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■ **BONE BIOLOGY**

Evaluation of common variants in the *CNR2* gene and its interaction with abdominal obesity for osteoporosis susceptibility in Chinese post-menopausal females

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Objectives

The objective of this study was to investigate the association of four single-nucleotide polymorphisms (SNPs) of the cannabinoid receptor 2 (*CNR2*) gene, gene-obesity interaction, and haplotype combination with osteoporosis (OP) susceptibility.

Methods

Chinese patients with OP were recruited between March 2011 and December 2015 from our hospital. In this study, a total of 1267 post-menopausal female patients (631 OP patients and 636 control patients) were selected. The mean age of all subjects was 69.2 years (SD 15.8). A generalized multifactor dimensionality reduction (GMDR) model and logistic regression model were used to examine the interaction between SNP and obesity on OP. For OP patient-control haplotype analyses, the SHEsis online haplotype analysis software (http://analysis.bio-x.cn/) was employed.

Results

The logistic regression model revealed that the C allele of rs2501431 and the G allele of rs3003336 were associated with increased OP risk, compared with those with wild genotype. However, no significant correlations were found when analyzing the association of rs4237 and rs2229579 with OP risk. The GMDR analysis suggested that the interaction model composed of two factors, rs3003336 and abdominal obesity (AO), was the best model with statistical significance (p-value from sign test (P_{sign}) = 0.012), indicating a potential gene-environment interaction between rs3003336 and AO. Overall, the two-locus models had a cross-validation consistency of 10/10 and had a testing accuracy of 0.641. Abdominally obese subjects with the AG or GG genotype have the highest OP risk, compared with subjects with the AA genotype and normal waist circumference (WC) (odds ratio (OR) 2.23, 95% confidence interval (CI) 1.54 to 3.51). Haplotype analysis also indicated that the haplotype containing the rs3003336-G and rs2501431-C alleles was associated with a statistically increased OP risk.

Conclusion

Our results suggested that the C allele of rs2501431 and the G allele of rs3003336 of the *CNR2* gene, interaction between rs3003336 and AO, and the haplotype containing the rs3003336-G and rs2501431-C alleles were all associated with increased OP risk.

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Article focus

■ The association of several single-nucleotide polymorphisms (SNPs) within the cannabinoid receptor 2 (*CNR2*) gene with osteoporosis (OP) risk.

■ The impact of additional gene-environment interaction on OP risk.

Key messages

■ This study suggests that obesity has an impact on the relationship between *CNR2* gene SNPs and OP risk.

Strengths and limitations

- Just four SNPs of the *CNR2* gene were chosen. The limited SNPs were not sufficient to capture most genetic information of the *CNR2* gene.
- The sample size in the study was relatively small, so the results obtained should be checked in future studies with a larger sample size in the different populations.

Introduction

Post-menopausal osteoporosis (OP) is a systemic bone disease characterized by low bone mass and microarchitectural deterioration of bone.[1] The World Health Organization (WHO) estimates that 200 million men and women suffer from OP worldwide.[2] In China, it is reported that there are 83.9 million people in this age range and that this number will rise to 212 million by the year 2050.[3] Some previous studies suggested that both environmental and genetic factors contributed to the susceptibility of OP.[4,5] To date, more than 70 genes/loci associated with bone mineral density (BMD) phenotypes have been reported in some genome-wide association studies (GWASs), such as GPR177, ESR1, WNT16, SOX6, LRP5, and FOXL1.[6-8]

In recent years, another candidate gene has gained increased attention: cannabinoid receptor 2 (*CNR2*), which contains six exons spanning a 90 kb region located on 1p36.11, which encodes a non-neuronal cannabinoid 2 (CB2) receptor mainly expressed in immune cells.[9] Previously, several studies have reported the association between the *CNR2* gene and the risk of some diseases, such as immune thrombocytopenia,[10] bipolar disorder,[11] obesity, and insulin resistance.[12] Additionally, some studies have reported the association between the *CNR2* gene and OP susceptibility in different populations.[13-16] However, these studies concluded with inconsistent results. In addition, OP is a disease caused by the interaction of genetic and environmental factors. The environmental factors can control gene expression and, accordingly, the process of the disease.[17] Recent studies suggested that obesity is an important risk factor for OP susceptibility.[18-20] However, until now, no study focused on the impact of the *CNR2* gene/obesity interaction on OP risk. Therefore, the aim of this study was to investigate the association between OP risk and several single-nucleotide polymorphisms (SNPs) within the *CNR2* gene, as well as the impact of additional gene-environment interaction on OP risk based on a Chinese population.

Patients and Methods

Patients. Chinese patients with OP were recruited between March 2011 and December 2015 from our hospital. In this study, 1267 post-menopausal female patients (631 OP patients and 636 control patients) were selected. The mean age of all subjects was 69.2 years (SD 15.8). Patients with OP were diagnosed according to WHO criteria, with OP being defined as BMD > 2.5 standard deviations (T-score) below the mean value of young adults, such as peak bone mass.[21] All selected subjects were Chinese Han and were not related to each other. Patients with no history of treatment for OP, nor of any disease or medication known to affect bone metabolism, were included in the control group. In addition, these control patients were randomly selected and matched by age and geographical location (within 3 km of residence) to OP patients at a ratio of nearly 1:1. Informed consent was obtained from all selected participants. Information on body weight, height, waist circumference (WC), and body mass index (BMI) was obtained. Cigarette smokers were classified as those who self-reported smoking cigarettes at least once a day for one year or more. Alcohol consumption was expressed as the sum of millilitres of alcohol per week from wine, beer, and spirits.

SNP selection and genotyping. The public-domain archive dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP) was used for SNP selection. We selected SNPs within the *CNR2* gene between 10 kb upstream and 10 kb downstream based on the following criteria: 1) located in a gene fragment that could have functional effects; 2) minor allele frequency (MAF) more than 5%; and 3) previously associated with BMD or OP. In this study, the SNPs were selected based on population usage. Due to the lack of manpower, material resources, and financial resources, we selected only four SNPs for genotyping: rs3003336, rs2501431, rs4237, and rs2229579. A genomic DNA extraction kit (Roche, Branchburg, New Jersey) was used to extract genomic DNA from peripheral blood samples of patients and healthy individuals, and was then stored at -20°C until further use. Polymerase chain reaction (PCR)-based restriction fragment length polymorphism was performed to identify the genotype for the four selected SNPs. The nucleotide sequence of primers and description for the four SNPs within the *CNR2* gene are shown in Table I. Genotyping for all SNPs was performed using the MassARRAY System platform (Sequenom, San Diego, California). We used high-throughput, matrix-assisted laser desorption/ionization–time of flight (MALDI–TOF) mass spectrometry. Next, the resulting spectra were processed using Typer Analyzer software (Sequenom), and genotype data were generated from the samples. The amplification protocol comprised an initial denaturation at 95°C for five minutes, 35 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 30 seconds, and a final extension at 72°C for two minutes.

Statistical analysis. All data analysis was performed on SPSS 22.0 software (IBM, Armonk, New York). The mean and standard deviation were measured for continuous variables and the difference was measured using Student's *t*-test; percentages were measured for categorical variables and the difference was measured using a chi-squared test. The genotype distribution difference among individuals with OP and healthy control patients

Table I. Description for the four single-nucleotide polymorphisms (SNPs)

SNP	Chromosome	Functional consequence	Prime sequences
rs2501431	1:23875153	Synonymous codon	F: 5′-ATGGGCATGTTCTCTGGAAG-3′; R: 5′-CCAACAGACTGTGTGCAGGT-3′
rs4237	1:23787639	UTR variant 3 prime	F: 5′-GGCCACATTAACTGGAAAAGCA-3′; R: 5′- GGGCTGGGTGGCATCTG-3′
rs3003336	1:23874958	Synonymous codon	F: 5′-ATGGGCATGTTCTCTGGAAG-3′; R: 5′-CCAACAGACTGTGTGCAGGT-3′
rs2229579	1:23874672	Missense	F: 5′-CTCTGCCCATCACTGCCGG-3′; R: 5′-GGGTCCGTGTCTAGGTGTCTGG-3′

F, forward; R, reverse; UTR, untranslated region

Table II. Best gene-gene and gene-environment interaction models, as identified by generalized multifactor dimensionality reduction (GMDR)

Locus number	Best combination	Cross-validation consistency	Testing accuracy	p-value*
Gene-gene interaction				
2	2, 3	6/10	0.522	0.324
3	2, 3, 1	7/10	0.514	0.255
4	2, 3, 1, 4	6/10	0.597	0.212
Gene-obesity interaction				
2	1, 5	7/10	0.488	0.536
3	1, 2, 5	6/10	0.523	0.706
4	1, 2, 3, 5	5/10	0.536	0.862
5	1, 2, 3, 4, 5	6/10	0.518	0.377
Gene-abdominal obesity interaction				
2	1, 6	10/10	0.641	0.012†
3	1, 2, 6	8/10	0.526	0.266
4	1, 2, 3, 6	9/10	0.514	0.545
5	1, 2, 3, 4, 6	5/10	0.634	0.372

*Adjusted for age, smoking, and alcohol consumption status
†Statistically significant
1, rs3003336; 2, rs2501431; 3, rs4237; 4, rs2229579; 5, obesity; 6, abdominal obesity

was analyzed using a chi-squared test. *In silico* analysis algorithm SHEsis was conducted to analyze OP patient-control haplotype (http://analysis.bio-x.cn/myAnalysis. php).[22,23] Generalized multifactor dimensionality reduction (GMDR)[24] was performed in order to investigate whether or not all of the interactions had been analyzed. We have provided some of the parameters in Table II, including cross-validation consistency, the testing balanced accuracy, and the sign test. These were calculated to assess each selected interaction. The cross-validation consistency score is a measure of the degree of consistency with which the selected interaction is identified as the best model among all possibilities considered. The testing balanced accuracy is a measure of the degree to which the interaction accurately predicts case-control status with scores between 0.50 (no better than chance) and 1.00 (perfect prediction). Finally, a sign test or a permutation test (providing empirical p-values) for prediction accuracy can be used to measure the significance of an identified model. The effects of the interaction between abdominal obesity (AO) and SNPs on the risk of OP were measured by logistic regression model. Statistical significance was set at $p < 0.05$.

Results

Participant characteristics stratified by OP cases and control patients are shown in Table III. The distributions of smoking, alcohol consumption, and mean number of years since menopause were not different between cases and control patients. The means of BMI and WC were higher in OP cases, but the means of lumbar BMD and femoral BMD were significantly lower in OP patients than in control patients.

All genotypes were distributed according to the Hardy–Weinberg equilibrium (all p-values were more than 0.05). The frequencies of the C allele of rs2501431 and the G allele of rs3003336 were higher in individuals with OP than in the healthy control patients (30.4% of OP patients and 22.2% of controls, $p < 0.001$ for C allele of rs2501431; 29.1% of OP patients and 21.2% of control patients, $p < 0.001$ for the G allele of rs3003336; Table IV). The logistic regression model revealed that the C allele of rs2501431 and the G allele of rs3003336 were associated with increased OP risk, compared with those with wild genotype (Table IV). However, no significant correlations were found when analyzing the association of rs4237 and rs2229579 with OP risk.

We employed the GMDR analysis to assess the impact of the *CNR2* gene-gene and gene-environment interaction on OP risk, after adjustment for covariates (Table II). The GMDR analysis suggested that the interaction model composed of rs3003336 and AO was the best model in terms of statistical significance (p-value from sign test (P_{sign}) = 0.012), indicating a potential gene-environment interaction between rs3003336 and AO. Overall, the two-locus models had a cross-validation consistency of 10/10, and had a testing accuracy of 0.641. In order to obtain the odds ratios (ORs) and 95% confidence

Table III. General characteristics of study participants in the osteoporosis group and the control group

Variable	Osteoporosis (n = 631)	Control (n = 636)	p-value
Mean age, yrs (SD)	68.8 (16.5)	69.6 (17.3)	0.400
Smoking, n (%)	41 (6.5)	49 (7.7)	0.403
Alcohol drinking, n (%)	78 (12.3)	86 (13.5)	0.538
Mean BMI, kg/m² (SD)	24.4 (5.9)	23.3 (5.6)	0.001*
Mean WC, cm (SD)	86.5 (16.1)	84.2 (15.6)	0.010*
Mean YSM, yrs (SD)	10.52 (5.34)	11.03 (5.15)	0.084
Mean lumbar BMD, g/cm² (SD)	0.91 (0.14)	1.06 (0.13)	< 0.001*
Mean femoral BMD, g/cm² (SD)	0.81 (0.10)	0.97 (0.11)	< 0.001*
Mean T-score (SD)			
Femoral neck	-2.75 (0.64)	0.01 (0.80)	< 0.001*
Total femoral	-1.50 (1.01)	0.02 (0.84)	< 0.001*
Lumbar spine	-0.72 (0.93)	0.43 (1.02)	< 0.001*
Calcium supplementation, n (%)	196 (31.1)	182 (28.6)	0.341

*Statistically significant
BMI, body mass index; WC, waist circumference; YSM, years since menopause; BMD, bone mineral density

Table IV. The association of genotype and allele within four single-nucleotide polymorphisms (SNPs) with osteoporosis (OP) risk

Genotypes and alleles	Frequency, n (%)		OR (95% CI)*	p-value	HWE test
	OP case (n = 631)	**Control (n = 636)**			
rs2501431					
TT	314 (49.8)	389 (61.2)	1.00		0.390
TC	250 (39.6)	212 (33.3)	1.36 (1.06 to 1.61)	0.028†	
CC	67 (10.6)	35 (5.5)	1.87 (1.26 to 2.50)	0.001†	
TC+CC	317 (50.2)	247 (38.8)	1.32 (1.11 to 1.69)	0.013†	
T	878 (69.6)	990 (77.8)			
C	384 (30.4)	282 (22.2)			
rs4237					
AA	332 (52.6)	365 (57.4)	1.00		0.125
AG	237 (37.6)	224 (35.2)	1.07 (0.94 to 1.39)	0.425	
GG	62 (9.8)	47 (7.4)	1.12 (0.85 to 1.60)	0.562	
AG+GG	299 (47.4)	271 (42.6)	1.09 (0.91 to 1.44)	0.513	
A	901 (71.4)	954 (75.0)			
G	361 (28.6)	318 (25.0)			
rs3003336					
AA	320 (50.7)	397 (62.4)	1.00		0.578
AG	255 (40.4)	208 (32.7)	1.62 (1.36 to 1.88)	0.0012†	
GG	56 (8.9)	31 (4.9)	2.01 (1.61 to 2.80)	< 0.001†	
AG+GG	311 (49.3)	239 (37.6)	1.71 (1.42 to 2.02)	< 0.001†	
A	895 (70.9)	1002 (78.8)			
G	367 (29.1)	270 (21.2)			
rs2229579					
CC	337 (53.4)	371 (58.3)	1.00		0.283
CT	243 (38.5)	223 (35.1)	1.08 (0.91 to 1.36)	0.287	
TT	51 (8.1)	42 (6.6)	1.04 (0.82 to 1.53)	0.628	
CT+TT	294 (46.6)	265 (41.7)	1.07 (0.89 to 1.39)	0.481	
C	917 (72.7)	965 (75.9)			
T	345 (27.3)	307 (24.1)			

*Adjusted for age, smoking, and alcohol consumption status
†Statistically significant
OR, odds ratio; CI, confidence interval; HWE, Hardy–Weinberg equilibrium

intervals (CI) for the joint effects of rs3003336 genotype and AO on OP, we conducted stratified analysis for rs3003336 genotype and AO. We found that abdominally obese subjects with the AG or GG genotype have the highest OP risk, compared with subjects with the AA genotype and a normal WC (OR 2.23, 95% CI 1.54 to 3.51), after adjustment for age, smoking, and alcohol consumption status (Table V).

Pairwise linkage disequilibrium analysis among four SNPs was performed, and the results show that the D′ value between rs2501431 and rs3003336 was more than 0.75. Therefore, haplotype analysis for rs2501431 and rs3003336 was also conducted using the SHEsis online haplotype analysis software. In all samples, the haplotype A-T was observed most frequently in both populations, with 49.22% and 52.87% in the patient and control groups of the population, respectively. The results also indicated that the haplotype containing the rs3003336-G and rs2501431-C alleles was associated with a statistically increased OP risk (OR 1.54, 95% CI 1.19 to 1.96; p = 0.000; Table VI).

Table V. Interaction analysis for rs3003336 and abdominal obesity on osteoporosis (OP) by using logistic regression

rs3003336	Abdominal obesity	OR (95% CI)*	p-value
AA	No	1.00	N/A
AG or GG	No	1.23 (1.07 to 1.49)	0.028[†]
AA	Yes	1.45 (1.12 to 1.87)	0.001[†]
AG or GG	Yes	2.23 (1.54 to 3.51)	< 0.001[†]

*Adjusted for age, smoking, and alcohol consumption status
[†]Statistically significant
OR, odds ratio; CI, confidence interval; N/A, not applicable

Table VI. Cannabinoid receptor 2 (*CNR2*) gene and osteoporosis (OP) risk association measured by haplotype analysis

Haplotype (rs3003336-rs2501431)	Frequency (ratio)		OR (95% CI)	p-value*
	OP patient	Control		
A-T	0.4922	0.5287	1.00	N/A
G-T	0.2386	0.2191	1.10 (0.76 to 1.67)	0.512
A-C	0.2068	0.218	1.23 (0.83 to 1.74)	0.421
G-C	0.0624	0.0342	1.54 (1.19 to 1.96)	< 0.001[†]

*Adjusted for sex, age, smoking status, and body mass index
[†]Statistically significant
OR, odds ratio; CI, confidence interval; N/A, not applicable

Discussion

The current study indicated that OP risk was significantly higher in individuals with the C allele of rs2501431 and the G allele of rs3003336. However, no significant correlations were found when analyzing the association of rs4237 and rs2229579 with OP risk after covariate adjustment. There were two receptors in the cannabinoid receptor system: cannabinoid receptors 1 (*CNR1*) and 2 (*CNR2*). The former, which is mainly located in the brain, has been effective for approaches in the treatment of obesity.[25] In contrast, *CNR2* has been referred to as the peripheral cannabinoid receptor isoform that is mainly expressed in cells of the immune system. The *CNR2* gene contains six exons spanning a 90 kb region located on 1p36.11, which encodes the non-neuronal CB2 receptor mainly expressed in immune cells.[9] Several articles have investigated the genetic aetiology of OP, implicating potential candidate genes associated with OP risk factors, which include SNP polymorphism within the *CNR2* gene;[13,16] however, the results of this association were inconsistent. A previous study showed that *CNR2* knockout mice had a decreased bone mass reminiscent of human OP.[26] A Chinese study[13] provided further supportive evidence for the association of the *CNR2* gene with BMD, and indicated that the *CNR2* gene is a susceptibility locus for BMD reduction. In this study, two SNPs (rs4237 and rs2501431) in the *CNR2* gene were associated with reduced BMD in post-menopausal Han Chinese women. Woo et al[14] conducted a study of Korean post-menopausal women and indicated that several SNPs, including rs2501431 and rs3003336 polymorphisms, in CNR genes may be genetic factors affecting BMD, and further highlighted that OP risk was higher in the subjects with minor alleles of the two SNPs. Similarly, Karsak et al[15] suggested a role for the peripherally expressed CB2 receptor in the aetiology of OP, and provided an interesting, novel, and therapeutic target for this severe and common disease. Another study by Yamada et al[16] indicated that *CNR2* loci were associated with reduced bone mass in Japanese women; therefore, this study concluded that *CNR2* may confer susceptibility to post-menopausal OP in women.

A combination of both genetic and environmental risks determines the development and progression of OP susceptibility. It has been proposed that genetic background is able to modulate individual response to environmental factors. Several metabolic abnormalities are implicated in the pathogenesis of OP, such as obesity, which has been reported in recent studies.[18-20] However, until now, no study focused on the effect of gene-obesity interaction on OP risk in the Chinese population. This study investigated the impact of additional *CNR2* gene-AO interaction on OP risk, based on a Chinese population, by using the GMDR model. We found a significant gene-environment interaction between rs3003336 and AO, and abdominally obese subjects with the AG or GG genotype have the highest OP risk, compared with subjects with the AA genotype and a normal WC. A previous study by Tamaki et al[27] has suggested that tobacco smoking is also a risk factor for OP. In this study, tobacco smoking has been adjusted in the analysis to reduce its influence on the results. In addition, in terms of the association between OP and obesity, previous studies failed to obtain a consistent result. Some studies[18,19] suggested that AO was a risk factor for OP, while other studies[28,29] concluded that obesity was a protective factor for OP. The implication of linkage disequilibrium in association studies is that knowledge of variation at a certain position also gives knowledge of variation at linked loci. In this study, we found that the D' value between rs2501431 and rs3003336 was more than 0.75; it showed a strong chain reaction. Thus, we also conducted haplotype analysis between rs2501431 and rs3003336. Furthermore, the results indicated that the haplotype containing the rs3003336-G and rs2501431-C alleles was associated with a statistically increased OP risk.

Several limitations of this study should be considered. First, just four SNPs of the *CNR2* gene were chosen. The limited number of SNPs was insufficient to capture most of the genetic information of the *CNR2* gene. Second, the sample size in the study was relatively small, so the results obtained should be checked in future studies with a larger sample size and in the different populations. Last, rs2501431 and rs3003336 showed a strong degree of linkage, suggesting that one could be a surrogate for the other, so a false positive may exist for the association between one SNP and OP risk.

In conclusion, our study provides further contributory evidence for the significant association of the rs2501431-C

allele, and rs3003336-G allele within the *CNR2* gene with increased susceptibility to OP. In addition, we also found that both interaction between rs3003336 and AO, and haplotype containing the rs3003336-G and rs2501431-C alleles were associated with increased OP risk.

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Author contributions

- W. Zheng: Designed and conceptualized the study, Reviewed the manuscript.
- C. Liu: Designed and conceptualized the study, Wrote, edited, and reviewed the manuscript.
- M. Lei: Performed experimental studies, Wrote the manuscript.
- Y. Han: Performed experimental studies, Wrote the manuscript.
- X. Zhou: Reviewed the literature, Wrote and edited the manuscript.
- C. Li: Performed experimental studies, Analyzed the data.
- S. Sun: Reviewed the literature, Analyzed the data.
- X. Ma: Reviewed the manuscript.