Purinergic P2X7 receptor functional genetic polymorphisms are associated with the susceptibility to osteoporosis in Chinese postmenopausal women

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Abstract Osteoporosis (OP) is a major public health problem worldwide. Genetic factors are considered to be major contributors to the pathogenesis of OP. The purinergic P2X7 receptor (P2X7R) has been shown to play a role in the regulation of osteoblast and osteoclast activity and has been considered as an important candidate gene for OP. A case–control study was performed to investigate the associations of functional single nucleotide polymorphisms (SNPs) in the P2X7R gene (rs2393799, rs7958311, rs1718119, rs2230911, and rs3751143) with susceptibility to OP in 400 Chinese OP patients and 400 controls. Results showed that rs3751143 was associated with OP; in particular, carriers of the C allele and CC/(AC + CC) genotypes were at a higher risk of OP, but no significant association of

rs2230911, rs7958311, rs1718119, and rs2393799 with OP risk was observed. Analysis of the haplotypes revealed one haplotype (rs1718119G-rs2230911G-rs3751143C) that appeared to be a significant “risk” haplotype with OP. The rs3751143 polymorphism was associated with osteoclast apoptosis; ATP-induced caspase-1 activity of osteoclasts with AC and CC genotypes is lower than that of osteoclasts with AA genotype in vitro. The findings suggest that the P2X7R rs3751143 functional polymorphism might contribute to OP susceptibility in Chinese postmenopausal women.

Keywords P2X7 receptor · Osteoporosis · Genetic polymorphisms · Postmenopausal women

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Introduction

As one of the most common metabolic bone diseases, osteoporosis (OP) is characterized by decreased bone mineral density (BMD), increased bone fragility, and bone tissue micro-architectural deterioration [1]. The true magnitude of the health problems associated with OP is osteoporotic fracture (OF) [2]. Annually, more than nine million osteoporotic fractures occur worldwide [3], and it is estimated that half of the world's fractures will occur in Asia by 2050 [4]. The incidence of hip fractures has risen two to threefold in China over the past 15 years [5] and produces a substantial economic burden on individuals and healthcare systems [2]. However, the pathogenesis of OP is still unclear.

Decades of extensive studies have demonstrated that the majority of the variation in BMD is determined by genetic factors [6]. In an attempt to identify the genes involved in the pathogenesis of OP, a number of candidate genes, such

as the vitamin D receptor (VDR) [7], estrogen receptor (ER) [8, 9], and collagen type Ia1 (COL1A1) [10, 11] genes, have been examined in association and/or linkage studies; however, they were found to be variably associated with BMD and the risk of OP. Together, these genetic variants only account for a small part of the total genetic variance in osteoporosis; other candidate genes require examination of their contribution to explain the genetic basis of OP.

The purinergic P2X7 receptor (P2X7R) is a member of the P2X subfamily of purinergic receptors activated by extracellular ATP. It is 595 amino acids long, with two transmembrane domains, an extracellular residue, and intracellular N and C termini. In contrast to other P2X receptors, the P2X7R C-terminal intracellular chain is about 240 amino acids longer and is essential for its function [12]. This carboxyl tail enables the P2X7R to generate a membrane pore and allows passage of larger molecules, resulting in apoptosis after extended ATP stimulation [12]. This unique property that long-term activation by ATP induces apoptosis in lymphocytes and osteoclasts while brief stimulation allows passage of cations makes P2X7R differ from other members of the P2XR family [12, 13]. Several evidence have pointed out P2X7R may be involved in regulating bone mass, although it has not been reported to be associated with bone mass or fracture in genome-wide association study (GWAS) studies so far. Expression of functional P2X7R has been reported in both osteoclasts and osteoblasts [14, 15]. The physiological role of P2X7R has been demonstrated to be involved in mechanically induced signaling between osteoblasts and osteoclasts [16]. Activation of P2X7R has been shown to induce osteoclast survival, apoptosis, and death [17–19]. In a P2X7R gene “knock-out” mouse model, loss of P2X7R function leads to increased osteoclast numbers and osteoclast surface, decreased trabecular bone mass, and responses to mechanical stimulation of the bones [20], supporting a role for the P2X7R in regulating bone mass, quality, and skeletal resistance to mechanical stress [16].

Recently, P2X7R gene has gained much attention in the field of research of OP. It is located in 12q24 and contains 13 exons spanning a 53-kb region [21]. Several single nucleotide polymorphisms (SNPs) in the P2X7R gene have been described to affect the function of this receptor, including loss of function or gain of function [21–24]. For example, three P2X7R SNPs with amino acid changes, rs3751143 (1513A > C, Glu496Ala), rs2230911 (1096 C > G, Thr357Ser), and rs7958311 (835G > A, Arg270His), were shown to lead to a loss of receptor function in both channel and pore function [22, 23]. Rs3751143 and rs2230911 were reported to be associated with a higher prevalence of OP [25–27], while rs7958311 was shown to be related with other diseases, including pulmonary tuberculosis, chronic pain, and anxiety [21, 28, 29]. On the other hand, the polymorphism of rs1718119 (1068G > A, Ala348Thr) causes a gain of function of the receptor via regulating channel and pore function [30]. It has been linked to less bone resorption and is probably protective against the development of OP in Danish women [30].

In addition, rs2393799, a polymorphism located in the promoter region of P2X7R gene (−762 C > T), was indicated to be related with pulmonary tuberculosis susceptibility in several previous studies [31].

Prompted by the importance of P2X7R gene in OP and no data about the association of P2X7R polymorphism with OP listed in Chinese, we conducted a case–control study in postmenopausal women of Han Chinese to determine whether P2X7R gene is associated with OP. This study provides further information regarding the use of P2X7R polymorphisms as markers of susceptibility to osteoporosis in postmenopausal women.

Material and methods

Subjects

Subjects recruited from four differently distributed communities in Nanchang, Jiangxi Province, China, were randomly invited to the First Affiliated Hospital of Nanchang University hospital for participation in this study between January 2014 and October 2016. They were all of the Han ethnicity which accounts for more than 93% of the total Chinese population. Only women who had been naturally postmenopausal for more than 1 year were eligible for the study. Then, they all gave written informed consent in official documents before inclusion. Subjects recruited from four differently distributed communities in Nanchang, Jiangxi Province, China, were randomly invited to the First Affiliated Hospital of Nanchang University hospital for participation in this study between January 2014 and October 2016. They all gave written informed consent in official documents before inclusion. All the subjects were required to fill out detailed questionnaires including demographics, body mass index (BMI), menarche age, menopause age, and medical history. They were also required to undergo physical examination, BMD measurements, and several biochemistry tests including complete blood count, serum calcium, phosphorus, creatinine, uric acid, urea nitrogen, fasting blood glucose, albumin, globulin, total protein levels, albumin/globulin ratio (A/G ratio), aspartate aminotransferase (AST), alanine aminotransferase (ALT), gammaglutamyl transferase (GGT), alkaline phosphatase (ALP), bilirubin, 25-hydroxyvitamin D level, parathyroid hormone (PTH), thyroid stimulating hormone (TSH), urine calcium, sodium, and creatinine. The criteria for exclusion are subjects with a history of bone disease, metabolic or endocrine disorders such as hyperthyroidism, hyperparathyroidism, diabetes mellitus, liver disease, renal disease and vitamin D deficiency, and subjects taking medications known to affect bone metabolism (e.g., corticosteroids, anticonvulsants, and heparin sodium) or such medicines for the treatment of OP as active vitamin D₃, bisphosphonates, selective estrogen receptor modulator (SERM), as well as calcium. Finally, a total of 400 had osteoporotic BMD (T score ≤ −2.5SD) at the lumbar spine and/or total hip and 400 had normal BMD

(T score ≥ -1.0 SD) at the lumbar spine and total hip were involved in the study for analysis. The study was approved by Ethnical Committee of Nanchang University.

BMD measurement

Dual-energy X-Ray absorptiometry (OSTEOCORE-3, MEDILINK, France) was used to measure the BMD. Left hip and posterior–anterior lumbar spine (L1–L4) scans were performed with the patient lying supine on the imaging table using the protocols recommended by the manufacturer. The BMD T score was calculated using the difference between the BMD value (in g/cm²) of an individual and the average BMD (expressed in standard deviation [SD] units) of a young adult in a reference population [32]. To calculate the T scores for our subjects, we used reference data for Chinese women provided by the manufacturer (MEDILINK, France). Corresponding T scores at the lumbar spine and total hip were calculated using the equipment's standard software (MEDILINK, France). OP was defined according to the conventional World Health Organization (WHO) definition [32].

P2X7R SNPs selection

The P2X7R SNPs with a minor allele frequency ≥ 0.05 were selected using the genotype data obtained from the HapMap database for Han Chinese in Beijing. The pairwise option of HaploView 4.2 (Broad Institute, Cambridge, MA) software was used to identify the tag-SNPs, and an r^2 of 0.8 was selected as the threshold for the analysis. SNPs located in promoter and coding regions, including 3' untranslated regions (UTRs), 5' UTRs, and non-synonymous substitutions were selected as possible SNPs in P2X7R gene. In addition, the existing literature about the P2X7R polymorphism was reviewed. Based on these, we selected five tag-SNPs (rs2393799, rs7958311, rs1718119, rs2230911, and rs3751143) in this present study. The positions of the 5 tag-SNPs were on 5' UTR, exon8, exon11, exon11, and exon13 regions respectively (Fig. 1).

Genotyping

Genomic DNA was isolated using the phenol–chloroform extraction method from whole blood. The rs1718119 (G > A), rs2393799 (C > T), and rs7958311 (G > A) polymorphisms were performed by allele-specific

polymerase chain reaction (PCR) method. PCR was performed in 20 μ l reaction volume containing 1 μ l of 100 ng template DNA, 25 pmol each primer 1 μ l, 10 μ l of 2 $\times$ Taq Master Mix, and 5 μ l of nuclease-free ddH₂O. Genotyping of P2X7R rs3751143 (A > C) and rs2230911 (C > G) was determined by PCR followed by restriction fragment length polymorphism procedures (PCR-RFLP). PCR was performed in 20 μ l reaction volume containing 1 μ l of 100 ng template DNA, 25 pmol each primer 1 μ l, 10 μ l of 2 $\times$ Taq Master Mix, and 7 μ l of nuclease-free ddH₂O. The PCR products were visualized by agarose gel electrophoresis with ethidium bromide staining and UV light. The primer sequences, PCR amplification condition, method, and fragment size were listed in Table 2.

In vitro osteoclast caspase-1 assay

Participants for this substudy were recruited from the main study on the basis of their rs3751143 genotype. Further, the eligible subjects were confirmed to have no rs1718119 and rs2230911 SNPs in the P2X7R gene. In total, 11 age-matched OP women were included in this substudy and were grouped into the AA (6 subjects) and AC (3 subjects)/CC (2 subjects) genotype groups. Peripheral blood was collected from P2X7R-genotyped volunteers. The mononuclear fraction of the cells was isolated by centrifugation using a gradient centrifugation. The cells were plated to 24-well plates with 1×10^6 cells/well and was added with osteoclast differentiation medium containing minimum essential medium with 10% fetal calf serum and penicillin/streptomycin supplemented with M-CSF (1 ml of 25 ng/ml M-CSF/ml medium) and RANKL (3 ml of 30 ng/ml RANKL/ml medium). Medium was changed every 5 days. After about 11 days (10–12), large multinucleated osteoclasts appeared and cells were then ready for further experiments [17]. Osteoclast differentiation medium was removed and cells washed once in minimum essential medium supplemented with 0.1% BSA (experimental medium). Caspase-1 activity in osteoclasts from P2X7R-genotyped volunteers was measured at baseline, and 4 h thereafter cells stimulated with 2 mM ATP by using human caspase-1 immunoassay (R&D Systems, Inc., Minneapolis, Minnesota, USA) [17]. Determinations were performed as duplicates.

Fig. 1 The structure of the P2X7R gene and the locations of the studied polymorphisms in the two genes. Exons are depicted as *filled boxes* and introns as *single lines* between *filled boxes*

Statistical analyses

The statistical analyses were conducted through SPSS Version 19.0 (SPSS Inc., Chicago, IL, USA). Comparisons of general characteristics between the two groups were performed using *t* tests. The Hardy-Weinberg equilibrium (HWE) for each SNP in both OP patients and controls was examined by the exact test. The phenotypic values were evaluated for the presence of a normal distribution by the Shapiro-Wilk test. Odds ratios (ORs) and 95% confidence interval (CIs) for individual SNP were calculated by non-conditional logistic regression analyses. Haplotype frequencies were estimated by using the software Haploview 4.2. Haplotype analysis was performed using Plink (<http://pngu.mgh.harvard.edu/~purcell/plink/>) [33], excluding rare haplotypes with frequencies <0.005. The Bonferroni correction was used to adjust for multiple testing in the analysis of individual SNP analyses and haplotype analyses.

Results

The characteristics of the case and the control groups

A total of 800 subjects (400 OP and 400 healthy controls) were included. The characteristics of the cases and controls are demonstrated in Table 1. As shown in Table 1, age, height, weight, BMI, waist circumference, hip circumference, age of menarche, and age of menopause were not significantly different between the control and case groups, implying that the associations between these clinical characteristics and OP

Table 1 The characteristics of the case and the control groups

	Control (<i>n</i> = 400)	OP (<i>n</i> = 400)	<i>P</i> value
Age (years)	60.36 ± 8.40	60.50 ± 7.20	0.50
Height (cm)	157.30 ± 5.10	155.40 ± 5.10	0.53
Weight (kg)	54.10 ± 9.90	53.2 ± 9.10	0.52
BMI (kg/m ²)	24.62 ± 8.40	21.90 ± 3.40	0.51
Waist circumference (in.)	24.30 ± 2.30	23.80 ± 2.80	0.65
Hip circumference (in.)	32.30 ± 3.70	28.90 ± 3.30	0.06
Age of menarche (years)	15.06 ± 3.40	15.08 ± 3.30	0.83
Age of menopause (years)	49.80 ± 3.60	48.60 ± 3.80	0.20
Lumbar spine BMD (g/cm ²)	0.951 ± 0.120	0.669 ± 0.042	0.03
Lumbar spine T score	−0.9 ± 1.0	−3.6 ± 1.2	0.005
Total hip BMD (g/cm ²)	0.933 ± 0.082	0.623 ± 0.044	0.02
Total hip T score	−0.2 ± 1.0	−2.7 ± 0.9	0.003

Values are means ± SD. *n* is the number of the study subjects in each group. *P* values for characteristics were the *t* test results between control and OP groups. *P* values ≤0.05 were defined as statistical significance. *BMI* body mass index, *BMD* bone mineral density

were not significant (all *P* > 0.05). However, BMD and T scores at the lumbar spine and total hip between the cases and controls were significantly different (*P* < 0.05) (Table 2).

Association between P2X7R polymorphisms and risk of OP

The success rate for calling genotypes on gels was 100%. In order to test the accuracy of genotype determination by PCR-RFLP and allele-specific PCR methods, 10% random samples of each genotype were verified by DNA sequencing (ABI3730XL DNA Analyzer, Applied Biosystems, Foster City, CA). No deviations from HWE were observed in both OP patients and controls in each polymorphism (*P* > 0.05). The allele frequencies and genotype frequencies of five SNPs of the P2X7R gene in OP patients and controls are shown in Table 3. Case-control comparison revealed a higher frequency of C allele at rs3751143 (*P* = 0.005, OR = 1.45, 95% CI 1.17–1.78) in OP. The genotype frequencies were significantly different between OP patients and controls; CC/(AC + CC) genotypes were at a higher risk of the disease (CC versus AA, *P* = 0.003, OR = 1.72, 95% CI 1.00–2.87; AC + CC versus AA, *P* = 0.002, OR = 1.41, 95% CI 1.13–1.67). However, no significant association of rs2230911, rs7958311, rs1718119, and rs2393799 with OP risk was observed (all *P* > 0.05).

Haplotype analyses

We analyzed the haplotypes of P2X7R determined by the three SNPs (rs1718119-rs2230911-rs3751143) and obtained five main haplotypes (ACA, GCA, GGA, GCC, and GGC) by using SHEsis (Table 4). Analysis of the haplotypes revealed one haplotype (GGC) was significantly associated with OP. The GGC haplotype appeared to be a significant “risk” haplotype (*P* = 0.002, OR = 3.76, 95% CI 1.21–11.68).

P2X7R SNP and ATP-mediated caspase-1 in osteoclast

Caspase-1 was used as quantitative measures of ATP-induced osteoclast apoptosis (Fig. 2). ATP-induced caspase-1 activity of osteoclasts with AC and CC genotypes is lower than that of osteoclasts with AA genotype (*P* < 0.05), while no difference was found for the basal caspase-1 activity (data not shown). Thus, ATP-induced osteoclast apoptosis is impaired in carriers with C allele.

Discussion

The incidence of OP and related fractures is increasing worldwide. It is urgent to identify the biomarkers able to identify patients at a high risk of OP. The genetic polymorphisms of P2X7R gene could affect purine signaling in the bone, which plays an

Table 2 PCR conditions used in genotyping P2X7R polymorphisms

SNPs	Primer sequence	PCR conditions	Genotyping method	Fragment size (bp)
rs1718119 (G > A)	A forward 5'-AGCGCTTGCTGCA TTCTCCCCAGGACA-3' A reverse 5'-ATGTCTTGGTGTCC TGTGAACACAGCGGC-3' G forward 5'-GCATGAGGCTCCGC TCCCTGATAGAACC-3' G reverse 5'-TGTCGATGAGGAAG TCGATGAACACCGC-3'	94 °C for 5 min; 36 cycles of denaturation at 95 °C for 45 s, annealing at 60.5 °C for 45 s, extension at 72 °C for 45 s; 72 °C for 10 min	Allele-specific PCR	208 bp for A; 282 bp for G
rs2393799 (C > T)	C forward 5'-GAAACAGGGCCCTG GGTCCTC-3' C reverse 5'-GGCAGTCCAACAAA GTTAGGTTTG-3' T forward 5'-GGTGTCCCTCACTG AATAGGTCAAT-3' T reverse 5'-TGGTGGGGGTGGAG GGGC-3'	94 °C for 5 min; 36 cycles of denaturation at 94 °C for 45 s, annealing at 58 °C for 45 s, extension at 72 °C for 45 s; 72 °C for 10 min	Allele-specific PCR	235 bp for C; 186 bp for T
rs7958311 (G > A)	A forward 5'-CGAGTTAGGTGGGG CTGTACATATGGTT -3' A reverse 5'-TCAAGGCGACGGAA ACTGTATTTGGTAT -3' G forward 5'-CTAGACCGTTGGTT CCATCACTGACG -3' G reverse 5'-TTTTTCATTCTCT TGTTGCCTTGAAA -3'	94 °C for 5 min; 36 cycles of denaturation at 94 °C for 45 s, annealing at 60 °C for 45 s, extension at 72 °C for 45 s; 72 °C for 10 min	Allele-specific PCR	239 bp for A; 201 bp for G
rs3751143 (A > C)	Forward 5'- AGACCTGCGATGGA CTTCACAG -3' Reverse 5'-AGCGCCAGCAAGGG CT-3'	94 °C for 5 min; 36 cycles of denaturation at 94 °C for 45 s, annealing at 59 °C for 45 s, extension at 72 °C for 45 s; 72 °C for 10 min	PCR-RFLP (<i>Hae</i> II)	199 and 117 bp for C; 316 bp for A
rs2230911 (C > G)	Forward 5'-ATGGGAGCGACAGC AGTTACTGGGTAA-3' Reverse 5'-TCTGAATTTACCT GAGTAACTCTCCC-3'	94 °C for 5 min; 36 cycles of denaturation at 95 °C for 45 s, annealing at 60.5 °C for 45 s, extension at 72 °C for 45 s; 72 °C for 10 min	PCR-RFLP (<i>Hap</i> I)	224 and 25 bp for G; 249 bp for C

SNPs single nucleotide polymorphisms, PCR polymerase chain reaction, PCR-RFLP PCR restriction fragment length polymorphism

important role in development of OP. The data of the present study showed an association of the P2X7R rs3751143 polymorphism with increasing OP susceptibility. However, we did not find the evidence of the association of other polymorphisms, including rs2230911, rs7958311, rs1718119, and rs2393799, with OP risk in a population of Chinese postmenopausal women. Moreover, the haplotype determined by the three SNPs (haplotype rs1718119G-rs2230911G-rs3751143C) was found to be associated with the raised OP risk. This study provides new evidence concerning associations between three functional genetic variations in the P2X7R gene and OP risk.

Rs3751143 (A > C) substitutes an alanine for a glutamic acid at amino acid position 496 (Glu496Ala) in the intracellular tail [22]. This SNP was reported to be associated with the impaired ATP-mediated pore formation. It was shown that the rs3751143-AC heterozygotes have decreased pore formation activity, and rs3751143-CC homozygotes have totally lost this function [22]. Rs3751143 was also indicated to impair ATP-induced opening of the cation-selective channel [24]. Rs3751143-C was proved to be related to low hip BMD

[25] and increased 10-year fracture incidence in Danish population [17]. Consistent with these previous studies, our study demonstrated rs3751143-C was associated with increased OP risk in Chinese postmenopausal women. Secondly, rs2230911 polymorphism, which changes serine to threonine at residue 35 (Ser357 to Thr), also resides in the region coding for the intracellular tail of P2X7R and thus was focused on. Mutation of Thr357Ser was shown to be associated with a partial function loss of P2X7R through affecting channel and pore function [23]. It was reported that rs2230911 was associated with a higher prevalence of OP in Scotland women [27]. However, rs2230911 investigated in the present study was not associated with the risk of OP. Thirdly, rs1718119 leads the substitution of histidine for tyrosine at position 348 localized in P2X7R ectodomain, which forms the channel. Rs1718119 was suggested to confer gain of function in P2X7R channel and pore formation [30]. In fact, this gain-of-function SNP was suggested to be associated with decreased 10-year fracture incidence, high lumbar BMD, and low fracture risk in Caucasian [25, 26, 30]. However, we do not see the protective pattern for

Table 3 Allele and genotype frequencies of five functional SNPs in the P2X7R gene in OP patients and controls

SNPs	OP, <i>n</i> (%)	Control, <i>n</i> (%)	OR (95% CI)	<i>P</i> value
rs1718119				
Allele frequency				
G	743 (92.88)	722 (90.25)	Reference	
A	57 (7.12)	78 (9.75)	0.73 (0.42–0.79)	0.370
Genotype frequency				
GG	345 (86.25)	326 (81.50)	Reference	
GA	53 (13.25)	70 (17.50)	0.79 (0.63–0.89)	0.570
AA	2 (0.50)	4 (1.00)	0.42 (0.32–0.57)	0.169
AG + AA	55 (13.75)	74 (18.50)	0.74 (0.52–0.79)	0.135
Rs2230911				
Allele frequency				
C	664 (83.00)	678 (84.75)	Reference	
G	136 (17.00)	122 (15.25)	1.63 (0.42–1.88)	0.340
Genotype frequency				
CC	278 (69.50)	284 (71.00)	Reference	
CG	108 (27.00)	110 (27.50)	1.44 (0.57–1.92)	0.740
GG	14 (3.50)	6 (1.50)	1.88 (0.73–1.94)	0.190
CG + GG	122 (30.50)	116 (29.00)	1.69 (0.47–1.89)	0.246
Rs3751143				
Allele frequency				
A	618 (77.25)	650 (81.25)	Reference	
C	182 (22.75)	150 (18.75)	1.45 (1.17–1.78)	0.005
Genotype frequency				
AA	249 (62.25)	260 (65.00)	Reference	
AC	120 (30.00)	130 (32.50)	1.32 (1.21–1.53)	0.145
CC	31 (7.75)	10 (2.50)	1.72 (1.00–2.87)	0.003
AC + CC	151 (37.75)	140 (35.00)	1.41 (1.13–1.67)	0.002
Rs2393799				
Allele frequency				
C	498 (62.25)	522 (65.25)	Reference	
T	302 (37.75)	278 (34.75)	0.70 (0.47–1.57)	0.210
Genotype frequency				
CC	165 (41.25)	168 (42.00)	Reference	
CT	168 (42.00)	186 (46.50)	0.89 (0.47–1.70)	0.720
TT	67 (16.75)	46 (11.50)	0.64 (0.24–1.53)	0.080
CT + TT	235 (58.75)	232 (58.00)	0.72 (0.53–1.64)	0.152
Rs7958311				
Allele frequency				
G	403 (50.38)	411 (51.38)	Reference	
A	397 (49.63)	389 (48.63)	1.41 (0.59–1.93)	0.691
Genotype frequency				
GG	104 (26.00)	109 (27.25)	Reference	
AG	195 (48.75)	193 (48.25)	1.31 (0.56–1.85)	0.173
AA	101 (25.25)	98 (24.50)	1.64 (0.82–2.30)	0.921
AG + AA	296 (74.00)	291 (72.75)	1.46 (0.64–2.01)	0.247

n is the number of the study subjects in each group. *P* values were calculated by non-conditional logistic regression model. *P* values ≤ 0.01 for the analyses of allele frequencies and ≤ 0.003 for the analyses of genotype frequencies of five SNPs in the P2X7R gene between OP patients and controls (after Bonferroni correction)

the rs1718119 polymorphism in our study. Fourthly, mutations of rs7958311 could also alter coding of amino acids (His357 to Arg) and was associated with a partial function loss of P2X7R through affecting channel and pore function [22, 23]. Rs7958311 was shown to be related with many disorders, including pulmonary tuberculosis, chronic pain, and anxiety [21, 28, 29]. However, the rs7958311 variant investigated in the present study was not associated with risk of OP. The last of the five identified polymorphisms is rs2393799.

Table 4 Haplotype analysis of P2X7R polymorphisms between OP patients and controls

Haplotype	OP, <i>n</i> (%)	Control, <i>n</i> (%)	<i>P</i> value	OR (95% CI)
ACA	65 (8.13)	77 (9.63)	0.455	0.83 (0.51–1.34)
GCA	429 (53.62)	447 (55.87)	0.508	0.91 (0.69–1.20)
GGA	108 (13.50)	96 (12.00)	0.539	1.13 (0.75–1.70)
GCC	166 (20.75)	166 (20.75)	0.976	1.01 (0.72–1.40)
GGC	25 (3.13)	6 (0.75)	0.002	3.76 (1.21–11.68)

Haplotypes were constructed by the rs1718119, rs2230911, and rs3751143 polymorphisms. Haplotype analysis was performed by using Plink. *n* is the number of the study subjects in each group. All the haplotypes with a frequency ≤ 0.005 were ignored in the analysis. *P* values ≤ 0.01 were defined as statistical significance after Bonferroni correction

This polymorphism is located in the promoter of P2X7R gene, which may alter P2X7R expression or function. Rs2393799 was generally applied to study its correlations with pulmonary tuberculosis susceptibility in several previous studies [31], yet it was reported to be unassociated with pulmonary tuberculosis susceptibility in Asians or whites in a previous meta-analysis [34]. Our study showed that there was no significant association of rs2393799 with OP risk.

The analysis of haplotypes indicated a strong association of GGC haplotype (rs1718119G-rs2230911G-rs3751143C) with susceptibility to OP in Chinese postmenopausal women. The significance of this association resisted Bonferroni correction for multiple comparison error ($P \leq 0.01$). As the analyses of individual SNPs showed rs3751143-C was associated with an increased risk of OP, whereas neither rs1718119-G nor rs2230911-G was associated with OP risk, it can be speculated that a presumed loss of P2X7R receptor function due to the presence of the rs3751143-C polymorphism is a high risk factor for OP if rs3751143-C is expressed together with the variant alleles of the rs1718119G-rs2230911G polymorphisms. Although the frequency of this haplotype was not high in the

Fig. 2 ATP-induced caspase-1 activity in osteoclasts. Human primary osteoclasts were stimulated with 2 mM ATP, and caspase-1 activity was measured after 4 h. Values are means $\pm$ SEM. *n* is the number of the study subjects in each group. #*P* value < 0.05

population, this finding suggests that studying the genetic markers at the haplotype level may resolve new aspects of genetic alterations.

To gain some insights on the mechanisms that might be involved in an increased OP risk in individuals carrying the CC genotype of the rs751143 SNP, we performed an in vitro substudy on ATP-induced caspase-1 activity in osteoclasts. It is demonstrated that ATP-induced caspase-1 activity of osteoclasts with AC and CC genotypes is lower than that of osteoclasts with AA genotype. This result indicated ATP-induced osteoclast apoptosis was significantly impaired in subjects with the C allele. This could potentially result in increased osteoclast numbers and osteoclastic bone resorption and thus a high risk of OP in carriers with C allele.

It has been suggested that a “systems biology approach” is needed to investigate the intricate genome–proteome–metabolome interaction in unraveling OP susceptibility [35]. We have conducted an exploratory case–control association study using a candidate gene approach to investigate whether genetic variants in P2X7R gene are associated with increased risk of OP, which, to our best knowledge, is the first study in Chinese population. Strength of the present study was mainly the case–control design, matched for age and gender. In terms of limitations, it was a hospital-based case–control study, restricted to Chinese Han populations. In fact, the genotype frequencies among controls fitting the HWE law suggest the randomness of subject selection, and we have achieved 90.7% study power (two-sided test, $\alpha = 0.05$) to detect an OR of 1.72 for the rs3751143CC genotype. Furthermore, although primary osteoporosis was evaluated via detailed questionnaires, physical examinations, several biochemistry tests, and BMD measurements to recruit eligible OP subjects, several secondary causes of osteoporosis may be clinically subtle (such as subclinical thyroid disease or even severe vitamin D deficiency) and thus may not be absolutely excluded in this study. In addition, the finding of an association between the rs3751143A > C polymorphism and risk of OP was supported by additional functional assays. Therefore, it appears that our finding that the rs3751143CC genotype and its estimated haplotype are associated with an increased risk of OP is unlikely to be due to chance. Also, we cannot exclude the possibility that there also exists a functional variant in the P2X7R gene or in a neighboring gene which is in linkage disequilibrium with the rs3751143 polymorphism.

In conclusion, we have shown that P2X7R rs3751143 polymorphism is associated with risk of OP in Chinese postmenopausal women. We have found that the P2X7R rs3751143 polymorphism is a functional SNP affecting osteoclast apoptosis in vitro. In order to replicate this finding and to pursue for further investigation, it is likely that large multicentre studies involving thousands of cases will be required, also including other ethnicities and potentially adopting a genome wide association study approach.

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Compliance with ethical standards

Conflicts of interest Hong Xu declares that she has no conflict of interest

Chengxin Gong declares that she has no conflict of interest

Luling He declares that she has no conflict of interest

Shenqiang Rao declares that he has no conflict of interest

Xingzi Liu declares that she has no conflict of interest

Yijun Nie declares that he has no conflict of interest

Changle Liu declares that he has no conflict of interest

Tao Li declares that he has no conflict of interest

Lu Ding declares that she has no conflict of interest

Yunming Tu declares that he has no conflict of interest

Yuping Yang declares that she has no conflict of interest

Fangfang Hu declares that she has no conflict of interest

Yongfang Fan declares that she has no conflict of interest

Hui Wang declares that she has no conflict of interest

Shuo Wang declares that he has no conflict of interest

Chaopeng Xiong declares that he has no conflict of interest

Peipei Zhong declares that she has no conflict of interest

Lan Tang declares that he has no conflict of interest

Shangdong Liang declares that he has no conflict of interest

Ethical approval Subjects recruited from four differently distributed communities in Nanchang, Jiangxi Province, China, were randomly invited to the First Affiliated Hospital of Nanchang University hospital for participation in this study between January 2014 and October 2016. They all gave written informed consent in official documents before inclusion. The study was approved by Ethnical Committee of Nanchang University.

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