be regarded as a trait that is polygenic in nature. Several studies of susceptibility genes and genome-wide linkages have estimated that multiple genetic loci are involved in BMD (Cheung et al. 2010; Hsu et al. 2010; Zhang et al. 2010). In populations of European ancestry, numerous candidate genes related to osteoporosis have been analyzed, including estrogen receptor 1 (*ESR1*), receptor activator of nuclear factor (*RANK*), major histocompatibility complex (*MHC*), and osteoprotegerin (*OPG*) (Liu et al. 2010; Stykarsdottir et al. 2008). However, there is some evidence indicating that genes regulating BMD differ between various gender and skeletal sites (Kaufman et al. 2008).

With the rapid advancement in single nucleotide polymorphism (SNPs) identifications and the development of databases, such as the genome-wide association study and International HapMap Project, genetic studies of osteoporosis are greatly facilitated (Koller et al. 2010). Although many osteoporosis-associated genes have been reported in the past 15 years (Li et al. 2010), only a few studies have directly examined the association between SNPs and osteoporosis in Taiwanese (Chao et al. 2010; Chen et al. 2001; Lin et al. 2008; Tsai et al. 2003). In the present study, we tested for an association between SNPs and BMD of the hip and lumbar spine by performing an analysis on a Taiwanese population containing 252 healthy persons (non-osteoporotic) and 193 persons with osteoporosis (including postmenopausal Taiwanese women and osteoporotic men). Using a double-blind test, we evaluated the feasibility of using the analyzed SNPs for determining osteoporosis in Taiwanese.

MATERIALS AND METHODS

Sample Collection

In this study, the Taiwanese population living on the area of Taiwan was divided into 2

groups: one group contained 252 healthy persons (non-osteoporotic) and another group had 193 osteoporotic persons (including postmenopausal Taiwanese women and osteoporotic men). Standardized BMD was calculated and corrected for sex, age, and weight. All blood samples were obtained from these 2 populations and kept at 4 °C. All of these study protocols were approved from The Institutional Review Board of Kaohsiung Veterans General Hospital (IRB No. VGHKS97-CT9-09).

Bone Mass Measurement

The BMDs of the lumbar spine and hip (a total hip or femoral neck measurement) of the study participants were measured by a dual energy X-ray absorptiometry machine (DELPHI QDR series, Hologic, USA.) (Flicker et al. 1995). A normal BMD is more than -1 standard deviation compared to a control matched for age, sex, and race; the BMD of a person with osteopenia is between -1 standard deviation and -2.5 standard deviation; and the BMD in an osteoporotic person is less than -2.5 standard deviation (Kanis et al. 1994).

DNA Extraction

DNA extraction was performed using the QIA amp DNA Blood Mini Kit (QIAGEN' Hilden' Germany) according to the manufacturer's recommendations. The blood was digested with 0.5 mg/mL proteinase K in 400 µL cell lysis solution for 24 h at 55 °C until the blood was completely lysed. Then, 200 µL absolute ethanol was added to the lysed sample. The mixture was transferred into the DNeasy mini column and centrifuged for 1 min at 8,000 rpm. The DNeasy mini column was washed with 500 µL washing buffer and centrifuged for 1 min at 8,000 rpm. Finally, the DNA was eluted into a clean 1.5-mL microcentrifuge tube.

Genotyping

Genotyping was conducted by the Allele-Specific PCR method (AS-PCR). Ten SNPs from 8 chromosomal regions were used in association studies with spinal BMD (Table 1). The SNPs was performed by As-PCR and used in this study were as follows: allelic SNPs on lp36 (rs7524102, rs6696981), one SNP on 6p21 (major histocom-

patibility complex, *MHC*) (rs3130340), one SNP on 2p16 (MutS homolog 6, *MSH6*) (rs3130340), one SNP on 3q13 (Intraflagellar transport protein 57 homolog, *IFT57*) (rs3130340), tri-allelic SNPs on 6q25 (estrogen receptor 1, *ESR1*) (rs9479055 C, rs326340 T, and rs1289759 C), one SNP on 8q24 (osteoprotegerin, *OPG*) (rs6993813), one SNP on 13q14 (receptor activator of nuclear factor kappa-B ligand, *RANKL*) (rs9594738), and one SNP on 18q21 (receptor activator of nuclear factor kappa-B, *RANK*) (rs3018362). These SNPs were selected based on previous genome-wide association studies (Lei et al. 2007).

Table 1: Ten SNPs from 8 chromosomal regions

Chromosomal regions	SNP and allele	
1p36	rs7524102 G	rs6696981 G
6p21(<i>MHC</i>)	rs3130340 C	
2p16(<i>SPTBN1</i>)	rs11898505 G	
6q25(<i>ESR1</i>)	rs9479055 C	
3q13(<i>IFT57</i>)	rs326340 T	rs1289759 C
8q24(<i>OPG</i>)	rs6993813 T	
13q14(<i>RANKL</i>)	rs9594738 C	
18q21(<i>RANK</i>)	rs3018362 G	

Double-blind Test

Using the double-blind test, we analyzed another 10 volunteers not including in the previous two groups. According to the previous integration of SNP sites, we scored the SNPs of these 10 volunteers. We used the integration of SNP sites to distinguish normal, osteopenia, and osteoporosis, respectively. These 10 volunteers were also measured at the lumbar spine and at the hip (a total hip or femoral neck measurement) by DXA. We followed the same definitions that the authors used in the previous study (Kanis et al. 1994) to identify normal, osteopenia, and osteoporosis with the BMD of these 10 volunteers. Then, we compared the score and the BMD to analyze the difference.

Statistical Analysis

The results of the genetic tests were expressed by genotype, and divided into several categories according to wild/mutant status for the SNPs determined. A 95% confidence interval for the odds ratio for subjects carrying a haplotype either above or below 1.0, or $p < 0.05$, was defined as constituting statistical significance. The

effect of the haplotypes of each gene on the BMD was evaluated by percentages of DXA measurements at the lumbar spine and at the hip in the subjects carrying the individual haplotypes. Data were compared with an analysis of variance (ANOVA). When the ANOVA results were statistically significant, multiple comparisons were performed using the Scheffé method. All data were analyzed using SPSS version 10.0 software (SPSS for Windows Inc., Chicago, IL, USA) and p values < 0.05 were defined as constituting statistical significance for every analysis.

RESULTS

Analysis of 10 SNPs Associated with Osteoporosis

According to BMD, our study sample was composed of 102 osteoporosis participants, 91 osteopenia participants, and 252 healthy participants. The data, evaluated by the SPSS software for analysis of variance (one-way ANOVA) (Table 2), were divided into 3 groups according to BMD analysis of 10 SNPs ANOVA (Table 3). The results of rs6993813 (T→C, $p = 0.049$) and rs9594738 (C→T, $p = 0.033$) showed significant association with BMD. *OPG* (rs6993813 T→C) and *RANKL* (rs9594738 C→T) were the 2 genes that were significantly associated with BMD among the 10 SNPs. These results show that family history affects BMD in a Taiwanese population. In addition to family history, our results showed that BMD changed with age, body mass index (BMI), coffee consumption, calcium supplements, vitamin D, and bisphosphonate.

Correlation of Integrated SNP Sites for Osteoporosis Analysis

For 7 SNPs (rs7524102, rs6696981, rs11898505, rs9479055, rs326340, rs1269759, rs9594738), the control group displayed the normal allele (wild type). For 3 SNPs (rs3130340, rs6993813, rs3018362) the control group displayed the mutant allele. We integrated the SNP sites into 3 groups: total SNPs, wild type, and mutant. We analyzed the median of the 3 groups (Table 4) and the wild type showed a significant difference when the value was 3.481 ($p = 0.002$) (Table 5). We used BMDs to evaluate the correlation of the key point for ag-

Table 2: Demographic data

	Healthy individuals	Osteopenia	Osteoporosis	F-value	p-value
	N=252	N=91	N=102		
Age	57.36±6.236	62.53±7.967	65.17±8.217	50.021	<0.001
BMI	24.4 ±3.07	24.1 ±3.30	22.6 ±3.43	11.444	<0.001
Postmenopausal age	48.85±5.682	48.96±5.267	47.78±6.673	1.416	0.244
Lumbar BMD	0.03±1.077	-1.13±0.899	-2.28±0.981	194.071	<0.001
Hip BMD	-0.46±0.867	-1.79±0.427	-2.98±0.673	429.304	<0.001
Hip & Neck bone	0.79±0.108	0.66±0.060	0.55±0.072	258.428	<0.001
Fracture	7.48±3.542	12.62±6.643	19.51±8.636	160.8	<0.001
Hip fracture	0.52±0.892	1.86±2.159	5.32±4.661	131.503	<0.001
	Case Number				
Smoke	0		1		
Family history	4		15	4.075	0.018
Soda	1		0		
Coffee	16		11	4.396	0.013
Medication					
Calcium supplement	35		48	7.106	0.001
Vitamin D	4		7	8.211	<0.001
Fosamax	10		23	30.838	<0.001
Estrogen	26		27	1.351	0.26
SSRI	4		9	1.001	0.369

Table 3: SNP analysis of osteoporosis by one-way ANOVA

	Healthy individuals	Osteopenia	Osteoporosis	F-value	p-value
1q36					
rs7524102 (G→A)	252	91	102	0.514	0.599
rs6696981 (G→T)				0.305	0.737
6p21 (MHC)					
rs3130340 (C→T)	252	91	102	0.661	0.517
2p16 (MSH6)					
rs11898505 (G→A)	252	91	102	0.639	0.528
6q25 (ESR1)					
rs9479055 (C→G)	252	91	102	0.284	0.753
3q13 (IFT57)					
rs326340 (T→G)				2.002	0.136
rs1269759 (C→T)				0.939	0.392
8q24 (OPG)					
rs6993813 (T→C)	252	91	102	3.035	0.049*
13q14 (RANKL)					
rs9594738 (C→T)	252	91	102	5.802	0.003*
18q21 (RANK)					
rs3018362 (G→A)	252	91	102	0.268	0.765

*Correlation is significant at the 0.05 level

Table 4: Integration of SNP sites for osteoporosis analyzed

	Num- bers	Mini- mum	Maxi- mum	Mean	Standard deviation
^a Total	445	1.0	13	7.8	2.106
^b Wild type	445	1.0	8.0	3.481	1.468
^c Mutant	445	0	6.0	4.32	1.229

The numbers were aggregated such that the wild type is homologous for zero units and heterologous for one unit, and the mutant is homologous for 2 units.

^aIncludes the total SNP sites analyzed.

^bIncludes 7 SNP sites (rs7524102, rs6696981, rs11898505, rs9479055, rs326340, rs1269759, rs9594738).

^cIncludes 3 SNP sites (rs3130340, rs6993813, rs3018362).

Table 5: Analyzed results of key point for aggregated numbers in total, wild-type, and mutant SNP sites

	Num- bers	Mean	p-value	95% CI
Total	445	7.8	0.197	-0.053 to 0.256
≥ Mean 7.8	238			
< Mean 7.8	207			
Wild type		3.48	0.002*	0.096 to 0.411
≥ Mean 3.48	185			
< Mean 3.48	260			
Mutant		4.32	0.141	-0.269 to 0.039
≥ Mean 4.32	223			
< Mean 4.32	222			

*Correlation is significant at the 0.05 level

gregated numbers with osteoporosis. The results showed a significant difference only between NS and OS ($p = 0.0106$, Table 6). As a consequence, neither the difference of osteoporosis and osteopenia nor the difference of osteoporosis and healthy individuals could be distinguished.

Double-blind Test

According to the correlation of integrated SNP sites for osteoporosis analysis, we randomly choose 10 volunteers to genotype the 7 wild-type SNPs associated with osteoporosis. Then, we compared the score from the integration of the 7 SNPs to BMDs measured by DXA (Table 7). Using the integrated score, we were able to identify 8 volunteers with osteopenia/osteoporosis and 2 volunteers with normal phenotype. The conformity showed 100% match between the integrated score and BMD. These results demonstrate that the evaluation score could accurately identify the osteopenia/osteoporosis from normal phenotype by genotyping the 7 wild-type SNPs.

Table 6: Correlation of analyzed results of key point for aggregated numbers with osteoporosis

	Num- bers	Total	Wild type	Mutant
Healthy individuals, osteopenia and osteoporosis correlation sig. (2-tailed)	445	0.065 0.173	0.123** 0.010	-0.036 0.452
Healthy individuals and osteopenia correlation sig. (2-tailed)	343	0.051 0.351	0.097 0.072	-0.025 0.650
Healthy individuals and osteoporosis correlation sig. (2-tailed)	354	0.065 0.223	0.128* 0.016	-0.037 0.491
Osteopenia and osteoporosis correlation sig. (2-tailed)	193	0.018 0.808	0.032 0.654	-0.015 0.841

*Correlation is significant at the 0.05 level (2-tailed).

**Correlation is significant at the 0.01 level (2-tailed).

DISCUSSION

Osteoporosis is considered to be associated with diet habits, such as coffee consumption (Barrett-Connor et al. 1994; Hallstrom et al. 2006) and cola intake (Tucker et al. 2006). Age and BMI have also been identified as risk factors for osteoporosis (van der Voort et al. 2000). Fox

et al. (1998) have reported that a positive family history is a potential risk factor for osteoporotic fractures. By analyzing environmental and medical factors, we found that age, BMI, family history, and consumption of coffee were associated with osteoporosis in our Taiwanese study population. Overall, our results also support the above-mentioned hypothesis that family history, age, BMI, and coffee are associated with osteoporosis in Taiwanese. In addition, our results showed that BMD changed with calcium supplements, vitamin D, and bisphosphonate.

In this study, we also identified genetic associations with osteoporosis. We tested 10 SNPs from 8 chromosomal regions. Two SNPs (rs6993813 of *OPG* and rs9594738 of *RANKL*) were significantly associated with osteoporosis in this Taiwanese population. We found that the key point of integration of 7 wild-type SNPs (rs7524102 and rs6696981 of 1p36, rs11898505 of *MSH6*, rs9479055 of *ESR1*, rs326340 and rs1289759 of *IFT57*, and rs9594738 of *RANKL*) is 3.48 ($p = 0.002$). Using BMD as a reference to determine the key point, we found significant differences between healthy individuals, osteopenia, and osteoporosis ($p = 0.010$), and healthy individuals and osteopenia ($p = 0.0106$). Using a double-blind test, we succeeded in distinguishing between normal and osteopenia/osteoporosis in 10 volunteers with 100% accuracy by following the key point of 7 wild-type SNPs.

Geographic ancestry is an important factor when evaluating the genetic risk factors of complicated diseases, especially osteoporosis (Styrkarsdottir et al. 2010). Several susceptibility genes for osteoporosis, including 1p36, *MHC*, *MSH6*, and *ESR1*, have recently been revealed and analyzed mainly in populations of European descent (Styrkarsdottir et al. 2008; Styrkarsdottir et al. 2009; Zhang et al. 2010). However, none of these SNPs had been fully investigated in Taiwanese populations. In contrast, a SNP in 1p36, *ESR1*, which had previously been shown to be associated with BMD in a Han Chinese population (Liu et al. 2010), did not show strong evidence of association in our study. Taiwanese population is composed of three subgroups, and three subgroups immigrated from China to Taiwan Island at different times; the Minnan (70% of the population; about 300-400 years ago), the Hakka (13%; about 200 years ago), and the Mainlanders (14%; about 50 years ago) (Yang et al. 2006). Rosenberg et al. (2010)

Table 7: Double-blind test

Case no.	Age	BMI	Meno-pausal Age	Lum-bar BMD	Hip BMD	Hip & Neck Bone	Fracture	Hip fracture	rs94 790	rs12 897	rs75 241	rs11 898	rs31 303	rs66 969	rs95 947	^a Score	^b BMD	^c Con-formity
									55 CA AA =2 AC =1 CC =0	59 CT CC =0 CT =1 TT =2	02 GA AA =2 AG =1 GG =0	505 GA AA =2 AG =1 GG =0	40 TG GG =2 GT =1 TT =2	81 GT GG =0 GT =1 TT =2	38 CT CC =0 CT =1 TT =2			
01	76	21	57	-2.1	-3.7	0.475	19	8.7	0	1	2	1	0	0	0	4 (osteoporosis/osteopenia)	-3.7 (osteoporosis)	+
02	74	23	47	-3.7	3.5	0.496	17	7.1	0	0	2	2	0	0	0	4 (osteoporosis/osteopenia)	-3.5 (osteoporosis)	+
03	65	32	46	-1.6	-2.2	0.629	12	2.3	0	2	2	2	1	0	0	7 (osteoporosis/osteopenia)	-2.2 (osteopenia)	+
04	63	26	54	-3.2	-3	0.547	11	3.1	0	0	2	2	0	0	0	4 (osteoporosis/osteopenia)	-3.2 (osteoporosis)	+
05	72	24	44	-1.5	-1.4	0.708	9	1.4	0	1	1	2	1	1	0	6 (osteoporosis/osteopenia)	-1.5 (osteopenia)	+
06	63	27	43	0.1	-0.5	0.907	5.1	0.1	0	0	1	2	0	0	0	3 (healthy individuals)	-0.5 (healthy individuals)	+
07	64	24	54	-3	-3.6	0.494	14	4.9	0	1	2	2	0	0	0	5 (osteoporosis/osteopenia)	-3.6 (osteoporosis)	+
08	64	24	48	0	-2.7	0.578	10	2.3	0	0	2	2	0	1	0	5 (osteoporosis/osteopenia)	-2.7 (osteoporosis)	+
09	57	23	45	-1.7	-3.2	0.526	9.6	3.1	0	2	2	0	1	0	0	5 (osteoporosis/osteopenia)	-3.2 (osteoporosis)	+
10	68	24	50	0.6	-2.1	0.64	15	2.7	0	0	2	1	0	1	0	4 (osteoporosis/osteopenia)	-2.1 (osteopenia)	+

^aScore is derived from the integration of the 7 wild-type SNPs. The groups are separated using the value of 3.481, such that a score higher than 3.481 is designated as OS/OA; and a score lower than 3.481 is determined to be NS.

^bBMD values greater than -1 are defined as control groups (NS); values between -2.5 and -1 are defined as osteopenia (OS); and values less than -2.5 are defined as osteoporosis (OA).

^cConformity represents a comparison between the BMD to the score integrated by the 7 wild-type SNPs. If the situation matched with each other, the conformity would be designated as "+". If the situation did not match with each other, the conformity would be designated as "-".

suggest that if two populations are separated by a barrier to gene flow, a marker allele might be found to be associated with a disease in one population, but might not in another population. This theory partially explains why a SNP in 1p36, *ESR1* are associated with BMD in a Han Chinese population but not in Taiwanese. That is the reason the previous study of Han Chinese population cannot be directly applied to Taiwanese. Our results showed similar findings to those from other studies evaluating different ethnic populations (Liu et al. 2010; Stykarsdottir et al. 2008; Stykarsdottir et al. 2009), in that we did not find any significant association between BMD and rs11898505 of *MSH6* or rs3130340 of *MHC* in our Taiwanese study population. Our results indicate that these variants represent osteoporosis susceptibility genes of Taiwanese that display different association profiles from either Han Chinese or European populations.

A variety of studies have suggested that polymorphisms in *RANKL*, *RANK*, and *OPG* may modulate bone density and turnover (Paternoster et al. 2010; Roshandel et al. 2010; Stykarsdottir et al. 2008). The *RANKL*/*RANK*/*OPG* signaling system plays a critical role in bone remodeling, and an imbalance of the *RANKL*/*RANK*/*OPG* system may cause osteoporosis (Sasaki et al. 2001; von Tirpitz et al. 2003). *RANKL* binds to *RANK*, increasing production, activation, and survival of osteoclasts (Hsu et al. 1999), but these effects of *RANKL* are blocked by *OPG* (Simonet et al. 1997). Recently, some studies found that SNPs located near *RANKL*, *RANK*, and *OPG* were associated with BMD in genome-wide association studies (Richards et al. 2008; Stykarsdottir et al. 2008; Stykarsdottir et al. 2009). In contrast to the previously mentioned study in Han Chinese population (Liu et al. 2010), rs3018362 of *RANK* is not significantly associated with BMD in our Taiwanese sample. Similar to previous studies, our results also indicated that *OPG* SNP (rs6993813 T→C) and *RANKL* SNP (rs9594738 C→T) were significantly associated with BMD in Taiwanese. These finding implicate *OPG* and *RANKL* as loci containing variations associated with BMD and provides further insight into the mechanism by which the *RANK*/*RANKL*/*OPG* pathway may affect the skeletal system. Our present study did not focus on the SNP interaction. However, the SNP interaction studies be-

come important (Lin et al. 2009; Meyers et al. 2010; Yang et al. 2011; Yen et al. 2008). Therefore, it is warranted that future studies could further explore and investigate the SNP interaction among these 10 SNPs in our study.

Several studies of susceptibility genes and genome-wide linkage have proven that multiple genetic loci are involved in modulation of BMD and the risk of osteoporosis (Zhang et al. 2010). Because many osteoporosis-associated genes are often located in different chromosomal regions, all the additive or synergistic influences among these candidate SNPs should be considered simultaneously instead of evaluating individually (Lin et al. 2008). Therefore, we divided 10 candidate SNPs into 3 groups (total, wild type, and mutant). We identified the key point of integration of 7 wild-type SNPs. Furthermore we employed the key point of 7 SNPs to perform a double-blind test of BMD on 10 Taiwanese volunteers. Using SNPs to detect osteopenia or osteoporosis, we were able to identify with 100% accuracy the OS patients that had been confirmed via the conventional DXA measurement method. This result reveals that the 7 wild-type SNPs may be useful in the clinical setting, not only in academic research. We suggest these 7 wild-type SNPs may serve as references for bone density testing of Taiwanese.

CONCLUSION

With the increase of the quality of medical care, the number of elderly persons is also growing. Although osteoporosis is not a lethal disease, the risk of fracture accompanying osteoporosis can be fatal to older persons. Therefore, osteoporosis becomes a major issue of modern medicine. Osteoporosis is a disease in which screening of asymptomatic individuals by BMD testing should be beneficial because osteoporosis has a long preclinical course before the onset of fracture. How this individualized practice of screening should be achieved still remains controversial, such as the issue of when and how often to test for BMD. How to detect or even predict the risk of osteoporosis as early as possible is an urgent issue. In this research, we proposed that the mean value of 3.48 in the integration of 7 wild-type SNPs can be used as a Taiwanese reference for bone density testing, and to achieve the effect of grading. The results of our present study may provide valuable mark-

ers in examining multiple genetic factors that cooperatively determine the phenotypic characteristics of osteoporosis.

REFERENCES

- Altkorn D, Vokes T 2001. Treatment of postmenopausal osteoporosis. *JAMA*, 285: 1415-1418.
- Barrett-Connor E, Chang JC, Edelstein SL 1994. Coffee-associated osteoporosis offset by daily milk consumption. The Rancho Bernardo Study. *JAMA*, 271: 280-283.
- Boonen S 2010. Addressing the age-related needs of osteoporotic patients with strontium ranelate. *Osteoporos Int*, 21 Suppl 2: S415-423.
- Brown LB, Streeten EA, Shapiro JR, McBride D, Shuldiner AR et al. 2005. Genetic and environmental influences on bone mineral density in pre- and post-menopausal women. *Osteoporos Int*, 16: 1849-1856.
- Burge R, Dawson-Hughes B, Solomon DH, Wong JB, King A et al. 2007. Incidence and economic burden of osteoporosis-related fractures in the United States, 2005-2025. *J Bone Miner Res*, 22: 465-475.
- Chao TH, Yu HN, Huang CC, Liu WS, Tsai YW et al. 2010. Association of interleukin-1 beta (-511C/T) polymorphisms with osteoporosis in postmenopausal women. *Ann Saudi Med*, 30: 437-441.
- Chen HY, Chen WC, Hsu CD, Tsai FJ, Tsai CH et al. 2001. Relation of BsmI vitamin D receptor gene polymorphism to bone mineral density and occurrence of osteoporosis in postmenopausal Chinese women in Taiwan. *Osteoporos Int*, 12: 1036-1041.
- Cheung CL, Xiao SM, Kung AW 2010. Genetic epidemiology of age-related osteoporosis and its clinical applications. *Nat Rev Rheumatol*, 6: 507-517.
- Crawford DC, Brown-Gentry K, Rieder MJ 2010. VKORC1 common variation and bone mineral density in the Third National Health and Nutrition Examination Survey. *PLoS One*, 5: e15088.
- Delmas PD 2002. Treatment of postmenopausal osteoporosis. *Lancet*, 359: 2018-26.
- Flicker L, Hopper JL, Rodgers L, Kaymakci B, Green RM et al. 1995. Bone density determinants in elderly women: a twin study. *J Bone Miner Res*, 10: 1607-1613.
- Fox KM, Cummings SR, Powell-Threets K, Stone K 1998. Family history and risk of osteoporotic fracture. Study of Osteoporotic Fractures Research Group. *Osteoporos Int*, 8: 557-562.
- Hallstrom H, Wolk A, Glynn A, Michaelsson K 2006. Coffee, tea and caffeine consumption in relation to osteoporotic fracture risk in a cohort of Swedish women. *Osteoporos Int*, 17: 1055-1064.
- Hsu H, Lacey DL, Dunstan CR, Solovyev I, Colombero A et al. 1999. Tumor necrosis factor receptor family member RANK mediates osteoclast differentiation and activation induced by osteoprotegerin ligand. *Proc Natl Acad Sci U S A*, 96: 3540-3555.
- Hsu YH, Zillikens MC, Wilson SG, Farber CR, Demissie S et al. 2010. An integration of genome-wide association study and gene expression profiling to prioritize the discovery of novel susceptibility Loci for osteoporosis-related traits. *PLoS Genet*, 6: e1000977.
- Huang QY, Recker RR, Deng HW 2003. Searching for osteoporosis genes in the post-genome era: progress and challenges. *Osteoporos Int*, 14: 701-715.
- Kanis JA, Melton LJ, 3rd, Christiansen C, Johnston CC, Khaltav N 1994. The diagnosis of osteoporosis. *J Bone Miner Res*, 9: 1137-1141.
- Kaufman JM, Ostertag A, Saint-Pierre A, Cohen-Solal M, Boland A et al. 2008. Genome-wide linkage screen of bone mineral density (BMD) in European pedigrees ascertained through a male relative with low BMD values: evidence for quantitative trait loci on 17q21-23, 11q12-13, 13q12-14, and 22q11. *J Clin Endocrinol Metab*, 93: 3755-3762.
- Koller DL, Ichikawa S, Lai D, Padgett LR, Doherty KF et al. 2010. Genome-wide association study of bone mineral density in premenopausal European-American women and replication in African-American women. *J Clin Endocrinol Metab*, 95: 1802-1809.
- Lei SF, Jiang H, Deng FY, Deng HW 2007. Searching for genes underlying susceptibility to osteoporotic fracture: current progress and future prospect. *Osteoporos Int*, 18: 1157-1175.
- Li WF, Hou SX, Yu B, Li MM, Ferec C et al. 2010. Genetics of osteoporosis: accelerating pace in gene identification and validation. *Hum Genet*, 127: 249-285.
- Lin GT, Tseng HF, Chang CK, Chuang LY, Liu CS et al. 2008. SNP combinations in chromosome-wide genes are associated with bone mineral density in Taiwanese women. *Chin J Physiol*, 51: 32-41.
- Lin GT, Tseng HF, Yang CH, Hou MF, Chuang LY et al. 2009. Combinational polymorphisms of seven CXCL12-related genes are protective against breast cancer in Taiwan. *OMICS*, 13: 165-172.
- Liu JM, Zhang MJ, Zhao L, Cui B, Li ZB et al. 2010. Analysis of recently identified osteoporosis susceptibility genes in Han Chinese women. *J Clin Endocrinol Metab*, 95: E112-120.
- Meyers KJ, Chu J, Mosley TH, Kardias SL 2010. SNP-SNP interactions dominate the genetic architecture of candidate genes associated with left ventricular mass in African-Americans of the GENOA study. *BMC Med Genet*, 11: 160.
- Murphy FT, Kivitz AJ, Sands EE 2003. Management of postmenopausal osteoporosis. *J Am Osteopath Assoc*, 103: S6-11.
- Paternoster L, Lorentzon M, Vandenput L, Karlsson MK, Ljunggren O et al. 2010. Genome-wide association meta-analysis of cortical bone mineral density unravels allelic heterogeneity at the RANKL locus and potential pleiotropic effects on bone. *PLoS Genet*, 6: e1001217.
- Peacock M, Turner CH, Econs MJ, Foroud T 2002. Genetics of osteoporosis. *Endocr Rev*, 23: 303-26.
- Ray NF, Chan JK, Thamer M, Melton LJ, 3rd 1997. Medical expenditures for the treatment of osteoporotic fractures in the United States in 1995: report from the National Osteoporosis Foundation. *J Bone Miner Res*, 12: 24-35.
- Richards JB, Rivadeneira F, Inouye M, Pastinen TM, Soranzo N et al. 2008. Bone mineral density, osteoporosis, and osteoporotic fractures: a genome-wide association study. *Lancet*, 371: 1505-1512.
- Rivadeneira F, Styrkarsdottir U, Estrada K, Halldorsson BV, Hsu YH et al. 2009. Twenty bone-mineral-density loci identified by large-scale meta-analysis of genome-wide association studies. *Nat Genet*, 41: 1199-1206.
- Rosenberg NA, Huang L, Jewett EM, Szpiech ZA, Jankovic I et al. 2010. Genome-wide association studies in diverse populations. *Nat Rev Genet*, 11: 356-366.
- Roshandel D, Holliday KL, Pye SR, Boonen S, Borghs H et

- al. 2010. Genetic variation in the RANKL/RANK/OPG signaling pathway is associated with bone turnover and bone mineral density in men. *J Bone Miner Res*, 25: 1830-1838.
- Sasaki N, Kusano E, Ando Y, Yano K, Tsuda E et al. 2001. Glucocorticoid decreases circulating osteoprotegerin (OPG): possible mechanism for glucocorticoid induced osteoporosis. *Nephrol Dial Transplant*, 16: 479-482.
- Simonet WS, Lacey DL, Dunstan CR, Kelley M, Chang MS et al. 1997. Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell*, 89: 309-19.
- Styrkarsdottir U, Halldorsson BV, Gretarsdottir S, Gudbjartsson DF, Walters GB et al. 2008. Multiple genetic loci for bone mineral density and fractures. *N Engl J Med*, 358: 2355-2365.
- Styrkarsdottir U, Halldorsson BV, Gretarsdottir S, Gudbjartsson DF, Walters GB et al. 2009. New sequence variants associated with bone mineral density. *Nat Genet*, 41: 15-17.
- Styrkarsdottir U, Halldorsson BV, Gudbjartsson DF, Tang NL, Koh JM et al. 2010. European bone mineral density loci are also associated with BMD in East-Asian populations. *PLoS One*, 5: e13217.
- Tsai FJ, Chen WC, Chen HY, Tsai CH 2003. The ALUI calcitonin receptor gene polymorphism (TT) is associated with low bone mineral density and susceptibility to osteoporosis in postmenopausal women. *Gynecol Obstet Invest*, 55: 82-87.
- Tucker KL, Morita K, Qiao N, Hannan MT, Cupples LA et al. 2006. Colas, but not other carbonated beverages, are associated with low bone mineral density in older women: The Framingham Osteoporosis Study. *The American Journal of Clinical Nutrition*, 84: 936-942.
- Van Der Voort DJ, Brandon S, Dinant GJ, Van Wersch JW 2000. Screening for osteoporosis using easily obtainable biometrical data: diagnostic accuracy of measured, self-reported and recalled BMI, and related costs of bone mineral density measurements. *Osteoporos Int*, 11: 233-239.
- Von Tirpitz C, Epp S, Klaus J, Mason R, Hawa G et al. 2003. Effect of systemic glucocorticoid therapy on bone metabolism and the osteoprotegerin system in patients with active Crohn's disease. *Eur J Gastroenterol Hepatol*, 15: 1165-1170.
- Yang CH, Chuang LY, Chen YJ, Tseng HF, Chang HW 2011. Computational analysis of simulated SNP interactions between 26 growth factor-related genes in a breast cancer association study. *OMICS*, 15: 399-407.
- Yang HC, Lin CH, Hsu CL, Hung SI, Wu JY et al. 2006. A comparison of major histocompatibility complex SNPs in Han Chinese residing in Taiwan and Caucasians. *J Biomed Sci*, 13: 489-498.
- Yen CY, Liu SY, Chen CH, Tseng HF, Chuang LY et al. 2008. Combinational polymorphisms of four DNA repair genes XRCC1, XRCC2, XRCC3, and XRCC4 and their association with oral cancer in Taiwan. *J Oral Pathol Med*, 37: 271-277.
- Zhang YP, Deng FY, Chen Y, Pei YF, Fang Y et al. 2010. Replication study of candidate genes/loci associated with osteoporosis based on genome-wide screening. *Osteoporos Int*, 21: 785-795.