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SNP Combinations in Chromosome-Wide Genes Are Associated with Bone Mineral Density in Taiwanese Women

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Abstract

Osteoporosis is a major public health problem, mainly quantified by low BMD. Eleven polymorphisms were investigated in this study; TNFα-857 (rs1799724), TGFβ1-509 (rs1800469), osteocalcin (rs1800247), TNFα-308 (rs1800629), PTH BstB I (rs6254), PTH Dra II (rs6256), IL-1ra (VNTR), HSP70 hom (rs2227956), HSP 70-2 (rs1061581), CTR (rs1801197), and BMP-4 (rs17563). The relationship between the combined polymorphisms in different genomic regions and BMD variation was

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investigated. Among the female subjects, the proportion of subjects with low BMD in low BMI group (≤ 18.50) was significantly higher than that of the middle (18.51-22.99) and high (≥ 23.00) BMI groups ($P < 0.05$). In post-menopausal women, there was a significant association between low BMD and genotypes ranging from 2 ~ 7 SNPs. For two combined SNPs, the portion of subjects with low BMD was significantly higher in those with CC-AA genotypes in rs1799724-rs1800629, compared to those with non-CC-AA genotypes in post-menopausal women and the combination of all women. Similarly, part of the combined SNPs with rs1799724-rs1800629-rs6254-rs6256-IL-1ra-rs2227956-rs1801197 was significantly associated with reduced BMD. After controlling for age and BMI, post-menopausal women with certain specific SNP combination had a 3.54- to 4.68-fold increased risk for low BMD, comparing to other SNP combinations. In conclusion, our data suggest that several gene polymorphisms may be cooperatively involved in the development of osteoporosis.

Key Words: SNP interaction, BMD, BMI, association, osteoporosis

Introduction

Osteoporosis is defined by World Health Organization (WHO) as a skeletal disorder characterized by compromised bone strength predisposing to an increase risk of fracture (33). Bone strength depends on the interaction between epigenetic and genetic factors (28). Epigenetic factors including aging (19, 34, 39), menopause (27, 39), and body mass index (BMI) (4, 34, 39, 43) had been reported. As genetic determinants of osteoporosis, several candidate genes including vitamin D receptor (VDR), estrogen receptor (ESR), parathyroid hormone (PTH) (17), glucocorticoid receptor, calcitonin receptor (CTR) (3, 10, 42), insulin-like growth factor-1, collagen 1-α-1 (COL1A1), interleukin-6, transforming growth factor-beta1 (TGF-β1) (44), and APOE had been investigated (14, 38). Recently, some other genes were also reported to be associated with osteoporosis. For example, TNFα is a proinflammatory cytokine that promotes osteoclastic bone resorption. The single nucleotide polymorphisms (SNPs) at -857 and -308 in TNFα gene promoter were found to be associated with osteoporosis in post-menopausal Japanese women (13, 30). Osteocalcin (also known as bone Gla protein, or BGP) gene polymorphism (rs1800247), detected with the Hind III restriction enzyme, was suggested to influence bone mineral density (BMD) and may serve as a possible genetic marker for bone metabolism in post-menopausal Japanese women (9). Noncarriage of the 240-base pair allele of the interleukin 1 receptor antagonist (IL-1ra) gene was reported to be associated with increased bone loss (40). In addition, the polymorphism of human bone morphogenetic protein-4 (BMP4) gene (rs 17563) affecting amino acid sequence was found to be associated with hip BMD in post-menopausal women (37).

Although the above-mentioned polymorphisms have been reported to be associated with osteoporosis, the integral role of these phenotypic and genetic factors on bone mass in pre- and post-menopausal women is still controversial. Carriers of risk alleles in two or more of these SNPs are likely to be at elevated risk of osteoporosis, because several partial deficiencies in these pathways may severely decrease the bone density. Interaction between the polymorphisms in relation to risk of osteoporosis may therefore occur. In this study, the relationships between age, BMI, and genetic factors on BMD in pre- and post-menopausal Taiwanese women were evaluated. The interactions between eleven polymorphisms in nine genes on the incidence of low BMD were examined in this study (Table 1). This is a novel analysis to investigate the association between osteoporosis and combined SNPs with genotypes.

Materials and Methods

Subjects

The study was approved by the Institutional Review Board of Kaohsiung Medical University, Kaohsiung, Taiwan. All subjects signed the informed consent. No individual was receiving or had previously received hormone replacement therapy. Women with surgical menopause were excluded. Clinical data, including body mass index, smoking history, and blood pressure, were collected. This teaching hospital had 1,500 beds and is located in Southern Taiwan. The characteristics of study subjects were randomly recruited from general health inspection in the Center of Health Examination, Department of Preventive Medicine, Kaohsiung Medical University. Fifty pre-menopausal (mean age 43 years) and 257 post-menopausal women (mean age 59 years) were involved in this study. Post-menopausal women were defined by the absence of menstruation for > 6 months or having attained an age ≥ 50 years. The subjects were categorized into underweight (BMI ≤ 18.5 kg/m^2), standard weight (BMI 18.51-22.99 kg/m^2) and overweight (BMI ≥ 23 kg/m^2) according to the re-defined WHO criterion for obesity in Asia Pacific Region (5). Blood samples were collected and stored at -70°C for further analysis.

Table 1. Single nucleotide polymorphisms (SNPs) investigated in this study and their restriction fragment length polymorphism genotyping information

SNP	Chr	Gene	Genotype			Primer pairs (5' to 3')	enzyme	Anneal	rs number
			1	2	3				
1	6	TNFα-857 (13)	TT	TC	CC	F: AAGTCGAGTATGGGGACCCCCCGTTAA R: CCCCAGTGTGTGGCCATATCTTCTT	Hinc II	68°C	rs1799724
2	19	TGFβ1-509 (44)	TT	TC	CC	F: AAGGCATGGCACCGCTTCTG R: GAAGGAGGGTCTGTCAACAT	Aoc I	58°C	rs1800469
3	1	Osteocalcin (9)	HH (CC)	Hh (CT)	hh (TT)	F: CCGCAGCTCCCAACCACAATAAGCT R: CAATAGGGCGAGGAGT	Hind III	59°C	rs1800247
4	6	TNFα-308 (30)	NN (AA)	Nn (AG)	nn (GG)	F: AGGCAATAGGTTTTGAGGGCCAT R: TCCTCCCTGCTCCGATTCCG	Nco I	65°C	rs1800629
5	11	PTH (BstB I) (17)	BB (GG)	Bb (AG)	bb (AA)	F: CATTCTGTGTACTATAGTTTG R: GAGCTTTGAATTAGCA	BstB I	61°C	rs6254
6	11	PTH (Dra II) (17)	DD (AA)	Dd (AC)	dd (CC)	F: CATTCTGTGTACTATAGTTTG R: GAGCTTTGAATTAGCA	Dra II	61°C	rs6256
7	2	IL1_ra* (40)	A1A1*	A1A2	A1A4	F: CTCAGCAAGACTCCTAT R: TCCTGGTCTGCAGGTAA	None	55°C	VNTR a
8	6	HSP70 hom (40)	NN (CC)	Nn (CT)	nn (TT)	F: GATCCAGGTGTATGAGGG R: GTAACTTAGATTCAGGTCTGG	Nco I	55°C	rs2227956
9	6	HSP 70-2 (40)	p1p1 (GG)	p1p2 (AG)	p2p2 (AA)	F: GTGCTCCGACCTGTTCCGAAG R: CGGAGTAGGTGGTGAAGATCTG	Pst I	57°C	rs1061581
10	7	CTR (32)	AA (CC)	Aa (CT)	aa (TT)	F: CTCAGTGATCACGATACTGTG R: ATTCAGTGGAACCAGCGTTGG	Alu I	57°C	rs1801197
11	14	BMP-4 (26)	HH (CC)	Hh (CT)	hh (TT)	F: CCTAACTGTGCCTAG R: CATAACCTCATAAATGTTTATATCGG	Hph I	56°C	rs17563

* IL1_ra genotype: A1, 410 bp; A2, 240 bp; A3, 500 bp; A4, 325 bp; and A5, 595 bp. Only one patient with A3 type is not included for further analysis. A5 type is absent in all patients.

[a] VNTR = variable number of tandem repeats.

Measurements of BMD and Definition of Low and Normal BMD Groups

Body height and weight were measured at the initial examination with the subjects in a standing position without shoes. BMD (in grams per square centimeter) was determined by dual-energy X-ray absorptiometry (XR36, Norland Corp., Fort Atkinson, WI, USA) at the lumbar spine (vertebrae L2, L3, and L4) in a posteroanterior projection. T-score was calculated according to the WHO classification using a locally derived reference range provided by the manufacturer. The subjects were divided into two BMD groups according to their T-score (22, 24, 41). The normal BMD group was defined as T-score > -1 and low BMD yggroup was defined as T-score ≤ -1.

DNA Preparation and SNP Genotyping by PCR-Restriction Fragment Length Polymorphism (RFLP)

Genomic DNA of the subjects was purified from peripheral leukocytes by QIAamp DNA Blood Kit (Qiagen, Valencia, CA, USA) (6). The polymerase chain reaction (PCR) primers and their annealing temperature and restriction enzymes as well as genotype numbering of the eleven SNP candidates investigated in this study are presented in Table 1. PCR reaction mixture (10 μl) containing 1 μl of 10x PCR buffer, 0.3 μl of 50 mM $MgCl_2$, 0.2 μl of 10 mM dNTP each, 0.6 μl of DMSO, 0.14 μl of Taq enzyme (5U/μl), 0.12 μl of 350 μg/ml primers mix (1:1), and 7.64 μl of DNA in water was performed as described (6, 8). PCR was performed in a single step with the following protocol: 94°C (3 min); 40 cycles of 94°C (30 s), 55 ~ 68°C (30 s), 72°C (10 s); 72°C (7 min); and 25°C (end). The annealing temperature for each primer set is listed in Table 1. Appropriate restriction enzymes for analysis of the restriction fragment length polymorphism (RFLP) were identified from the established web-tool, SNP-RFLPing (7). After digesting with corresponding restriction enzymes (New England Biolabs, UK) overnight, the DNA was electrophoresed for genotype determination using agarose gel.

Statistical Analysis

Independent *t*-test was used to analyze differences in age, BMI, and BMD (T-score) between the low BMD and normal BMD groups. The significance for the distribution of BMD status among the three BMI levels (under-, standard-, and over-weight) was analyzed by Chi-squared test. The largest difference in the occurrence of low and normal BMD were determined from the combinations of two to ten SNPs from eleven SNP candidates in this study. Each of the same number of SNP selection was summarized. Logistic regression was used to estimate the odds ratio and 95% confidence interval (CI), corresponding to the effect of each specific SNP combination on the occurrence of low BMD, controlling for age and BMI. All of the analyses were performed separately by menopausal status as well as all subjects combined. Statistical software used in data management and analysis was SPSS 10.0 for Windows. The pairwise Linkage disequilibrium (LD) statistics D' and P value were calculated for markers located at chromosomes 6 or 11 using the HelixTree (tm) version 4.4.1 (Golden Helix, Inc, Bozeman, MT, USA) software packages.

Results

Characteristics of the Study Population

T-score for BMD, age, and BMI of the subjects, separated by menopausal status, are presented in Table 2. The average T-score, age, and BMI between normal and low BMD groups were significantly different in pre-menopausal as well as post-menopausal women. In all women combined, the proportions of subjects with low BMD in low (≤ 18.50), middle (18.51-22.99), and high (≥ 23.00) BMI groups were 90.9%, 65.9%, and 57.3%, respectively ($P < 0.05$).

Association between Combined Polymorphisms and BMD

Table 3 showed the proportion of subjects with low BMD in women with specific SNP combination and other combinations, separated by menopausal status. In pre-menopausal women, the proportions of subjects with low BMD seem to show no significant difference between the subjects with the specific SNP combination and other combinations. However, there were only a few pre-menopausal women in our analysis, and thereby the power of finding significant association in this group was low. In post-menopausal women, the proportions of subjects with low BMD in the group with certain specific SNP combinations were significantly higher than those in their counterparts with other combinations. For example, the proportion of subjects with low BMD in post-menopausal women with a 3, 3 (CC-AA) genotype combination in 1, 5 SNPs (rs1799724-rs6254) was 82.61%, comparing to 57.75% among those with other combination (Chi-square=18.32, $P < 0.05$ in Table 3). The proportions of subjects with low BMD in post-menopausal women with certain specific SNP combinations (two to seven SNPs) were 20-25% higher than those in their counterparts other combinations.

Association between Combined Polymorphisms and BMD after Controlling for Age and BMI

Table 4 presented the estimated effect (Odds ratio

Table 2. T-score for bone mineral density (BMD), age, and BMI distributions in pre- and post-menopausal Taiwanese women

	Pre-menopausal (n = 50)		Post-menopausal (n = 257)		Combined (n = 307)	
	BMD (T-score)		BMD (T-score)		BMD (T-score)	
	Normal (T>-1) n = 37	Low (T ≤ -1) n = 13	Normal (T > -1) n = 80	Low (T ≤ 1) n = 177	Normal (T > -1) n = 117	Low (T ≤ -1) n=190
Average T-score (S.D.)[a]	1.59 (1.71)	-2.31 (1.23)[c]	0.91 (2.35)	-3.28 (1.28)[c]	1.13 (2.18)	-3.21 (1.30)[c]
Average age (S.D.)[a]	43.43 (5.80)	41.92 (6.37)[c]	53.78 (8.63)	61.52 (7.92)[d]	50.50 (9.19)	60.18 (9.26)[c]
Average BMI (S.D.)[a]	23.26 (2.83)	21.25 (1.97)[c]	24.21 (2.73)	23.52 (2.93)[c]	23.94 (2.78)	23.36 (2.90)[d]
BMI groups[b]						
≤ 18.50	1 (50.0%)	1 (50.0%)	0 (0.0%)	9 (100.0%)	1 (9.1%)	10 (90.9%)[d]
18.51-22.99	14 (60.9%)	9 (39.1%)	28 (28.0%)	72 (72.0%)	42 (34.1%)	81 (65.9%)
≥ 23 22 (88.0%)	3 (12.0%)	51 (34.9%)	95 (65.1%)	73 (42.7%)	98 (57.3%)	

a. S.D.: Standard deviation

b. Two post-menopausal women did not have BMI data

c. $P < 0.01$ between normal and low BMD groups

d. $P < 0.05$ between normal and low BMD groups

Table 3. SNP combinations and proportion of low BMD (T score ≤ -1) among pre- and post-menopausal Taiwanese women[b]

Combined SNP #	Individual SNPs	Combination	Pre-menopausal			Post-menopausal			Combined		
			Number of subjects	Number of low BMD (%)	Chi-square	Number of subjects	Number of low BMD (%)	Chi-square	Number of subjects	Number of low BMD (%)	Chi-square
2SNPs	1,5	3,3	26	7 (26.92%)	0.024	115	95 (82.61%)	18.32[a]	141	102 (72.34%)	12.08[a]
		Other	24	6 (25.00%)		142	82 (57.75%)		166	88 (53.01%)	
3SNPs	1,5,7	3,3,1	25	7 (28.00%)	0.104	102	83 (81.37%)	12.33[a]	127	90 (70.87%)	7.40[a]
		Other	25	6 (24.00%)		155	94 (60.65%)		180	100 (55.56%)	
4SNPs	1,4,5,7	3,3,3,1	19	4 (21.05%)	0.390	90	67 (74.44%)	9.05[a]	99	71 (71.72%)	5.98[a]
		Other	31	9 (29.03%)		197	110 (55.84%)		208	119 (57.21%)	
5SNPs	1,4,5,7,10	3,3,3,1,1	10	1 (10.00%)	1.663	58	50 (86.21%)	10.50[a]	68	51 (75.00%)	6.37[a]
		Other	40	12 (30.00%)		199	127 (63.82%)		239	139 (58.16%)	
6SNPs	1,4,5,7,8,10	3,3,3,1,3,1	3	0 (0.00%)		37	32 (86.49%)	6.26[a]	40	32 (80.00%)	6.40[a]
		Other	47	13 (27.66%)		220	145 (65.91%)		267	158 (59.18%)	
7SNPs	1,4,5,6,7,8,10	3,3,3,3,1,3,1	2	0 (0.00%)		28	24 (85.71%)	4.16[a]	30	24 (80.00%)	4.62[a]
		Other	48	13 (27.08%)		229	153 (66.81%)		277	166 (59.93%)	
8SNPs	1,4,5,6,7,8, 9,10	3,3,3,3,1,3,2,1	1	0 (0.00%)		20	16 (80.00%)	1.25	21	16 (76.19%)	1.96
		Other	49	13 (26.53%)		237	161 (67.93%)		286	174 (60.84%)	
9SNPs	1,4,5,6,7,8,9,10,11	3,3,3,3,1,3,2,13	1	0 (0.00%)		11	9 (81.82%)	0.90	12	9 (75.00%)	0.91
		Other	49	13 (26.53%)		246	168 (69.29%)		295	181 (61.36%)	
10SNPs	1,3,4,5,6,7,8,9,10,11	3,3,3,3,3,1,3,2,1,3	1	0 (0.00%)		4	4 (100.00%)		5	4 (80.00%)	0.71
		Other	49	13 (26.53%)		253	173 (68.38%)		302	186 (61.59%)	

a. $P < 0.05$; b. The detailed SNP information is summarized in Table 1.

and 95% CI) of certain specific SNP combinations on the occurrence of low BMD. No specific SNP combination was associated with the risk for low BMD in pre-menopausal women. However, after controlling for age and BMI, post-menopausal women with certain specific SNP combination (two to seven SNPs) had a 3.54- to 4.68-fold increased risk for low BMD, comparing to those with other SNP combinations.

The Pairwise Linkage Disequilibrium (LD) for SNPs Located in Chromosomes 6 and 11

In chromosome 6, a strong pairwise linkage disequilibrium was noted between rs2227956, rs1061581, and rs1800629 ($D' > 0.8$, Table 5). Moderate LD was found between rs1061581 and rs2227956 ($D' > 0.66$). LD was weak in other situations. In chromosome 11, rs6256 and rs6254 are found to have strong pairwise LD ($D' > 0.999$) (data not shown). The relationship between LD value and their association to osteoporosis will be discussed later.

Discussion

The importance of the relationship among multigene polymorphism combinations, epigenetic factors, and multifactorial disease risk had been reviewed (25). In this study, we introduced this idea to examine the important role of phenotype and genotype factors in osteoporosis. BMD was reported to be related negatively to age and positively to body size (19, 35). In this study, all of the underweight (BMI ≤ 18.5) post-menopausal women had low BMD (n = 9), while only 65.1% overweight and obesity (BMI ≤ 23) post-menopausal women had low BMD.

Among the eleven SNP candidates listed in Table 1, we found there are complicated network and possibly have the chance of cross-talk directly or indirectly. For example, estrogens modulate the catabolic effects of PTH on bone *in vivo* and *in vitro* and also may enlist TGF-β in its proapoptotic action on osteoclasts (18). Receptor activity modifying protein-3 (RAMP3), a PTH-induced primary response gene in osteoblastic cells, is a coactivator that directs calcitonin receptor (CTR) for glycosylation, trafficking, and ligand-binding specificity (36). BMP4 is a multifunctional growth factor belonging to the TGF-β super family which is known to play an important role in skeletal development and bone formation (15). Moreover, TGF-β-induced signaling protein Smad7 links the kinase TAK1 to upstream regulators in the proinflammatory TNF signaling pathway (16). Inhibition of SAPK/JNK restored TNFα effects on BMP-induced osteoblast differentiation had demonstrated by Id-1-promoter activity as well as Runx2 and osteocalcin mRNA levels (31). The absence of IL-1ra may suppress TGF-β-mediated signaling pathway (20). HSP70 induction by ING proteins sensitizes cells to TNFα receptor-mediated apoptosis (12).

Accordingly, many osteoporosis-associated genes are located in different chromosome. All the additive or synergistic effect among these SNPs should be considered at the same time rather than evaluating individually from chromosome to chromosome (haplotype-based method). To test this hypothesis, these eleven SNPs from different chromosomes were selected to address the inter-chromosomal relationship of SNPs in osteoporosis at the same time. This study showed that the proportions of subjects with low BMD in post-menopausal women with specific SNP combinations (two to seven SNPs) were higher than those in their counterparts with other combinations. After controlling for age and BMI, post-menopausal women with certain specific SNP combinations (two to seven SNPs) still had increased risk for low BMD, comparing to those with other SNP combinations. Part of the combined SNPs with rs1799724-rs1800629-rs6254-rs6256-IL-1ra-rs2227956-rs1801197 was significantly associated with reduced BMD.

Similarly, gene-to-gene interaction had been applied to analyze the association for complex traits, which are under the influence of multiple and possibly interacting genes. For example, the gene-to-gene interactions between PPARδ (rs2016520 located in chromosome 6) and PPARα (rs1800206 located in chromosome 22) and between PPARδ and PPARγ (rs1801282 located in chromosome 3) in association with clinical parameters were demonstrated (1). The interaction between IL-10 -1082A and IL-6 174C alleles, which is located in chromosome 1 and 7, respectively, on nosocomial blood stream infections were addressed (2). One SNP in CYP19 (3UTR in chromosome 10), two SNPs in CYP1B1 (R48G and A119S in chromosome 2) and one in CYP1A1 (T461N in chromosome15) were significantly differently distributed between the high- and low- level metabolic groups (23). Furthermore, some gene polymorphisms with less interaction with age had been reported. For example, the ADR ApaI polymorphism was significantly associated with normal BMD with or without adjusting for age. However, this significant association vanished after correcting for both age and BMI (43).

Some SNPs were reported to be associated or un-associated with osteoporosis in both pre- and post-menopausal women. For example, the CD38-PvuII polymorphism was significantly associated with femoral neck BMD in pre- and post-menopausal women (11). On the other hand, insulin-like growth factor I gene polymorphisms were reported to be unassociated with BMD in pre-menopausal Chinese women (21) and post-menopausal Japanese women (29). It is possible that some polymorphisms are associated with BMD in pre-menopausal women but unassociated with post-

Table 4. Estimated effects of specific SNP combinations on the occurrence of low bone mineral density in pre- and post-menopausal Taiwanese women

Combined SNP	Individual SNPs	Combination	Pre-menopausal (n=50)		Post-menopausal (n=255)		Combined (n=305)	
			Odds ratio[a]	95% CI	Odds ratio[a]	95% CI	Odds ratio[b]	95% CI
2SNPs	1,5	3,3	1.13	0.28-4.51	3.81	1.96-7.41	2.93	1.64-5.24
		Other	1.0		1.0		1.0	
3SNPs	1,5,7	3,3,1	1.35	0.34-5.32	3.54	1.79-7.00	2.83	1.57-5.10
		Other	1.0		1.0		1.0	
4SNPs	1,4,5,7	3,3,3,1	0.81	0.19-3.46	4.68	2.13-10.30	3.02	1.59-5.72
		Other	1.0		1.0		1.0	
5SNPs	1,4,5,7,10	3,3,3,1,1	0.26	0.03-2.54	4.06	1.61-10.20	2.44	1.18-5.04
		Other	1.0		1.0		1.0	
6SNPs	1,4,5,7,8,10	3,3,3,1,3,1	NE[c]		3.75	1.26-11.15	2.80	1.11-7.09
		Other	1.0		1.0		1.0	
7SNPs	1,4,5,6,7,8,10	3,3,3,3,1,3,1	NE		4.17	1.22-14.24	3.03	1.06-8.70
		Other	1.0		1.0		1.0	
8SNPs	1,4,5,6,7,8,9,10	3,3,3,3,1,3,2,1	NE		2.66	0.73-9.70	2.35	0.71-7.76
		Other	1.0		1.0		1.0	
9SNPs	1,4,5,6,7,8,9,10,11	3,3,3,3,1,3,2,1,3	NE		4.96	0.78-31.45	3.57	0.74-17.25
		Other	1.0		1.0		1.0	
10SNPs	1,3,4,5,6,7,8,9,10,11	3,3,3,3,3,1,3,2,1,3	NE		NE		6.53	0.54-79.65
		Other	1.0		1.0		1.0	

a. Odds ratio estimated with age and body mass index as covariates in the logistic regression model.

b. Odds ratio estimated with age, body mass index, and menopausal status as covariates in the logistic regression model.

c. NE: Not Estimate.

Table 5. Pairwise linkage disequilibrium coefficients (D′) and related chromosome position among four polymorphisms in chromosome 6

SNP ID (chromosome position)	rs1799724 (22400737)	rs1800629 (22401282)	rs2227956 (22636523)	rs1061581 (22642837)
rs1799724		0.295	0.003	0.040
rs1800629	0.295		0.859[a]	0.940[b]
rs2227956	0.003	0.859[a]		0.668[b]
rs1061581	0.040	0.940[b]	0.668[b]	

[a, b] Chi-square *P*-value < 0.005, 0.001, respectively. $D' = 1$ indicated the complete linkage disequilibrium (LD) and $D' = 0$ indicated the absence of LD.

menopausal women, or vise versa. Accordingly, we found that certain specific combined SNPs is unassociated with BMD in pre-menopausal Taiwanese women, while these SNPs were significantly associated with BMD in post-menopausal Taiwanese women with or without the adjustments for age and BMI. These results raised the possibility that these specific combined SNPs are cooperated in hormone-dependent manner. However, we cannot exclude the possibility that the different combined effects may exist in pre- and post-menopausal women. Further research in this area is needed.

Linkage disequilibrium (LD) is a statistical measure of the non-independence of alleles at adjacent loci. Although LD makes tightly linked variants strongly correlated producing cost savings for association studies, such loci are generally in close physical proximity. The relationship can vary dramatically. High or low LDs don't mean they are closely associated with target diseases. A strong pairwise linkage disequilibrium was noted between SNPs rs2227956, rs1061581, and rs1800629 within chromosome 6 ($D' > 0.8$ shown in Table 5) and SNPs rs6256 and rs6254 within chromosome 11 ($D' > 0.999$, data no shown). However, most of these SNPs were not significantly associated with osteoporosis individually in this study except the rs1799724 with low LDs located in chromosome 6 (data not shown). Usually, the haplotype association studies included several SNPs in the same chromosome. This criterion limits the usage numbers of SNPs from many chromosomes. Some of these studies may use many haplotypes from several chromosomes, but the interrelationship among different haplotypes from different chromosomes was less addressed. In contrast, we provided the SNP-SNP interaction for chromosome-wide genes to improve these problems.

The results of this study suggested that specific SNP combination may be a risk factor for post-menopausal osteoporosis in Taiwanese. These results indicated that the specific SNP combination, BMI, and age were independently associated with BMD in post-menopausal Taiwanese women. The methods of this study may provide a valuable tool in examining multiple low-penetrance genetic factors that cooperatively determine the phenotypic traits like osteoporosis.

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