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ORIGINAL ARTICLE

The association of common polymorphisms in the *QPCT* gene with bone mineral density in the Chinese population

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Abstract Evidence of the linkage of chromosome 2p to bone mineral density (BMD) has previously been reported in multiple populations. However, the identification of the BMD quantitative trait loci (QTL) gene at chromosome 2p remains a challenge. We performed a gene-wide and tag single nucleotide polymorphism (SNP)-based association study of four positional and functional candidate genes (*CALM2*, *CYP1B1*, *QPCT*, and *POMC*) in a sample of 1,243 cases and matched controls. Thirteen HapMap tag SNPs were selected and genotyped by using the high-throughput Sequenom genotyping platform. Binary logistic regression analyses were performed to test for associations between each SNP genotype and BMD. Haplotype association analyses were performed by WHAP. The rs3770748 within the *QPCT* gene showed a significant association with spine BMD in both single-marker ($P = 0.002$) and haplotype association analyses ($P = 0.0482$ for the global test; $P = 0.00092$ for the haplotype-specific test). Subgroup analysis revealed that the effect was primarily driven by an association in the postmenopausal women, presumably suggesting that the rs3770748 affects postmenopausal bone loss rather than peak bone mass. Our results suggest that *QPCT* may be the QTL gene at chromosome 2p for spine BMD variation in the Chinese population.

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Introduction

Osteoporosis is characterized by low bone mineral density (BMD) and the development of fractures, particularly of the spine, hip, and wrist. Although the genetic basis of osteoporosis/BMD has been well documented, the genetic causes of osteoporosis remain poorly defined. Genetic linkage analysis and candidate gene association studies have been extensively employed to hunt for osteoporosis susceptibility genes. To date, more than 20 genome-wide linkage scans across multiple populations have been launched (Huang and Kung 2006). Several significant or suggestive chromosomal regions of linkage to BMD have been identified and replicated in genome-wide linkage screens. However, genes that are responsible for these linkage signals remain to be identified.

Several lines of evidence strongly suggest that chromosome 2p is implicated in the regulation of BMD. Devoto et al. (1998) reported a multi-point logarithm of the odds (LOD) score of 2.25 on 2p23 for spinal BMD in a gemome wide screen of 74 independent sibpairs from 7 large pedigrees. Niu et al. (1999) found linkage evidence of 2p21-23 with forearm BMD with LOD scores of 2.15 in a Chinese population. Kammerer et al. (2003) found significant evidence for quantitative trait loci (QTL) influencing the femoral neck BMD in men on chromosome 2p near D2S1780 (maximum LOD = 3.98, genomic $P = 0.013$) in a study of 29 two- and three-generation Mexican–American families using a genome-wide scan. Peacock et al. (2005) identified a QTL for spine BMD with a LOD score of 3.7 on chromosome 2p in 323 pairs of brothers (264 pairs of white

and 59 pairs of black). Potential functional candidate genes residing on 2p21-23 include the pituitary glutaminyl cyclotransferase (*QPCT*), cytochrome P450, subfamily 1B, polypeptide 1 (*CYP1B1*), pro-opiomelanocortin (*POMC*), and calmodulin 2 (*CALM2*). To identify QTL gene(s) underlying BMD variation at chromosome 2p, we performed a gene-wide and tag single nucleotide polymorphism (SNP)-based association study of the four positional and functional candidate genes in a sample of 1,243 cases and matched controls. Association with SNPs within the *QPCT* gene was observed.

Materials and methods

Subjects

The study subjects came from an expanding database being created at the Osteoporosis Center at Queen Mary Hospital, the University of Hong Kong, Hong Kong, to determine the genetic and environmental risk factors for osteoporosis. All of the study subjects were community-dwelling subjects of southern Chinese descent. These subjects were recruited when they passed by road shows and health talks on osteoporosis held in various districts of Hong Kong between 1998 and 2003. Individuals with disease known to affect bone metabolism, premature menopause (age < 40), bilateral oophorectomy, or drug use that could affect bone turnover and BMD were excluded. All subjects underwent a physical examination and were interviewed by a trained research assistant using a structured questionnaire for their ethnicity, social, medical and reproductive histories, dietary and lifestyle factors, and family history of osteoporosis. A total of 5,872 subjects were invited to the Osteoporosis Center at Queen Mary Hospital for BMD assessment. BMDs (g/cm^2) at the L1–4 lumbar spine, femoral neck, trochanter, and total hip were measured by dual-energy X-ray absorptiometry (DXA; Hologic QDR 4500 Plus, Hologic Waltham, MA, USA). The in vivo precision of the machine for the lumbar spine, femoral neck, and total hip regions was 1.2, 1.5, and 1.5%, respectively (Kung et al. 1998). The hip and spine were chosen because they are the most common osteoporotic fracture sites. Weight and height was measured at the same visit when the BMD measurements were taken. To increase the power of the studies, subjects with low and high BMD were identified from these population-based volunteers. A case–control association approach was used, in which the cases were arbitrarily defined as subjects having a low BMD of Z scores ≤ -1.28 (equivalent to the lowest 10% of the population) at either the lumbar spine or femoral neck, while the controls were age- and sex-matched subjects with a high BMD of Z scores $> +1$ at the corresponding sites. We identified 1,243 case–control subjects, with 909 spine case–control subjects and 792 femoral neck case–control subjects. Of those, 286 case subjects and 190 control subjects overlapped in both spine and hip analyses. All participants gave informed consent and the study was approved by the Ethics Committee of the University of Hong Kong and conducted according to the Declaration of Helsinki.

Tag SNP selection and genotyping

Thirteen tag SNPs (tSNP) of the *CALM2*, *CYP1B1*, *QPCT*, and *POMC* genes are identified using HapMap (three in *CALM2*, three in *CYP1B1*, five in *QPCT*, and two in *POMC*) (Table 2). The criterion for tagging was set at $r^2>0.8$. SNPs were genotyped by using the high-throughput Sequenom genotyping platform. Briefly, the genotypes were determined with the Homogenous Mass EXTEND assay (Sequenom, San Diego, CA, USA). After polymerase chain reaction (PCR) amplification, nonincorporated dNTPs were removed by shrimp alkaline phosphatase. A detecting primer immediately upstream from the polymorphic site was added, together with a specific combination of deoxy dTTP and di-deoxy dATP, dCTP, dGTP, and thermosequenase (Amersham, Bioscience, Piscataway, NJ, USA). The extension products were then analyzed by mass spectrometry (Sequenom Mass Array System). DNA from case and control subjects were randomly assigned to the 96 well plates, and genotyping was performed blind to the status of the samples. Genotyping was repeated in 5% of the samples for verification and quality control. In the quality control exercises, the genotype data were confirmed to have an error rate of less than 0.1%.

Statistical analyses

The genotyping quality of each SNP was first checked for the call rate, minor allele frequency, and Hardy–Weinberg equilibrium. SNPs which did not pass the quality control checks were excluded from further consideration. Binary logistic regression analyses were performed to test for associations between each SNP genotype and BMD, which is implemented in SPSS version 13.0 for Windows (SPSS Inc., Chicago, IL, USA). Haplotype associations were tested by WHAP (http://pngu.mgh.harvard.edu/~purcell/whap/) (Purcell et al. 2007). Haplotype-specific and global tests were performed to assess the association of haplotypes with BMD. Haplotype global tests assess the effects of all haplotypes (if there are H haplotypes, a single H-1 degrees of freedom test). Haplotype-specific tests evaluate each specific haplotype versus all other haplotypes (i.e., H 1 degrees of freedom tests).

Results

The basic characteristics of the study subjects are summarized in Table 1. For the 13 HapMap tSNPs selected, genotyping call rate >90%, minor allele frequency >1%, duplicate error rate <2%, and Hardy–Weinberg equilibrium *P* values > 1% were achieved for nine tSNPs. Of these, six tSNPs were mapped within introns and three were in exons. Four tSNPs (rs6713532, rs1693869, rs2302651, and rs2551188) did not pass quality control checks. Of those, three had significant discrepancy with the Hardy–Weinberg equilibrium and one tSNP had genotyping call rates <90%. Those four tSNPs were not included in further analyses. Details for all tSNPs (including genomic and genic position, minor allele frequency, Hardy–Weinberg equilibrium, and call rate) are shown in Table 2.

Binary logistic regression analyses were performed to test for associations between each SNP genotype and BMD. The results of the single-marker association tests are shown in Table 3. For femoral neck BMD, no significant association was found in our population. For spine BMD, the rs3770748 genotypes showed a significant association with spine BMD ($P = 0.002$). The result remained significant, even after a Bonferroni correction for 18 tests (nine SNPs and two BMD measurements). The genotype frequencies for TT, TC, and CC are 30.7, 43.6, and 25.7% in control samples, and 26.6, 54.9, and 18.5% in case samples. Notably, frequencies of the T/C heterozygotes were significantly higher among case than control subjects ($P = 0.001$; OR = 1.26; 95% CI = 1.1–1.44). None of the other polymorphisms were associated with spine BMD (Table 4). We further studied the relationship between rs3770748 genotypes and spine BMD in relation to menopausal status. The rs3770748 genotypes were significantly associated with spine BMD in postmenopausal women ($P = 0.023$). The genotype frequencies for TT, TC, and CC are 35.2, 42.9, and 21.9% in control samples, and 26.6, 55.5, and 17.9% in case samples. Frequencies of the T/C heterozygotes were significantly higher among case than control subjects ($P = 0.006$; OR = 1.3; 95% CI = 1.07–1.57). No association was detected between rs3770748 genotypes and spine BMD in premenopausal women (Table 4).

We further conducted haplotype association analysis across the *QPCT* gene using WHAP. We identified seven haplotypes, each with the minor allele frequency greater than 3%. Combined, these seven haplotypes accounted for 94.7% of the chromosomes in this population. We then examined the association of these common haplotypes with spine BMD. The global test of all common haplotype effects revealed a marginally significant association between the *QPCT* gene and spine BMD ($P = 0.0482$). Haplotype-specific tests revealed a strong association between the haplotype GCCT and spine BMD ($P = 0.00092$) (Table 5).

Discussion

Previous studies in different populations have reproducibly shown that chromosome 2p has a QTL that contributes to BMD variation (Devoto et al. 1998; Niu et al. 1999;

Table 1 Basic characteristics of the 1,243 case–control subjects

	Spine		Femoral neck	
	Case	Control	Case	Control
Subject number	476	433	427	365
Postmenopausal women	270	214	235	166
Premenopausal women	137	153	160	162
Men	69	66	32	37
Age (years)	53.3 ± 14.4	53.7 ± 14.6	51.4 ± 14.9	51.0 ± 14.0
Height (m)	1.54 ± 0.08	1.58 ± 0.07	1.53 ± 0.08	1.58 ± 0.07
Weight (kg)	49.7 ± 8.2	63.2 ± 10.7	48.6 ± 7.7	64.1 ± 10.9
BMD (g/cm^2)				
Spine	0.67 ± 0.10	1.13 ± 0.11	0.72 ± 0.14	1.07 ± 0.13
Femoral neck	0.56 ± 0.09	0.80 ± 0.13	0.52 ± 0.08	0.89 ± 0.10
Trochanter	0.47 ± 0.08	0.71 ± 0.11	0.46 ± 0.08	0.75 ± 0.10
Total hip	0.64 ± 0.10	0.92 ± 0.13	0.61 ± 0.10	0.97 ± 0.11
Z-score				
Spine	−1.99 ± 0.48	1.73 ± 0.63	−1.62 ± 0.79	1.15 ± 0.94
Femoral neck	−1.42 ± 0.70	0.87 ± 0.91	−1.80 ± 0.41	1.60 ± 0.52
Trochanter	−1.44 ± 0.72	1.03 ± 0.90	−1.63 ± 0.64	1.38 ± 0.83
Total hip	−1.61 ± 0.78	1.01 ± 0.92	−1.89 ± 0.63	1.49 ± 0.78

Data are expressed as mean ± SD

Table 2 Tag single nucleotide polymorphism (SNPs) genotyped in case–control association analyses

Gene	tSNP ID	Genomic position (bp)[a]	Genic position	Alleles (major/minor)	MAF	HDW (*P*)	Call rate
POMC	rs6713532	25359368	Intron 3	C/T	0.43	**0.007**	0.83
	rs934778	25363759	Intron 1	T/C	0.11	1.00	0.93
CALM2	rs1027478	47364560	Intron 3	A/G	0.29	0.14	0.90
	rs815815	47373598	Intron 2	A/G	0.11	0.83	0.97
	rs1693869	47376598	Intron 2	G/C	0.05	**0.007**	0.88
QPCT	rs2255991	37554506	Exon 3	G/A	0.33	0.87	0.97
	rs2302651	37561216	Intron 3	T/C	0.37	0.076	**0.78**
	rs2373000	37567163	Intron 4	T/C	0.34	0.65	0.96
	rs3770748	37570060	Intron 5	T/C	0.47	0.71	0.96
	rs2373001	37572956	Intron 6	T/C	0.45	0.83	0.97
CYP1B1	rs1056836	38272738	Exon 4	C/G	0.10	0.053	0.97
	rs1056827	38276712	Exon 3	G/T	0.19	0.018	0.98
	rs2551188	38277329	Intron 1	C/T	0.17	**0.004**	0.96

MAF minor allele frequency, *HDW* Hardy–Weinberg equilibrium

[a] Positions are based on the NCBI web site

Table 3 Results of the single-marker association tests

Gene	tSNP ID	Spine BMD	Femoral neck BMD
POMC	rs934778	0.099	0.443
CALM2	rs1027478	0.971	0.482
	rs815815	0.678	0.867
QPCT	rs2255991	0.815	0.807
	rs2373000	0.674	0.884
	rs3770748	**0.002**	0.178
	rs2373001	0.683	0.918
CYP1B1	rs1056836	0.054	0.614
	rs1056827	0.281	0.867

Kammerer et al. 2003; Peacock et al. 2005). One approach to follow up the linkage data is to investigate the association between polymorphisms in the candidate genes within the regions of linkage and BMD. To identify the QTL gene underlying BMD variation at chromosome 2p, we have targeted four positional and functional candidate genes (*CALM2*, *CYP1B1*, *QPCT*, and *POMC*), and performed a gene-wide and tSNP-based association study in a sample of 1,243 cases and matched controls. Associations with rs3770748 genotypes within the *QPCT* gene for spine BMD were observed in the single-marker analyses ($P = 0.002$) and haplotype association analyses ($P = 0.0482$ for the global test; $P = 0.00092$ for the haplotype-specific test). Subgroup analysis revealed that the effect was primarily driven by an association in the postmenopausal women, presumably suggesting that the rs3770748 affects postmenopausal bone loss rather than peak bone mass. However, because the genotype frequency of rs3770748 in the premenopausal control subjects is very different from that of postmenopausal controls (Table 4), it is likely that there may be a sampling bias in the premenopausal women, resulting in the failure of detecting significant association. A larger sample size will be needed to confirm the findings in premenopausal women.

Table 4 Case–control genotypic association analysis of rs3770748 and spine bone mineral density (BMD)

Sample	Genotypic frequency (%)			*P*	Odds ratio (OR)
	TT	TC	CC		
All subjects					
Case ($n = 455$)	26.6	54.9	18.5	**0.002**	1.26
Control ($n = 417$)	30.7	43.6	25.7		
Postmenopausal women					
Case ($n = 263$)	26.6	55.5	17.9	**0.023**	1.3
Control ($n = 210$)	35.2	42.9	21.9		
Premenopausal women					
Case ($n = 129$)	25.6	52.7	21.7	0.205	
Control ($n = 143$)	25.2	44.1	30.8		

Table 5 Association analysis of *QPCT* gene haplotypes with spine BMD

Haplotype	Frequency	*P*
Global test		0.0482
Specific test		
ATTC	0.276	0.604
GTCT	0.273	0.729
GCCT	0.183	**0.0009**
GCTC	0.091	0.179
GCTT	0.046	0.221
GTTC	0.044	0.398
ATCT	0.034	0.253

Estrogen is essential for skeletal maturation, as well as for the maintenance of bone mass in adulthood, and a lack of estrogen is the main cause of postmenopausal osteoporosis (Riggs and Melton 1986; Armamento-Villareal et al. 1992; Riggs et al. 2002). Hence, factors involved in estrogen homeostasis may affect bone status. The hypothalamic/pituitary gonadal axis controls the serum levels of sex hormones (Herbison and Pape 2001; Kang et al. 2001; Burns and Matzuk 2002). Gonadotropin-releasing hormone is a primary regulator of the hypothalamic/pituitary gonadal axis. The *QPCT* gene encodes pituitary glutaminyl cyclotransferase that can convert active forms of gonadotropin-releasing hormone peptides to the protected forms by converting the N-terminal glutamine of glutamine peptides into the pyroglutamate, forming a cyclic amide structure by dehydration (Busby et al. 1987; Fisher and Spiess 1987; Schilling et al. 2002). The protective function of the *QPCT* gene for hypothalamic/pituitary peptide hormones makes it a potential candidate gene. Ezura et al. (2004) previously reported that the *QPCT* gene affects BMD among postmenopausal women in the Japanese population. In this study, we independently replicated the association between the variant rs3770748 and BMD in the Chinese population. Our results indicated that the *QPCT* gene is one of the osteoporosis susceptibility genes.

The rs3770748 is located in intron 5 of the *QPCT* gene. The association of the rs3770748 heterozygotes with lower BMD is puzzling. The most conventional explanation is that the associations with the rs3770748 heterozygotes might simply reflect linkage disequilibrium with a functional marker, elsewhere in the gene, that affects BMD. A systematic evaluation of other *QPCT* SNPs throughout coding and noncoding regions of the gene would be required to investigate this. Another possibility is that this is a true example of heterozygote disadvantage (underdominance). Functional analysis of allelic interactions of the *QPCT* would be required to investigate this. Underdominance and overdominance are considered to be rare phenomena, but have been observed in model organisms (Peters et al. 2003; Kim et al. 2004; Swanson-Wagner et al. 2006).

The *CYP1B1*, *POMC*, and *CALM2* genes are also located on chromosome 2p. The CYP450 enzymes metabolize estrogen. The C allele of the *CYP1B1* G1294C polymorphism is associated with increased estrogen catabolism and lower BMD (Napoli et al. 2004). *POMC*, a precursor for adrenocorticotropic hormone, acts on the cortex of the adrenal glands, leading to the production of glucocorticoid. Glucocorticoids diminish calcium absorption and increase renal calcium excretion. A significant association of three variations in the promoter region of the *POMC* gene (-2353G/A, -2345G/A, and -2313A/C) with radial BMD was recently observed among adult Japanese women (Sudo et al. 2005). Calmodulin is involved in the differential control of osteoblast proliferation and differentiation (Siddhanti and Quarles 1994). In this study, we also investigated common polymorphisms of these genes, and no significant associations have been found between these gene variants and BMDs at all of the sites examined.

The genome-wide haplotype maps developed by the International HapMap Project provide important resources for the selection of tSNPs in association studies (International HapMap Consortium 2005). Based on detailed information about validated SNPs and haplotype structure, we have chosen 13 tSNPs within four positional and functional candidate genes to use in our association study. Minor allele frequencies of these 13 tSNPs are comparable to those of 45 unrelated Han Chinese people from Beijing. Notably, T allele of the rs3770748 within the *QPCT* gene has a frequency of 0.522 in Han Chinese in Beijing, 0.398 in Japanese in Tokyo, and 0.9 in Caucasian CEPH samples (Utah residents with ancestry from northern and western Europe). Frequencies of the T/C heterozygotes were 0.6 in Han Chinese in Beijing, 0.614 in Japanese in Tokyo, and 0.167 in Caucasians. Ethnic differentiation of the genetic determination of osteoporosis has previously been observed. For example, the frequencies of the high-fracture-risk allele 's' at the Sp1 site of the collagen IA1 gene were elevated in Caucasians, but absent in East Asian populations (Lambrinoudaki and Kung 2001). Hence, whether the variant rs3770748 within the *QPCT* gene is associated with spine BMD in Caucasians remains to be documented.

In this study, we did not attempt to systematically genotype the chromosome 2p region; rather, we prioritized four obvious positional and functional candidate genes to test the associations of their gene polymorphisms with BMD variation. Faced with the absence of complete functional information for the majority of genes in this susceptibility locus and limited knowledge of the link between gene function and osteoporosis, we cannot

exclude other genes in this locus that might contribute to the genetic risk of osteoporosis. This would require high-density SNP genotyping and comprehensive/unbiased analysis of all candidate genes in this genomic region. However, our results pinpoint *QPCT* as a QTL gene at chromosome 2p for spine BMD variation in an Asian population.

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