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Association of a *RUNX2* Promoter Polymorphism with Bone Mineral Density in Postmenopausal Korean Women

Hee-Jung Lee · Jung-Min Koh · Joo-Yeon Hwang · Kang-Yell Choi · Seung Hun Lee · Eui Kyun Park · Tae-Ho Kim · Bok Ghee Han · Ghi Su Kim · Shin-Yoon Kim · Jong-Young Lee

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Abstract Osteoporosis is characterized by impaired osteoblastogenesis. Bone mineral density (BMD) is a major determinant of bone strength. RUNX2 is an osteoblast-specific transcription factor involved in osteoblast differentiation and ossification. To determine whether *RUNX2* is associated with BMD in an ethnically distinct population, we investigated SNPs within the two *RUNX2* promoters (P1 and P2) using the Illuminar GoldenGate system in 729 postmenopausal Korean women. Subjects bearing the minor homozygote genotype (*CC*) at the *RUNX2* $-1025\ T > C$ SNP (rs7771980) located in P2 showed a significant association with reduced lumbar spine BMD ($p = 0.02$) and BMDs at proximal femur sites (trochanter, $p = 0.05$; total femur, $p = 0.04$) compared with subjects carrying the major homozygote genotype (*TT*) or the heterozygote genotype (*TC*), respectively. These results present an interesting genotype association complementary to the previously reported association of BMD with the *RUNX2* $-1025\ T > C$ P2 SNP in Spanish and Australian cohorts. Therefore, we suggest that the *RUNX2* P2 polymorphism ($-1025\ T > C$) may be a useful genetic marker for bone metabolism and may play an important role in BMD in postmenopausal Korean women.

Keywords Osteoporosis · Bone mineral density · Promoter · RUNX2 · Polymorphism

Osteoporosis is a systemic disease characterized by low bone mineral density (BMD), low bone strength, microarchitectural deterioration of bone tissue, and a consequent increase in fragility. BMD is the primary determinant of fracture risk and accounts for approximately 70% of bone strength. Bone loss results from an imbalance between osteoclastic bone resorption and osteoblastic bone formation, the latter of which is important for osteoblastogenesis and ossification. Although several environmental risk factors influence bone loss, genetic factors are mostly

Hee-Jung Lee, Jung-Min Koh and Joo-Yeon Hwang are equal contributors to this work.

H.-J. Lee · J.-Y. Hwang · B. G. Han · J.-Y. Lee (✉)
Center for Genome Science, National Institute of Health, 5 Nokbun-dong, Eunpyung-gu, Seoul 122-701, Republic of Korea
e-mail: leejy63@nih.go.kr

J.-M. Koh · S. H. Lee · E. K. Park · T.-H. Kim · G. S. Kim · S.-Y. Kim
Skeletal Diseases Genome Research Center, Kyungpook National University Hospital, 44-2, Samduk 2-ga, Jung-gu, Daegu 700-412, Republic of Korea

J.-M. Koh · S. H. Lee · G. S. Kim
Division of Endocrinology and Metabolism, University of Ulsan College of Medicine, Asan Medical Center, Seoul 138-736, Republic of Korea

K.-Y. Choi
National Research Laboratory of Molecular Complex Control and Department of Biotechnology, Yonsei University, Seoul 120-749, Republic of Korea

E. K. Park
Department of Pathology and Regenerative Medicine, School of Dentistry, Kyungpook National University, 188-1 Samduk 2-ga, Jung-gu, Daegu 700-412, Republic of Korea

S.-Y. Kim (✉)
Department of Orthopedic Surgery, Kyungpook National University School of Medicine, 50, Samduk 2-ga, Jung-gu, Daegu 700-412, Republic of Korea
e-mail: syukim@knu.ac.kr

implicated and account for 50%–85% of the variance in BMD based on twin and family studies [1–6].

The gene *RUNX2* is located at 6p21, spanning 124.7 kb, and contains two separate promoters, designated P1 and P2, and seven exons. These two promoters give rise to the expression of two distinct isoforms, RUNX2-I and RUNX2-II. RUNX2-I contains 514 amino acid residues, is driven by promoter P2, and begins with the residues MRIPVP derived from exon 2. RUNX2-II contains 528 residues, is driven by the P1 promoter, and begins with the first 19 residues of exon 1 [7]. Both type I and type II isoforms are found in human osteoblast cells and osteoblast precursors [8, 9].

RUNX2 is an osteoblast-specific transcription factor that normally stimulates bone formation and osteoblast differentiation. *Runx2* knockout mice display complete ossification failure [10, 11]. Interestingly, overexpression of a dominant negative form of RUNX2 in mice results in less active osteoblasts, reduced bone formation, and short stature [12]. In humans, mutations in *RUNX2* can cause cleidocranial dysplasia, patent fontanelles, supernumerary teeth, short stature, and other changes in skeletal patterning and growth [13]. These findings indicate that *RUNX2* is necessary for endochondral bone formation and regulation of skeletogenesis and polymorphic changes within this gene may be markers for common bone disorders. Association studies with BMD have been performed using genome-wide scans to identify causal genomic regions and genetic markers [14, 15]. Several studies have reported the association between *RUNX2* polymorphisms and BMD. In a study of 495 Australian women (Geelong Osteoporosis Study; GOS), a polymorphism in exon 1 of *RUNX2* was significantly associated with higher BMD in the lumbar spine (LS; L2–L4), proximal femur (femoral neck [FN], Ward's triangle, and trochanter), whole body, ultradistal, and mid-forearm. In addition, an allelic association between the polymorphism and Colles' fracture in elderly women was demonstrated [16]. In a subsequent study on a subgroup of 312 postmenopausal Scottish women, the same polymorphism was associated with significantly higher FN BMD, validating the GOS association result [17]. These studies clearly underscore the role of polymorphisms and rare variants of *RUNX2* in BMD and osteoporosis. In a separate study, on 264 Australian females taken from the GOS study with extremely high or low FN BMD values, three *RUNX2* P2 polymorphisms were identified in which the minor alleles were overrepresented in the high-FN BMD group and corresponded to a haplotype block that was significantly associated with increased FN BMD. Furthermore, when RUNX2 proteins synthesized from the polymorphic genes were assessed for DNA binding affinity, the $-1025\ T > C$ polymorphism caused the most significant increase in binding affinity compared to the wild-type promoter [18]. The relevance of P2 $-1025\ T > C$ SNP on BMD was also captured in another study examining 821 Spanish postmenopausal women in whom the $-1025\ T > C$ polymorphism was associated with higher FN BMD but not with LS BMD changes [19]. These results suggest that the *RUNX2* P2 promoter may be a candidate marker for adult BMD abnormalities. In particular, the P2 promoter $-1025\ T > C$ polymorphism may be important in the determination of FN BMD.

In this study, we performed a candidate gene-based association study of 729 postmenopausal Korean women to determine the association of the *RUNX2* -1492 and *RUNX2* -1025 P2 promoter polymorphisms with osteoporosis risk and BMD.

Materials and Methods

Subjects

The study population comprised apparently postmenopausal Korean women ($n = 729$) who visited the Asan Medical Center in Seoul. Informed consents were obtained from all participants. This study was approved by the Asan Medical Center Ethics Committee. Menopause was defined as the absence of menstruation for at least 1 year and was confirmed by measurement of serum follicle-stimulating hormone levels. Women displaying premature menopausal (age, <40 years) and those taking drugs possibly affecting bone metabolism for more than 6 months or within the previous 12 months, such as glucocorticoid (GC), bisphosphonate, or other treatment for osteoporosis, were also excluded.

BMD Measurement

Areal BMD (g/cm^2) at the LS (L2–L4) and FN was measured in 476 of the study subjects using dual-energy x-ray absorptiometry (Expert XL; Lunar, Madison, WI). In the remaining 253 subjects, BMD was measured using Hologic equipment (QDR 4500-A; Waltham, MA). The precision of the Lunar and Hologic equipment, presented as the coefficient of variation, was 0.82% and 0.85%, respectively, for the LS and 1.12% and 1.20%, respectively, for the FN. Precision values were obtained using 17 volunteers who were not part of the study; each volunteer underwent five scans on the same day, getting on and off the table between scans. To derive cross-calibration equations between the two systems, BMD was measured by the two machines for 109 healthy Korean women (mean age, 55 ± 11 years; range, 31–75 years), and cross-calibration equations were calculated as follows [20].

$$\text{For L2}-\text{L4 BMD}\left(\text{g}/\text{cm}^2\right);\ \text{Lunar BMD} = 1.1287 \times \text{Hologic BMD} - 0.0027$$

$$\text{For femoral neck BMD}\left(\text{g}/\text{cm}^2\right);\ \text{Lunar BMD} = 1.1556 \times \text{Hologic BMD} - 0.0182$$

The normal T-score was calculated using the software installed in the apparatus, and the mean BMD ± SD was established using healthy young Korean individuals. According to the WHO definition, osteoporosis was defined as a lowest T-score less than or equal to –2.5 SD at any bone site.

Sequencing of the *RUNX2* Gene

We sequenced all *RUNX2* exons, including exon-intron boundaries, and the promoter region (ca. 1.5 kb), to identify SNPs from 24 Korean DNA samples using an ABI PRISM 3730 DNA analyzer (Applied Biosystems, Foster City, CA). Sixteen primer sets for amplification and sequencing analyses were designed based on GenBank sequences (reference sequence of *RUNX2* mRNA, NM_004348.3; contig, NT_007592.14). Sequence variants were verified by automated sequencing chromatograms. SNPs were detected by multiple sequence alignment using the Phred/Phrap/Consed package and polyphred [21–23].

Golden Gate Assay for Genotyping

Genotyping was performed at a multiplex level using the Illumina Golden Gate genotyping system [24], and data quality was assessed using duplicate DNAs ($n = 10$). The genotype quality score for data retention was set at 0.25. SNPs that could not satisfy the following criteria were excluded: (i) a minimum call rate of 90%; (ii) no duplicate error; and (iii) Hardy–Weinberg equilibrium greater than $p > 0.001$.

Statistics

The chi-square test was used to determine whether individual variants were in Hardy–Weinberg equilibrium at each locus in the population. We determined Lewontin's D' ($|D'|$) and the linkage disequilibrium (LD) coefficient, r^2, between all pairs of biallelic loci. Genotypes were assigned codes of 0, 1, and 2 for the additive model, 0, 1, and 1 for the dominant model, and 0, 0, and 1 for the recessive model. The multivariate linear regression analyses of BMD at the LS and FN were performed using age, years since menopause (YSM), weight, and height as covariates. Associations of BMD values at other proximal femur sites, such as the total femur, the trochanter, Ward's triangle, and the shaft were determined after further statistical adjustments were made necessary by the choice of bone densitometer; cross-calibration data were not available at the time of the study for these values in Korean women. The genotype distributions between participants with and those without nonvertebral fractures were analyzed using a logistic regression model controlled for age, YSM, weight, and height. All statistical analyses were conducted using the SAS package (SAS Institute, Cary, NC).

Results

To locate Korean specific polymorphic loci of the *RUNX2* gene, we first sequenced all *RUNX2* exons and intron/exon boundaries including 1500-bp 5′ flanking regions of 24 independent Korean individuals directly. We detected three SNPs, two in the promoter region and one in exon 4, and examined DNA samples from 729 healthy Korean postmenopausal subjects to identify potential relationships between the detected *RUNX2* polymorphisms and the BMD.

The clinical profiles of the postmenopausal Korean women are reported in Table 1. The mean age of the participants was 58.69 ± 7.52 years (range, 43–84 years), and the mean YSM was 9.44 ± 7.77 years (range, 1–34 years). Multiple linear regression analysis showed that age and YSM correlated negatively with BMD at the LS ($\beta = -0.005$, $p = 0.0004$). Weight and height correlated positively with BMD ($\beta = 0.005$, $p < 0.001$, and $\beta = 0.003$, $p = 0.02$, respectively). BMDs measured by the Lunar equipment at the LS (0.887 ± 0.145 g/cm^2) were significantly higher than those measured by the Hologic equipment (0.785 ± 0.092 g/cm^2) (data not shown). Because the cross-calibration data were not available for these female Koreans at other sites (total femur, trochanter, femur shaft, and Ward's triangle), bone densitometry was used as an additional covariate when analyzing associations between genetic variation and BMD values.

Table 1 Clinical profiles of postmenopausal Korean women ($n = 729$)

Parameter	*N*	Mean ± SD
Age (yr)	729	58.69 ± 7.52
Weight (kg)	729	56.24 ± 7.08
Height (cm)	728	155.04 ± 5.29
YSM	729	9.44 ± 7.77
BMD (g/cm^2)		
Lumbar spine	729	0.85 ± 0.17
Trochanter	320	0.58 ± 0.12
Total femur	320	0.79 ± 0.14

Note: *YSM* years since menopause, *BMD* bone mineral density

The genotype frequencies of three detected SNPs tested in our subjects are reported in Table 2. Two promoter SNPs (in LD), *–1492 A > T* (rs7746992) and *–1025 T > C* (rs7771980), were still polymorphic, with respective genotype frequencies of A/A 99.8% and A/T 0.1% for *–1492 A > T* and T/T 83.6%, T/C 15%, and C/C 1.2% for *–1025 T > C*, but the exon SNP *+69430 C > T* (rs11498200) was monomorphic in our subjects (Fig. 1, Table 2). Therefore, *–1492 A > T* and *–1025 T > C* polymorphisms were examined further.

The association between polymorphic genotypes at detected *RUNX2* promoter SNP loci and BMD values was assessed by multiple logistic regression analysis after controlling age, YSM, weight, and height of enrolled subjects. In this analysis, the *RUNX2 −1025 T > C* polymorphism displayed a significant association with low LS BMD ($p = 0.02$) in the recessive model (Table 3). Subjects carrying the C/C minor homozygote genotype had lower LS BMD values (0.71 ± 0.13 g/cm^2) than subjects carrying the T/T major homozygote genotype (BMD = 0.85 ± 0.17 g/cm^2) or the T/C heterozygote genotype (BMD = 0.86 ± 0.19 g/cm^2). Furthermore, the proximal femur BMD values among subjects with the *−1025 T > C* polymorphism were also associated with decreased trochanter ($p = 0.05$) and total femur ($p = 0.04$) BMD in the recessive model (Table 3). Individuals bearing the *RUNX2 −1025 T > C* minor homozygote genotype (C/C) had a lower trochanter BMD (0.47 ± 0.16 g/cm^2) than those bearing the common T/T homozygote genotype (BMD = 0.59 ± 0.11 g/cm^2) or the T/C heterozygote genotype (BMD = 0.58 ± 0.16 g/cm^2). Also, subjects carrying the rare C/C homozygote genotype had a lower total femur BMD (0.64 ± 0.18 g/cm^2) than those bearing the T/T genotype (BMD = 0.79 ± 0.14 g/cm^2) or the T/C genotype (BMD = 0.78 ± 0.15 g/cm^2).

In summary, subjects with the *−1025 T > C* polymorphism minor homozygote genotype (C/C) had a consistent reduction of BMD (quantitative trait) in the LS ($\sim$0.15 g/cm^2), the trochanter ($\sim$0.12 g/cm^2), and the total femur ($\sim$0.15 g/cm^2), compared with subjects bearing the common T/T homozygote genotype or the T/C heterozygote genotype.

Discussion

Identification and characterization of specific loci or genes affecting BMD variation are important for understanding the genetic basis of osteoporosis and for determining pathophysiological therapies. Promoter variations in the 5′ UTR of regulatory genes have potential for affecting transcription factor binding and, as such, are potential osteoporosis risk indicators.

RUNX2 is a member of the runt homology domain family of transcription factors and is essential for osteoblast differentiation and bone formation. RUNX2-I transcription from the P2 promoter is related to BMD in selective Runx2-II in knockout mice [25]. In this regard, the P2 promoter of *RUNX2* polymorphisms is expected to provide genetic information for osteoporosis and susceptibility to fracture. However, the candidate genes that contribute to the pathogenesis of osteoporosis remain largely unknown. Several studies on diverse ethnic groups such as Australians, Scottish women, and Spanish women have provided evidence for a significant association between BMD and *RUNX2* polymorphisms. [16–19].

To investigate the potential genetic association of *RUNX2* polymorphisms with BMD, we analyzed two *RUNX2* polymorphisms (*−1492 A > T* and *−1025 T > C* within P2) among postmenopausal Korean women. The SNP *−1025 T > C* was previously shown to be associated with femoral neck BMD in Australian and Spanish postmenopausal women [18, 19]. We hypothesized that the *−1025 T > C* polymorphism within the P2 promoter might also have a validated association with BMD in the Korean population. In contrast, the SNP *−1492 A > T* has not been analyzed for associated bone phenotypes. We did not find a significant association between the *−1492 A > T* SNP and BMD in this study. Using regression analyses of the recessive model, we determined that the *RUNX2*

Table 2 Frequencies of *RUNX2* polymorphisms in postmenopausal women ($n = 729$)

Locus	Position	Residue change	rs ID	Genotype (*n*)				MAF	Heterozygosity	HWE[a]
−1492A > T	Promoter		rs7746992	A (728)	AT (1)	T (0)	N (729)	0.001	0.001	0.985
−1025T > C	Promoter		rs7771980	T (610)	CT (110)	C (9)	N (729)	0.088	0.160	0.118
+69430C > T	Exon4	R237C	rs11498200	C (729)	CT	T	N	0.000		

Note: *MAF* minor allele frequency

[a] *p*-values represent deviation from Hardy–Weinberg equilibrium (HWE) among all subjects

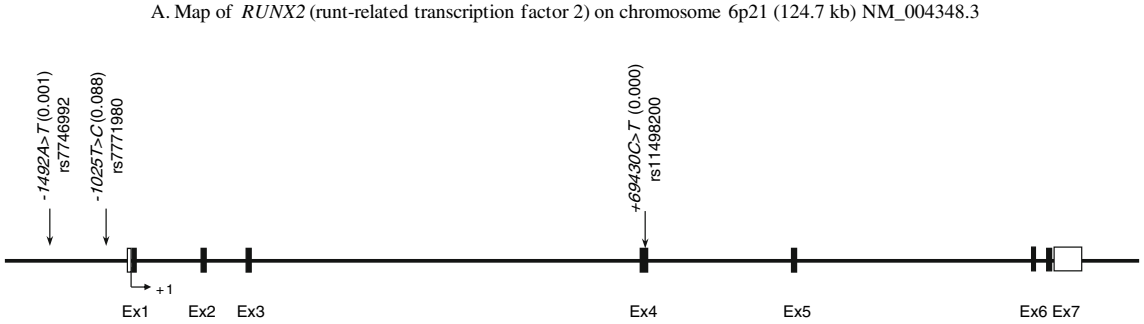

Fig. 1 Gene maps, haplotypes, and LD coefficients of *RUNX2*. Coding exons, black blocks; 5′- and 3′-UTRs, white blocks. The first base of the translation start site is denoted nucleotide "+1." Polymorphisms genotyped in a larger population ($n = 729$). The frequencies of polymorphisms not subject to larger-scale genotyping were based on sequence data ($n = 24$)

Table 3 Regression analyses of lumbar spine, trochanter, and total femur bone mineral density (BMD; g/cm^2, mean ± SD) with *RUNX2* polymorphisms in postmenopausal women

Position	C/C BMD	C/R BMD	R/R BMD	p_a	p_b	p_c
–1492 A > T						
Lumbar spine	727 (0.85 ± 0.17)	1 (0.78)		0.69	0.69	
Trochanter	318 (0.58 ± 0.12)	1 (0.52)		0.76	0.76	
Total femur	318 (0.79 ± 0.14)	1 (0.71)		0.99	0.99	
–1025 T > C						
Lumbar spine	609 (0.85 ± 0.17)	110 (0.86 ± 0.19)	9 (0.71 ± 0.13)	0.68	0.81	**0.02**
Trochanter	269 (0.59 ± 0.11)	45 (0.58 ± 0.16)	5 (0.47 ± 0.16)	0.38	0.74	**0.05**
Total femur	269 (0.79 ± 0.14)	45 (0.78 ± 0.15)	5 (0.64 ± 0.18)	0.20	0.44	**0.04**

Note: *C/C* common allele homozygotes, *C/R* rare allele heterozygotes, *R/R* rare allele homozygotes. p_a, p_b, and p_c are multiple regression analysis p-values for the additive, dominant, and recessive models, respectively. BMD values and p-values for regression analysis of three alternative models (additive, dominant, and recessive) controlling for age, weight, height, and years since menopause as covariates

−1025 T > C polymorphism was significantly associated with BMD values at the LS ($p = 0.02$) and proximal femur (trochanter, $p = 0.05$; total femur, $p = 0.04$). Specifically, subjects carrying the minor homozygote genotype (R/R) had lower LS and proximal femur BMD values compared with subjects carrying the major homozygote genotype (C/C) or the heterozygote genotype (C/R).

It could be argued that the Bonferroni correction should be applied to the p-values obtained in this study. However, when the Bonferroni correction was stringently adjusted, associated p values did not retain significance (data not shown). In fact, multiple comparisons at various bone sites were not totally independent of each other due to strong LDs among SNPs and related phenotypes. The significant association of the *−1025 T > C* polymorphism with BMD in this study is consistent with the reported role of this site in regulating the *RUNX2* P2 promoter and influencing adult BMD. An intriguing aspect of our results is that the genotype association of the *–1025 T > C* polymorphism can lead to diverse consequences on BMD values. The genotyping results at *–1025 T > C* polymorphic sites in a Spanish population showed that TC genotypes had a significantly higher FN BMD than those bearing TT and CC genotypes [19]. Basically congruent results were also observed in Australian populations, where the C allele was overly represented in the group that had a high FN BMD (allele frequency, 0.117) compared to the group with a low FN BMD (0.064) [18], since C alleles were mainly from heterozygotes due to their low frequency. Interestingly, however, close inspection of results from the Spanish population actually reveals that the CC genotype is associated with a lower LS and FN BMD compared with the TC or TT genotype, though not at a statistically significant level, possibly due to the small sample size. Although we were not able to confirm this in the Australian results due to the absence of genotype information, our results support the same genotype association of rare allele homozygotes. We contend that the association between CC genotype and lower BMD for the *RUNX2* –1025 T > C polymorphism has escaped proper appreciation because of the rarity of the genotype. For instance, only ∼1.5% of our subjects (9 of 729) had this rare variant. Studies with larger populations are imperative for full exposition of the roles played by *RUNX2* SNPs in determining BMD values.

To investigate the potential functions of *RUNX2* SNPs significantly associated with decreased BMD, we used the FASTSNP program (http://fastsnp.ibms.sinica.edu.tw) to identify and prioritize high-risk polymorphisms according to phenotypic parameters and putative functional effects. The biological implications of SNPs identified by FASTSNP were further analyzed using information from the Transcriptional Regulatory Element Database (TRED; http://rulai.cshl.edu/TRED), a platform for gene transcriptional regulation information that includes transcription factor binding sites. The analysis revealed that *RUNX2* $-1025\ T > C$ (rs7771980) is a risk factor for promoter/regulatory region malfunction: the T-to-C substitution may result in the alteration of binding site affinity by the transcription factor NFκB, which has been implicated in osteoblast suppression [26]. Subsequently, the promoter variation at the $-1025\ T > C$ locus may potentially have differential effects on mature osteoblasts as a mediator by recruiting transcriptional proteins to bind to DNA and regulating the specificity and kinetics of the transformational process. Therefore, this approach provides useful information for identifying interrelationships between promoter sequences that modulate transcription factor binding efficacy and biological function.

In conclusion, we show that *RUNX2* P2 promoter SNPs are significantly associated with decreased LS and proximal femur BMD in postmenopausal Korean women. This study suggests that the $-1025\ T > C$ promoter polymorphism may be a useful marker for osteoporosis among Korean women. Replication of these findings using a large cohort remains to be accomplished.

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