Full Length Research Paper

Cannabinoid receptor 2 gene polymorphism was associated with bone mineral density and t-score in Chinese postmenopausal women

Xilian Hu[1,2], Haibao Xie[1,2], Quanshen Yang[1], Yazhen Wang[1,2], Gengxiang Mao[1,2,] Weihong Xu[1,2], Yuandong Lv[1,2], Xiaolin Wen[1,2], Pinghua Tao[1,2], Bing Lin[1,2], Guofu Wang[1,2]* and Jing Yan[1,2]

[1]Zhejiang Provincial Key Lab of Geriatrics, Zhejiang Hospital, 12 Lingyin Road, Hangzhou, 310013, China.
[2]Zhejiang Hospital, 12 Lingyin Road, Hangzhou, 310013, China.

The objective of the study is to investigate the association of rs2229579 polymorphism of cannabinoid receptor 2 gene (CNR2) with bone mineral density (BMD) in Chinese Han postmenopausal women. 490 Chinese Han postmenopausal women were randomly recruited and their BMD were measured by dual-energy X-ray absorptiometry (DEXA) at total body, lumbar spine (L2-L4), femoral neck and trochanter. Genotypes for the CNR2 rs2229579 were determined by polymerase-chain-reaction with the confronting-two-primer (PCR-CTPP). The genotypes distribution of CNR2 rs2229579 was met with Hardy-Weinberg equilibrium (P>0.05). The allelic frequencies for the 490 Chinese Han postmenopausal women were 82.8% for C and 17.2% for T in CNR2 rs2229579 polymorphisms. The prevalence of each genotype in the study population was 67.8% CC, 30.0% CT, and 2.2% TT. BMD for both of femoral neck and total body in women with TT genotype was significantly higher than those with CC, CT and combined group of CC and CT genotypes, respectively (all the *P* values <0.05). T-score at femoral neck in women with TT genotype was higher than those with combined group of CC and CT genotypes (*P*<0.05). T-score at total body in women with CC genotype was lower than those with combined group of CT and TT genotypes (*P*<0.05). There were no genotypes or alleles distribution differences in CC, CT and TT genotypes between osteoporosis and control group (χ^2 = 0.750, *P* > 0.05; χ^2 = 1.804, *P* > 0.05). Our results suggest that there is a significant association between CNR2 rs2229579 genotype polymorphisms and BMD, as well as t-score in Chinese Han postmenopausal women. This suggests that CNR2 rs2229579 may play a role in osteoporosis pathogenesis.

Key words: Association, bone mineral density, cannabinoid receptor 2 gene, osteoporosis, polymorphism, rs2229579.

INTRODUCTION

Osteoporosis is a common age-related skeleton disease, characterized by low bone mass, disturbed micro-architecture of bone tissue, and increased fracture risk with high morbidity and mortality. The multiple factors contributing to the pathogenesis of osteoporosis include genetic and environmental factors. BMD is closely related to genetic factors, where as much as 60 to 85% of the variance in BMD has been attributed to genetic factors according to heritability studies in twins and families (Yamada et al., 2007). A significant decrease in BMD leads to an enhanced liability to bone fractures and is a major clinical denominator of human osteoporosis.

Since BMD decrease is an important clinical indicator and a useful quantitative character, many association and linkage studies of BMD have been conducted. Although the molecular regulation of BMD is largely unknown, variants in a few genes, including the collagen 1A1-gene (Grant et al., 1996), the vitamin D receptor gene (Morrison

_____
*Corresponding author. E-mail: zpklg@yahoo.com.cn or zjicu@vip.163.com. Tel: 86-571-87987373-5421. Fax: 86-571-87985354.

et al., 1994), and the low density lipoprotein receptor-related protein 5 gene (Gong et al., 2001), have repeatedly been shown to be associated with bone mass. Some new candidate genes including the procollagen lysyl hydroxylase (PLOD) and methylenetetrahydrofolate reductase (MTHFR) genes (Huang et al., 2009), also showed significant genotypic/allelic associations with BMDs at all sites measured. Moreover, the bone morphogenetic protein 2 gene (Styrkarsdottir et al., 2003) has been identified as a human osteoporosis susceptibility gene by a positional cloning approach.

Recently, the role of the endocannabinoid system in the regulation of bone mass has been demonstrated in mice. The endogenous cannabinoids bind to and activate the type 1 (CNR1) and type 2 (CNR2) cannabinoid receptors. Cannabinoid receptors have been implicated in bone mass, bone loss and osteoclast and osteoblast activities (Idris et al., 2005; Ofek et al., 2006; Sophocleous et al., 2011). However, the genes that indicate susceptibility to osteoporosis continue to be identified definitively. Recently, research about the association of polymorphisms of CNR2 gene and BMD appears as new explorations, which demonstrates a role for the peripherally expressed CNR2 receptor in the etiology of osteoporosis and provide a new therapeutical target for osteoporosis (Idris, 2010). A significant association of single polymorphisms of CNR2 rs2229579 implies osteoporosis in a case-control study in French postmenopausal women (Karsak et al., 2005). CNR2 rs2229579 is not associated with radiographic hand BMD but associated with breaking bending resistance index (BBRI) in Chuvashians (Karsak et al., 2009). Huang et al. (2009) obtained a result of no association of BMD and CNR2 2229579 in Chinese population of Hong Kong. As far as we know, no more related studies were performed. Considering ethnic diversities in gene polymorphisms exist as well in lifestyle and other environmental factors, it is necessary to investigate polymorphisms related to BMD in different ethnic groups. China is a country of mutilple ethnic groups. In Huang et al's (2009) study, there was no exact information about the ethnic group of the investigated Chinese population. Meanwhile, Z-score of BMD was selected as the diagnostic standard of osteoporosis in Huang et al's study, which is not appropriate for an elderly population. Therefore, we selected the rs2229579 to examine the association of its polymorphism with BMD in postmenopausal women of Chinese Han population.

MATERIALS AND METHODS

Clinical data

All subjects were randomly recruited from the region of Hangzhou and investigated in Zhejiang Hospital. They were all postmenopausal women of Chinese Han population and ≥ 50 years old, more than one year after natural menopause. All participants underwent a routine clinical and biochemical assessment to exclude

secondary causes of osteoporosis and signed an informed consent. The study was approved by Zhejiang hospital Internal Ethical Review Board.

Subjects with diseases known to cause abnormalities of bone metabolism, including chronic renal failure, rheumatoid arthritis, diabetes mellitus, as well as thyroid, parathyroid, adrenal, and other endocrine disorders, or those who had received treatment known to affect bone metabolism for more than a month, such as estrogen, glucocorticoids, bisphosphonates, and vitamin D, were excluded from the present study. Menopausal status was evaluated with a detailed questionnaire, and menopause was defined as complete cessation of menstruation. Individuals whose genotypes were not successfully determined were also excluded from the analysis. Finally 490 women's data was used in the statistical analysis.

Measurement of BMD

The subjects underwent BMD measurements of total body, lumbar spine (L2 to L4), femoral neck and trochanter determined by dual energy X-ray absorptiometry (DEXA) (GE LUNAR EXPERT-XL, Lunar Corporation, U.S.A.). Further details of the subjects' clinical data are given in Table 1.

Body mass index

The participants' body mass index (BMI) was calculated by dividing weight (kg) by the square of height (m^2).

DNA extraction

Genomic DNA was isolated from whole blood using the QIAamp DNA blood protocol according to the manufacturer's instruction (Qiagen Ltd., UK).

Genotyping of the rs2229579 of CNR2

Genotyping was based upon PCR-CTPP method, which does not require a step to digest DNA products for single nucleotide polymorphism genotyping (Tamakoshi et al., 2003). Briefly, the sequences of four primers (Shanghai Chaoshi Biotech, Ltd, China) used for CNR2 polymorphism are F1:5'-CCCATCACTGCCTGGCTC-3', R1:5'-CATCAGCCTCTGTCTCGGTG-3', F2: 5'-CCCGAATGAGGCTGGATG-3' and R2: 5'-CCCCTCACACACTTCTTCCAGTA-3'.

Each 25 µl reaction mixture contained 1U CS^{TM} Taq CE DNA polymerase (Shanghai Chaoshi Biotech, Ltd, China), 1.5 mmol/L Mg^{2+}, 0.2 mmol/L dNTPs (Shanghai Chaoshi Biotech, Ltd, China), 2 primers separately for each set of CTPP primers (F1R1 and F2R2), 20 pmol of each primer and 1 µl template. The PCR (Bio-Rad Laboratories, Inc, U.S.A) conditions were as follows: Initial denaturation at 95°C for 5 min, followed by 40 cycles at 95°C for 30 s, at 59°C for 30 s, at 72°C for 40 s, and a final extension at 72°C for 5 min. After transient centrifugation, 2% agarose gel electrophoresis was performed. All PCR products were visualized with ethidium bromide staining. All samples were tested twice by different examiners and the results were concordant for all duplicate sets.

Statistical analysis

Data presented as means ± SE. SPSS 13.0 statistical software (SPSS Inc., Chicago, IL, USA) was used to analyze the data. Data were compared among three genotype groups by one-way analysis

Table 1. BMD and other characteristics for postmenopausal women (n = 490) according to the CNR2 genotype.

Characteristic	CC	CT	TT	CC+CT	CT+TT
Number (%)	332 (67.8%)	147 (30.0%)	11 (2.2%)	479 (97.8%)	158 (32.2 %)
Age (years)	63.6±0.3	63.1±0.4	62.0±1.0	63.4±0.3	63.0±0.4
Height (cm)	157.0±0.3	157.1±0.4	157.5±1.4	157.0±0.2	157.1±0.4
Weight (kg)	57.8±0.4	57.5±0.7	57.5±2.4	57.7±0.4	57.5±0.7
BMI (kg/m^2)	23.4±0.2	23.3±0.3	23.2±1.0	23.4±0.1	23.3±0.3
Years after menopause	12.7±0.4	12.1±0.5	11.7±1.6	12.5±0.3	12.1±0.5
Age at menopause	50.9±0.2	51.0±0.3	50.3±1.2	50.9±0.2	50.9±0.3
BMD (g/cm^2)					
L2-4	0.989±0.009	0.995±0.012	1.018±0.057	0.991±0.007	1.000±0.012
Femoral neck	0.803±0.007[a]	0.816±0.011[b]	0.908±0.031	0.807±0.006[c]	0.823±0.011
Trochanter	0.646±0.006	0.647±0.008	0.691±0.025	0.646±0.005	0.650±0.008
Total body	0.856±0.007[d]	0.866±0.010[e]	0.956±0.035	0.859±0.006[f]	0.873±0.009
T-score					
L2-4	-0.876±0.083	-0.752±0.119	-0.845±0.476	-0.838±0.068	-0.758±0.115
Femoral neck	-0.532±0.064	-0.37±0.103	0.109±0.251	-0.482±0.055[g]	-0.337±0.098
Trochanter	-0.600±0.067	-0.612±0.095	-0.364±0.257	-0.601±0.055	-0.594±0.090
Total body	-0.327±0.065	-0.117±0.093	0.282±0.291	-0.263±0.053	-0.089±0.089[h]

BMD is adjusted for age, height, and body weight. Data are means ± SE. aP = 0.006 bP = 0.018, cP = 0.007, dP = 0.008, eP = 0.020, fP = 0.010, gP = 0.042 versus TT, hP = 0.031 versus CC.
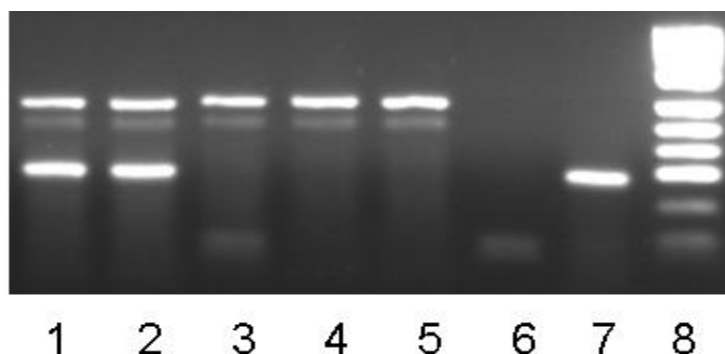

Figure 1. Agarose gel electrophoresis of PCR products. Lane 1 and 2 are the mixture of 102 and 265 bp products, which means C plus T alleles. Lane 3, 4, 5 is the 265 bp PCR product, respectively, which means T allele of rs2229579. Lane 6 is the negative control. Lane 7 is the 102 bp PCR product, which means C allele of rs2229579. Lane 8 is the DNA marker.

of variance (ANOVA) and the LSD (least-significant difference) post hoc test, and between two groups (Combined genotype and the rested group) by the Independent-Samples T-test. Genotype frequencies were compared and tested for Hardy-Weinberg Equilibrium (HWE) by Pearson's χ^2 test or Fisher's exact test when appropriate. When the assumption of the χ^2 test was violated (that is, when > 1 cell had an expected count of <1 or >20% of the cells had an expected count of <5), Fisher's exact test was used. A *P* value of <0.05 was considered statistically significant. Distribution of allele and genotype of CNR2 in osteoporosis and control groups was determined by Pearson's χ^2 test and Fisher's exact test, respectively.

RESULTS

Genotypes frequencies of CNR2 rs2229579

Agarose gel electrophoresis of PCR-CTPP products (Figure 1) showed that 102 bp fragments represented the C allele while 265 bp fragments represented the T allele of CNR2 rs2229579. All 490 subjects fall within three genotypes (CC, CT and TT) at rs2229579 site. Genotype CC was the most common genotype in these participants
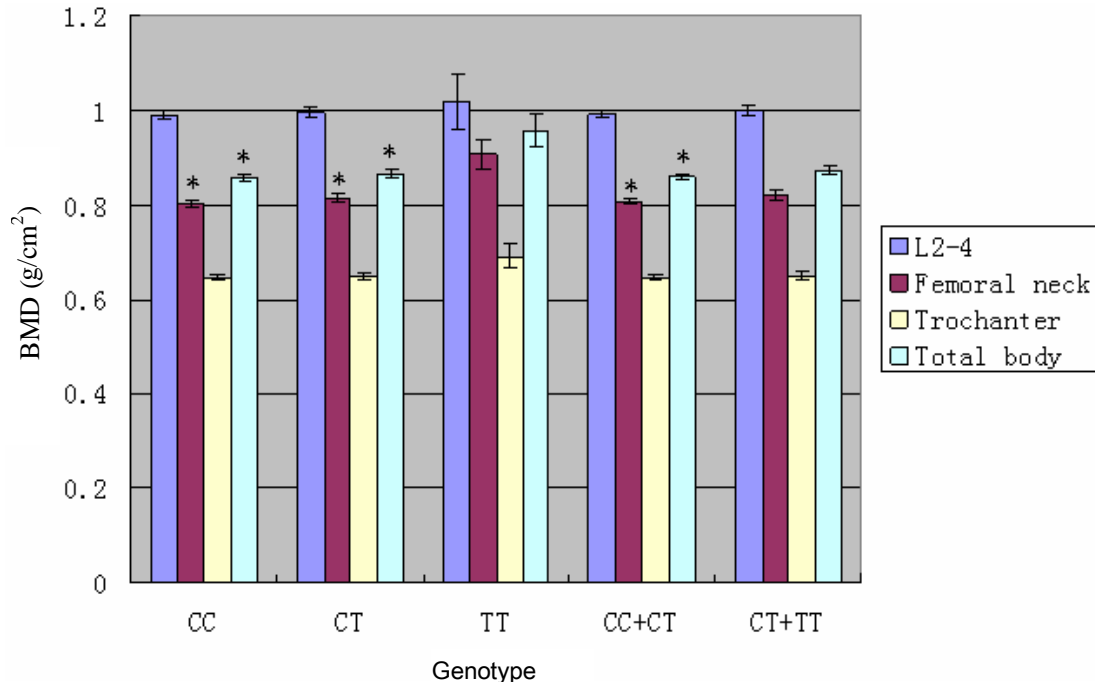
Figure 2. BMD of the lumbar spine (L2-4), femoral neck, trochanter and total body with different genotypes and combined genotypes in 490 postmenopausal women. *Significant difference compared with TT genotype (P<0.05).

(n = 332, 67.8%), genotype CT was the medium one (n = 147, 30.0%), and genotype TT was rare (n = 11, only 2.2%). The genotype frequencies satisfied the Hardy-Weinberg equilibrium (χ^2 = 1.279, P = 0.258).

Association of the CNR2 rs2229579 polymorphism to BMD and T-score

Age, height, weight, BMI, years after menopause and age at menopause did not differ among genotypes for all women (Table 1). BMD for femoral neck in women with TT genotype was significantly higher than those with CC, CT and combined group of CC and CT genotypes (P = 0.006, P = 0.018 and P = 0.007, respectively); and BMD for total body in women with TT genotype was also significantly higher than those with CC, CT and combined group of CC and CT genotypes (P = 0.008, P = 0.020 and P = 0.010, respectively). T-score at femoral neck in women with TT genotype was significantly higher than those with combined group of CC and CT genotypes (P = 0.042) (Figure 2). T-score at total body in women with CC genotype was significantly lower than those with combined group of CT and TT genotypes (P = 0.031) (Figure 3). Besides, there were no significant differences between BMD or t-score and rs2229579 genotypes polymorphism. No significant distribution difference existed in CC, CT and TT genotypes or alleles between osteoporosis and control group (χ^2 = 0.750, P = 0.734; χ^2 =

1.804, P = 0.179) (Table 2).

DISCUSSION

Genotyping is a common technique used in association studies of diseases. Many high throughput platforms of SNP genotyping have been developed in recent years, such as single nucleotide polymorphism (SNP) array and real-time PCR using TaqMan probes. However, compared to these high throughput platforms of SNP genotyping, polymerase chain reaction- Restriction Fragment Length Polymorphism (PCR-RFLP) (PCR-RFLP) is an economic way (Yang et al., 2010). It is still a most frequently used method of genotyping in many laboratories (Chang et al., 2006; Aomori et al., 2009). One shortcoming of PCR-RFLP is its long digestion time for restriction enzymes. Recently, a new genotyping technique called "PCR with confronting two-pair primers (PCR-CTPP)" was developed without restriction reaction (Hamajima et al., 2000) and has been applied successfully in SNP genotyping (Abu-Amero et al., 2006; Togawa et al., 2005). PCR-CTPP has the advantage of simplicity, and is also applicable for detection of association of disease study, which means that it should be superior to PCR-RFLP for genotyping. DNA microarrays will eventually be cheaper, but may not be so effective for genotyping of a limited number of SNPs with a large sample size.

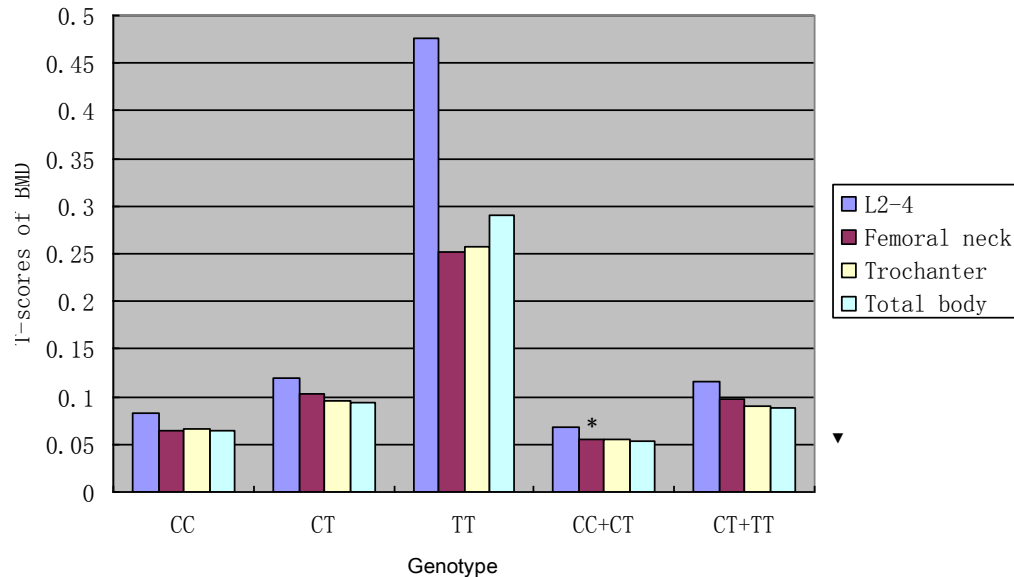In a study of postmenopausal Caucasian of French

Figure 3. T-scores of BMD for the lumbar spine (L2-4), femoral neck, trochanter and total body with different genotypes and combined genotypes. *Significant difference compared with TT genotype (P<0.05), ▼Significant difference compared with CC genotype (P<0.05).

Table 2. The distribution of different CNR2 genotypes and alleles on the development of osteoporosis in 490 postmenopausal women.

Osteoporosis	CNR2 genotype			CNR2 allele	
	CC	CT	TT	C	T
Osteoporosis patients/total patients (%)	216 (66.5%)	101 (31.1%)	8 (2.5%)	533 (80.8%)	127 (19.2%)
Control/total control (%)	116 (70.3%)	46 (27.9%)	3 (1.8%)	278 (84.2%)	52 (15.8%)
Total	332	147	11	811	179

Statistical analysis method	Fisher's exact test	Pearson Chi-Square
χ^2	0.750	1.804
P value	0.734	0.179

origin (Karsak et al., 2005), a significant association of single allele polymorphisms (*P* = 0.038) and genotypes (*P* = 0.046) of CNR2 2229579 with osteoporosis was found, However, the research did not analyze BMD at trochanter and total body, which is specially useful to diagnose osteoporosis and predict the fracture risk of osteoporosis. The relationship of genotypes polymorphism of CNR2 rs2229579 and BMD at trochanter or total body was not clear. Another study (Karsak et al., 2009) showed that CNR2 rs2229579 was significantly associated with radiographic breaking bending resistance index (BBRI) while not associated with hand BMD in Chuvashian population. Huang et al. (2009) investigated association of BMD including spine, femoral neck, trochanter and total body sites with CNR2 rs2229579 in Chinese Hong Kong population, but the SNP rs2229579 did not show significant allelic or genotype association with BMD for all measured sites. Besides, Huang et al's (2009) studies did

not analyze the BMD information of participants with bone loss (-1.28 < Z-score ≤ 1.0) (Huang et al., 2009), while participants with bone loss also have a risk of osteoporosis and are worth analyzing.

In our study, we found that BMD for women with TT genotype at femoral neck and total body sites was significantly higher than those with CC, CT and combined group of CC and CT genotypes, respectively. T-score at femoral neck in women with TT genotype was significantly higher than those with combined group of CC and CT genotypes, while t-score at total body in women with CC genotype lower than those with combined group of CT and TT genotypes. The percentage of TT genotype was rare. The distributions of genotypes and alleles were not significant different between osteoporosis and control group. This suggests that the polymorphism at CNR2 rs2229579 would possibly be used as a predictive factor for reduced BMD and t-score in Chinese Han

postmenopausal women. Obviously, the results of previous studies (Huang et al., 2009; Karsak et al., 2005, 2009) between CNR2 rs2229579 genotypes and BMD or osteoporosis are slightly different from ours. Ethnic diversities contribute to these genetic diversities. Chinese population is different from Caucasian, Chuvashian and other populations in genetics. Han population is the largest one of 56 ethnic groups in China, which possesses the majority of Chinese people (at least > 90%). Therefore, it requires special study. In Huang's (2009) study, no exact Chinese ethic group was given. By the way, selecting Z-score of BMD as the diagnostic standard of osteoporosis is not quite suitable for elderly persons. Lifestyle, environmental factors, and dietary habits, labor intensity, sunshine time are also factors affecting the BMD. Besides, the not big sample size limited the reliability of study results. Furthermore, the occurring mechanisms of osteoporosis are complicated and regulated by multiple genes.

Rs2229579 and His316Tyr is a missense variant among the large number of associated polymorphisms in CNR2 with amino acid exchanges in non-conserved regions, the alone or in combination with other linked polymorphisms, may contribute for the observed association by altering protein function (Karsak et al., 2005).

In summary, our study demonstrated significant association of the CNR2 rs2229579 genotype polymorphisms with BMD and t-score in Chinese Han postmenopausal women. Further researches with multiple centers, large number sample in different areas and ethnic groups will be essential to address the association of CNR2 polymorphisms and BMD, to reveal the functional role of CB2 receptors in osteoporosis and may eventually open novel therapeutic strategies. The principal endogenous ligand for the CB2 receptor is 2-archidonoylglycerol (2-AG). Available agonists include HU-308, JWH-015, JWH-133, L-759,633, L-759,656, and Echinacea purpurea. The antagonists include BML-190 and JTE-907. We are interested in testing these compounds on animal model of osteoporosis in the future studies.

ACKNOWLEDGEMENTS

REFERENCES

Abu-Amero KK, Al-Boudari OM, Mohamed GH, Dzimiri N (2006). The Glu27 genotypes of the beta2-adrenergic receptor are predictors for severe coronary artery disease. BMC Med. Genet., 7: 31.

Aomori T, Yamamoto K, Oguchi-Katayama A, Kawai Y, Ishidao T, Mitani Y (2009). Rapid single-nucleotide polymorphism detection of cytochrome P450 (CYP2C9) and vitamin K epoxide reductase (VKORC1) genes for the warfarin dose adjustment by the SMart-amplification process version 2. Clin. Chem., 55: 12- 804.

Chang HW, Yang CH, Chang PL, Cheng YH, Chuang LY (2006). SNP-RFLPing: restriction enzyme mining for SNPs in genomes. BMC Genomics, 7: 30.

Gong Y, Slee RB, Fukai N, Rawadi G, Roman-Roman S, Reginato AM (2001). LDL receptor-related protein 5 (LRP5) affects bone accrual and eye development. Cell, 107: 23-513.

Grant SF, Reid DM, Blake G, Herd R, Fogelman I, Ralston SH (1996). Reduced bone density and osteoporosis associated with a polymorphic Sp1 binding site in the collagen type I alpha 1 gene. Nat. Genet., 14: 5- 203.

Hamajima N, Saito T, Matsuo K, Kozaki K, Takahashi T, Tajima K (2000). Polymerase chain reaction with confronting two-pair primers for polymorphism genotyping. Jpn. J. Cancer Res., 91: 8- 865.

Huang QY, Li GH, Kung AW (2009). Multiple osteoporosis susceptibility genes on chromosome 1p36 in Chinese. Bone, 44: 8-984.

Idris AI (2010). Cannabinoid receptors as target for treatment of osteoporosis: A tale of two therapies. Curr. Neuropharm., 8(3): 243-53.

Idris AI, van't Hof RJ, Greig IR, Ridge SA, Baker D, Ross RA (2005). Regulation of bone mass, bone loss and osteoclast activity by cannabinoid receptors. Nat Med., 11: 9-774.

Karsak M, Malkin I, Toliat MR, Kubisch C, Nürnberg P, Zimmer A (2005). Cannabinoid receptor type 2 gene is associated with human osteoporosis. Hum. Mol. Genet., 14: 96-3389.

Karsak M, Malkin I, Toliat MR, Kubisch C, Nürnberg P, Zimmer A (2009). The cannabinoid receptor type 2 (CNR2) gene is associated with hand bone strength phenotypes in an ethnically homogeneous family sample. Hum Genet., 126: 36-629.

Morrison NA, Qi JC, Tokita A, Kelly PJ, Crofts L, Nguyen TV (1994). Prediction of bone density from vitamin D receptor alleles. Nature, 367: 7- 284.

Ofek O, Karsak M, Leclerc N, Fogel M, Frenkel B, Wright K (2006). Peripheral cannabinoid receptor, CB2, regulates bone mass. Proc. Natl. Acad. Sci. U S A, 103: 696-701.

Sophocleous A, Landao-Bassonga E, Van't Hof RJ, Idris AI, Ralston SH (2011). The Type 2 Cannabinoid Receptor Regulates Bone Mass and Ovariectomy-Induced Bone Loss by Affecting Osteoblast Differentiation and Bone Formation. Endocrinol., 2011 Jun; 152(6): 2141-2149.

Styrkarsdottir U, Cazier JB, Kong A, Rolfsson O, Larsen H, Bjarnadottir E (2003). Linkage of osteoporosis to chromosome 20p12 and association to BMP2. PLoS Biol., 1: E69.

Tamakoshi A, Hamajima N, Kawase H, Wakai K, Katsuda N, Saito T (2003). Duplex polymerase chain reaction with confronting two-pair primers (PCR-CTPP) for genotyping alcohol dehydrogenase beta subunit (ADH2) and aldehyde dehydrogenase 2 (ALDH2). Alcohol, 38: 10- 407.

Togawa S, Joh T, Itoh M, Katsuda N, Ito H, Matsuo K (2005). Interleukin-2 gene polymorphisms associated with increased risk of gastric atrophy from Helicobacter pylori infection. Helicobacter, 10: 8-172.

Yamada Y, Ando F, Shimokata H (2007). Association of candidate gene polymorphisms with bone mineral density in community-dwelling Japanese women and men. Int. J. Mol. Med., 19: 791-801.

Yang CH, Cheng YH, Chuang LY, Chang HW (2010). Confronting two-pair primer design for enzyme-free SNP genotyping based on a genetic algorithm. BMC Bioinforma., 11: 509.