CLINICAL STUDY

Allelic variations of RANKL/OPG signaling system are related to bone mineral density and *in vivo* gene expression

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Abstract

Objective: Receptor activator of nuclear factor- κ B ligand/osteoprotegerin (RANKL/OPG) signaling system plays a crucial role in the regulation of bone resorption. Polymorphic variations in the genes may have an influence on gene expression and bone metabolism. In the present study, we aimed to investigate the influence of RANKL/OPG allelic variations on the *in vivo* human gene expression of five genes, bone mineral density (BMD), and fracture incidence in Hungarian postmenopausal women.

Methods: Three hundred and sixty postmenopausal women (61.6 ± 7.9 years) were genotyped. All together, five single nucleotide polymorphisms (SNPs) in the two genes have been investigated. In addition, bone samples from 17 examined subjects were acquired for gene expression studies. Bone densities and fracture data have also been collected.

Results: All two SNPs in OPG gene and three SNPs in RANKL gene showed correlation with BMD. Haplotype analysis of these genes gave similar results. The 'CCT' haplotype of RANKL promoter region, which was associated with decreased BMD, exhibited a significantly upregulated expression of RANKL mRNA, while the other haplotypes of RANKL or OPG 15 genes did not. No correlation between genetic variations and fracture data was found.

Conclusion: We have demonstrated associations between RANKL and OPG haplotypes and BMD as well as between RANKL haplotypes and *in vivo* RANKL expression in a Hungarian postmenopausal population. Moreover, we have found a new RANKL haplotype associating with reduced BMD and increased *in vivo* RANKL expression in human bone tissue.

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Introduction

Osteoporosis (OP) is a systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fractures (1). The major determinants of the risk for OP in later life are peak bone mass achieved during adulthood and the subsequent rate of bone loss (2). The peak bone mineral density (BMD) of adult women is mostly determined genetically (3). Twin studies in postmenopausal and elderly women also support a persistent and significant genetic influence on bone mass in later life (4). OP is a complex disease that is presumably influenced by multiple genes (5). Identification of genes that affect postmenopausal OP will permit early detection of individuals who are at risk for OP and early institution of preventive measures.

TNFRSF11A/TNFSF11/TNFRSF11B signaling system plays a crucial role in the regulation in bone resorption

(6, 7). The receptor activator of nuclear factor- κ B (TNFRSF11A/RANK) and its ligand TNFSF11 are the key proteins in osteoclastogenesis (8). The TNFRSF11A/TNFSF11 interaction is negatively controlled by TNFRSF11B (osteoprotegerin (OPG)), a known inhibitor of osteoclastogenesis, which acts as a soluble decoy receptor for TNFSF11 (8, 9).

In vivo studies confirm the bone-protective role of TNFRSF11B. TNFRSF11B knockout mice developed severe OP (10, 11), while overexpression of TNFRSF11B in transgenic mice resulted in osteopetrosis (6). Polymorphisms of the TNFRSF11B gene may contribute to the genetic regulation of bone mass as suggested by several recent publications, but the results are somewhat contradictory (12–16). The ambiguous results include the frequently investigated rs3102735 single nucleotide polymorphism (SNP) effects on BMD and fracture risk (14, 17). Recently, an association between BMD and SNPs of OPG gene has been demonstrated during a genome-wide screening (18, 19).

TNFSF11 expressed by osteoblasts directly activates cells of osteoclast lineage by connecting to the membrane receptor TNFRSF11A (20). TNFSF11-deficient mice show severe osteopetrosis with no tartrate-resistant acid phosphatase-positive osteoclasts (21). Soluble form of TNFSF11 rapidly induced hypercalcemia and bone loss (22). SNPs identified in the promoter and 5' UTR region of the gene have shown an association with BMD (10, 23–25). The quantitative trait analysis also found a correlation between BMD, low-trauma fractures, and genomic regions of TNFSF11 (19).

Based on previous data, we hypothesized that the allelic variations of the *TNFRSF11B* and *TNFSF11* genes – individually, in haplotypes, and in interaction – are associated with both osteoporotic phenotypes and *in vivo* gene expressions of the two genes. Our study was aimed at investigating the influence of *TNFRSF11B*/*TNFSF11* gene allelic variations on the *in vivo* human gene expression of these genes, BMD, and fracture incidence in Hungarian postmenopausal women.

Materials and methods

Study population and BMD measurement

Three hundred and sixty Hungarian unrelated postmenopausal women were recruited into the study through our metabolic bone disease outpatient clinic. One thousand and eight patients presenting to our clinic between January and July 2005 were invited to participate in our study. All subjects underwent physical examination and completed a detailed questionnaire on family and medical histories as well as on lifestyle habits including calcium intake. The calcium intake was determined by a validated European questionnaire (26). BMD values at the lumbar spine (L2–L4) and total hip were measured using a Lunar Prodigy DXA (GE Medical Systems, Diegem, Belgium). Covariance was below 1% at both sites. OP was defined according to the WHO criteria, i.e. *T*-score less than -2.5 s.d. at any measured site. Detailed fracture history was obtained from each subject. Nonvertebral osteoporotic fracture was defined as low-trauma fractures after the age of 45 years excluding fractures of the face, skull, fingers, toes, and spine. Vertebral compression fractures were not investigated in this study. Exclusion criteria were history of bone, metabolic or endocrine diseases; any chronic illness; previous hormone-replacement or steroid therapy or any medication known to influence bone metabolism; premature menopause (before 40 years of age); and alcohol consumption (more than two units/day). Subjects with biochemical abnormalities such as increased level of serum alkaline phosphatase, TSH, and parathyroid hormone, or reduced level of 25-OH vitamin D₃ (<30 ng/ml) were not included in the study. The population characteristics are presented in Table 1. The study was approved by the local ethics

Table 1 Characteristics of study population ($n=353^a$).

Variable	Mean $\pm$ s.d.
Age (years)	61.6 $\pm$ 7.9
Menopausal age (years)	13.5 $\pm$ 8.3
Body mass index (kg/m ²)	27.6 $\pm$ 4.5
Smokers	45 (12.5%)
Alcohol consumption ^b	16 (4.44%)
Calcium intake (mg/day)	642 $\pm$ 113
Serum 25-OH vitamin D ₃ (ng/ml)	44.6 $\pm$ 6.7
Lumbar spine BMD (g/cm ²)	0.887 $\pm$ 0.176
Total hip BMD (g/cm ²)	0.785 $\pm$ 0.168
Subjects with osteoporosis at either site	198 (55%)
Subjects with nonvertebral osteoporotic fractures	76 (21.5%)

^aThis table shows the parameters of the final study cohort. Seven subjects were excluded from the statistical analyses.

^bMore than one drink per week but less than one drink per day.

committee, and written informed consent was obtained from all participants.

Seventeen of the studied subjects had recently undergone total hip arthroplasty due to osteoarthritis. During the operation, nonosteoarthritic bone tissue samples from the femoral neck were collected from these patients for gene expression analyses. This study was separately approved by the ethics committee, and separate informed consent was obtained from these women.

Genotyping

Two SNPs in *TNFRSF11B* (rs1564858 and rs3102735 (A163G)) and three SNPs in *TNFSF11* (rs9533156 (T643C), rs9525641 (T290C), and rs3742257) were genotyped in this study (Table 2). The rationale for selecting the SNPs was that either these polymorphisms had been suggested to play a role in bone metabolism or they had been shown to be located physically close to one of the previously examined SNPs. In this way, we hoped to identify haplotypes demonstrating a strong relationship with gene expression and BMD as well as with fracture incidence.

Genomic DNA was extracted from venous blood samples using High Pure PCR Template Purification kit (Roche). DNA quality and quantity were determined using NanoDrop B-100 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Genotypes were determined using a GenomeLab SNPstream Genotyping System (Beckman Coulter, Fullerton, CA, USA) at the SNP Core Facility of Semmelweis University. The automated, ultra-high-throughput genotyping system utilizes multiplexed PCR in conjunction with tagged-array, multiplexed, single-base primer extension technology. PCR primers and tagged extension primers were designed on Autoprimer.com, the web-based primer design tool of Beckman Coulter (<http://www.autoprimer.com>). Ten random samples were genotyped in three parallel runs for testing technical errors.

Table 2 Selected single nucleotide polymorphisms (SNPs) for genotyping.

Gene	SNP number	Alleles	SNP position	SNP site	Success (%)	HW	MAF
<i>TNFRSF11B</i>	rs1564858	A/G	chr8:120014347	Intron	99.7	0.069	0.140
	rs3102735	C/T	chr8:120034251	Promoter	100	0.954	0.146
<i>TNFSF11</i>	rs9533156	C/T	chr13:42045671	Promoter	99.4	0.396	0.460
	rs9525641	C/T	chr13:42046024	Promoter	99.4	0.453	0.459
	rs3742257	C/T	chr13:42071198	Intron	99.4	0.215	0.486

Success rate means the call percentage of genotypes, HW, P value of the χ^2 test for Hardy–Weinberg equilibrium; MAF, minimal allele frequency.

Gene expression assays

Removed bone samples were extensively washed in PBS for eliminating blood and marrow contamination and were stored in liquid nitrogen. Human bone samples were cryoground under liquid nitrogen using a freezer-mill 6750 (SPEX Certiprep Inc., Metuchen, NJ, USA). Direct polyA-RNA was isolated with Dynabeads Oligo (dT)₂₅ paramagnetic particles (DynaL Biotech, Oslo, Norway) using the buffer set and protocol recommended by the manufacturer as it was previously described (27). After DNase treatment, mRNA was purified with NucleoSpin RNA Clean-up kit (Macherey-Nagel, Düren, Germany). The quantity and quality of mRNA were checked by spectrophotometry at 260/280 nm. One hundred nanograms of human mRNA were reverse transcribed at 55 °C using 200 U Superscript III RNase H reverse transcriptase (Invitrogen Life Technologies) with 125 ng random hexamer (Promega) and 40 U RNaseOUT Ribonuclease Inhibitor (Invitrogen Life Technologies) in 30- μ l reaction volumes. cDNAs for *TNFRSF11B* and *TNFSF11* were amplified by quantitative real-time PCR in a 25- μ l reaction volume containing 1 μ l cDNA, 12.5 μ l TaqMan 2 $\times$ Universal PCR Master Mix NoAmpErase UNG, 1.25 μ l predesigned and validated glyceraldehyde-3-phosphate (GAPDH) and *TNFRSF11B*- and *TNFSF11*-specific TaqMan gene expression assay 20 $\times$ (Hs02758991_g1, Hs00900359_m1, and Hs00243519_m1) from Applied Biosystems (Foster City, CA, USA). ABI Prism 7500 Real-Time PCR system was used to amplify the gene-specific cDNA from each sample in three parallel runs using a 96-well optical reaction plate following the protocol of 10-min denaturation at 95 °C, and 50 cycles of 15-s denaturation at 95 °C, 1-min annealing, and extension at 60 °C. Housekeeping gene of GAPDH was used as endogenous control. Threshold cycle numbers were analyzed by Relative Quantification Study of 7500 System SDS Software 1.3. The relative quantity (RQ) of *TNFRSF11B*- and *TNFSF11*-specific mRNA was calculated from the averaged value of ΔC_{t-s} .

Statistical analysis

Descriptive analyses of genotyped SNPs were performed using Haploview 4.0 software (28) (Table 3). Missing genotypes were imputed using a 10-nearest neighbor method (29). Kolmogorov–Smirnov test was applied to

analyze the distribution of the study phenotypes. Stepwise regression analyses were applied to spine and total hip BMDs (linear regression) and nonvertebral osteoporotic fracture (logistic regression), as phenotypes, and age, menopausal age, body mass index (BMI), smoking status, and alcohol consumption as covariates were used to identify significant covariates with phenotypes. Spine and total hip BMD values were also entered into a logistic regression model to determine their influence on fracture risk. Computed regression residuals, representing covariate-adjusted study phenotypes, were used in subsequent analyses (30). Genotype–phenotype associations were tested in the three common genetic models (additive, dominant, and recessive) applying nonparametric tests (Jonckheere–Terpstra trend test for the additive model, as well as Mann–Whitney U test for dominant and recessive models). To assess the reliability of the results, 10 000 Monte Carlo permutations were performed to generate exact P values. In individual analyses, permuted P values were corrected with the number of the genotyped SNPs, thus here only results with a P value of <0.01 were considered significant. The power of the study was calculated with the Quanto 1.1 software (31).

Haplotype analyses were performed using the ‘haplo-stats’ R-package (<http://cran.r-project.org/>). Score statistics were applied to test association between *TNFSF11* haplotypes and study phenotypes. This method computes permuted P values of the associations. Effect of haplotypes on covariate-adjusted study phenotypes was calculated in a generalized linear model (GLM) using the ‘haplo.glm’ function built in the software. Gene–gene interaction between the two genes was tested by performing multivariable linear regression analyses. Relative expression of *TNFRSF11B* and *TNFSF11* in bone tissue samples was compared between genotypes and haplotypes with nonparametric tests applying 10 000 Monte Carlo permutations. Regression and nonparametric tests were performed using the SPSS for Windows, release 13.0.1 (SPSS Inc., Chicago, IL, USA). In haplotype, interaction, and gene expression studies, a P value of <0.05 was considered nominally significant.

Sequence analysis

Genomic sequences of the regions next to the SNPs were obtained from the NCBI dbSNP database (<http://www.ncbi.nlm.nih.gov/SNP/>). Two sequences containing the

Table 3 Effect of *TNFRSF11B* and *TNFSF11* polymorphisms on bone mineral density in three genetic models.

<i>TNFRSF11B</i>	Genotypes (number)	Additive model			Dominant model			Recessive model		
		A/A (13)	A/G (73)	G/G (267)	P value ^a	A/A + A/G (86)	G/G (267)	P value ^b	A/A (13)	A/G + G/G (340)
<i>rs1564858</i>	Spine BMD (g/cm ²)	-0.013 ± 0.159	-0.039 ± 0.149	0.011 ± 0.165	0.021	-0.035 ± 0.150	0.011 ± 0.165	0.017	-0.013 ± 0.159	0.001 ± 0.163
	Hip BMD (g/cm ²)	-0.018 ± 0.154	-0.044 ± 0.130	0.013 ± 0.155	0.0002 ^c	-0.040 ± 0.133	0.013 ± 0.155	0.0001	-0.018 ± 0.153	0.001 ± 0.152
	Genotypes (number)	C/C (8)	C/T (87)	T/T (267)	P value ^a	C/C + C/T (95)	T/T (267)	P value ^b	C/C (8)	C/T + T/T (345)
	Spine BMD (g/cm ²)	-0.073 ± 0.111	-0.003 ± 0.167	0.003 ± 0.163	0.173	-0.010 ± 0.164	0.003 ± 0.162	0.403	-0.073 ± 0.111	0.002 ± 0.164
<i>rs3102735</i>	Hip BMD (g/cm ²)	-0.129 ± 0.083	-0.014 ± 0.131	0.009 ± 0.158	0.009 ^d	-0.024 ± 0.132	0.009 ± 0.158	0.035	-0.129 ± 0.083	0.003 ± 0.151
<i>TNFSF11</i>	Genotypes (number)	Additive model			Dominant model			Recessive model		
		C/C (95)	C/T (185)	T/T (73)	P value ^a	C/C (95)	C/T + T/T (258)	P value ^b	C/C + C/T (280)	T/T (73)
<i>rs9533156</i>	Spine BMD (g/cm ²)	0.004 ± 0.178	-0.017 ± 0.156	0.040 ± 0.154	0.109 ^e	0.004 ± 0.178	-0.001 ± 0.157	0.880	-0.010 ± 0.163	0.040 ± 0.154
	Hip BMD (g/cm ²)	0.024 ± 0.174	-0.017 ± 0.137	0.013 ± 0.152	0.466	0.024 ± 0.157	-0.009 ± 0.142	0.356	-0.003 ± 0.151	0.013 ± 0.152
	Genotypes (number)	C/C (96)	C/T (184)	T/T (73)	P value ^a	C/C (96)	C/T + T/T (257)	P value ^b	C/C + C/T (280)	T/T (73)
	Spine BMD (g/cm ²)	0.004 ± 0.177	-0.017 ± 0.157	-0.040 ± 0.154	0.057 ^e	0.004 ± 0.177	-0.001 ± 0.158	0.898	-0.010 ± 0.176	0.040 ± 0.154
<i>rs9525641</i>	Hip BMD (g/cm ²)	0.024 ± 0.174	-0.017 ± 0.137	0.013 ± 0.152	0.449	0.024 ± 0.174	-0.009 ± 0.142	0.352	-0.003 ± 0.151	0.013 ± 0.152
	Genotypes (number)	C/C (90)	C/T (189)	T/T (74)	P value ^a	C/C (90)	C/T + T/T (263)	P value ^b	C/C + C/T (279)	T/T (74)
	Spine BMD (g/cm ²)	0.032 ± 0.151	-0.020 ± 0.156	0.013 ± 0.185	0.080 ^d	0.032 ± 0.151	-0.010 ± 0.165	0.016	-0.004 ± 0.156	0.013 ± 0.185
	Hip BMD (g/cm ²)	0.020 ± 0.180	-0.019 ± 0.137	0.027 ± 0.164	0.363	0.020 ± 0.167	-0.007 ± 0.146	0.112	-0.007 ± 0.148	0.027 ± 0.164

Adjusted spine and hip mean BMD $\pm$ S.D. related to genotypes and *P* values of nonparametric tests (^aJonckheere–Terpstra and ^bMann–Whitney *U*) are represented. In additive model, *post-hoc* tests were also performed, and significant associations between genotypes are also represented (^chomozygote recessive versus homozygote dominant, ^dhomozygote dominant versus heterozygote, and ^ehomozygote recessive versus heterozygote).

two allelic variants of each SNP were used to search for putative transcription factor binding sites in the TRANSFAC database using the PROMO web-based software (<http://alggen.lsi.upc.es/>).

Results

Descriptive analyses

The average age of the subjects whose gene expression was analyzed from the bone sample was 63.6 ± 12.2 years (no significant difference from the rest of the subjects, $P=0.29$). Other clinical parameters of this subgroup were not different either. Altogether, seven subjects were excluded from the statistical analyses because their genotyping success rate was lower than 4/5 SNPs. The reproducibility of the high-throughput genotyping method called SNPstream was 100% in our study (genotypes were the same in each parallel run). The overall success rate of genotyping process was 99.7%, only five missing genotypes were imputed in the final database. All the genotyped SNPs were in Hardy–Weinberg equilibrium, and the minimal allele frequency was higher than 5% in each case. Linkage disequilibrium was also determined in Haploview. The three SNPs (rs9533156, rs9525641, and rs3742257) of TNFSF11 were in almost complete LD constructing a haplotype, while the two SNPs of TNFRSF11B were not significantly linked with each other. Age, menopausal age, and BMI proved to be significant covariates of BMD in the regression model. Fracture risk was significantly influenced by age, BMI, and BMD. The power of the study was higher than 75% (0.75) for hip BMD (mean $\pm$ s.d.: 0.785 ± 0.168) at a significance level of 0.05, MAF of 0.140, and 353 individuals to detect a difference in BMD of 0.054 g/cm^2 under the dominant

model. For spine BMD (mean $\pm$ s.d.: 0.887 ± 0.176) and MAF of 0.460, there would be a power of 65% to detect a difference of 0.053 g/cm^2 under the recessive model.

Association analyses

Table 3 shows the distribution of genotypes and the unadjusted mean BMD $\pm$ s.d. values. Permuted P values are also presented for the three genetic models. TNFRSF11B polymorphisms were significantly associated with the spine and hip BMDs. Carriers of the 'A' allele of rs1564858 had significantly lower BMD at the hip in the dominant model (covariate-adjusted differences in BMD $\pm$ s.d. were $-0.040 \pm 0.133 \text{ g/cm}^2$ for 'A/A + A/G' and $0.013 \pm 0.155 \text{ g/cm}^2$ for 'G/G' genotypes at the hip, $P=0.0001$). The rs3102735 was significantly related to hip BMD in the recessive model (adjusted hip BMD $\pm$ s.d. values were -0.129 ± 0.083 and 0.003 ± 0.151 in the cases of 'C/C' and 'C/T + T/T' genotypes, $P=0.004$). Two SNPs of TNFSF11 were significantly associated with spine BMD (adjusted spine BMD $\pm$ s.d. values were -0.010 ± 0.163 and 0.040 ± 0.154 in the groups of 'C/C + C/T' and 'T/T' of the two strongly linked rs9533156 and rs9525641). None of the studied SNPs were related to nonvertebral fractures.

The gene–gene interaction analyses did not show any significant interaction between the studied SNPs of the two genes.

Haplotype analyses

We performed score tests and GLMs to analyze the association between the TNFSF11 haplotypes and BMD as well as nonvertebral fracture risk (Table 4). The TNFSF11 haplotypes proved to be significantly associated with spine BMD (permuted global $P=0.022$

Table 4 Effects of TNFSF11 haplotypes on adjusted spine BMD and TNFRSF11B haplotypes on adjusted hip BMD.

		Score test		GLM model	
Haplotypes	Frequencies	Hap score	<i>P</i> value ^a	Coefficient ± s.e.m.	<i>P</i> value ^b
(A) Effect of <i>TNFSF11</i> haplotypes on adjusted spine BMD					
TTC	0.453	−0.398	0.711	0.037 ± 0.019	0.048
CCC	0.063	−0.428	0.679	−0.025 ± 0.028	0.423
CCT	0.471	−2.432	0.013	−0.049 ± 0.021	0.018
		Score test		GLM model	
Haplotypes	Frequencies	Hap score	<i>P</i> value ^a	Coefficient ± s.e.m.	<i>P</i> value ^c
(B) Effect of <i>TNFRSF11B</i> haplotypes on adjusted hip BMD					
GT	0.789	2.846	0.006	0.016 ± 0.010	0.102
GC	0.069	−1.182	0.245	−0.034 ± 0.023	0.138
AT	0.063	−1.487	0.148	−0.037 ± 0.022	0.106
AC	0.078	−1.987	0.041	−0.044 ± 0.021	0.039

Haplotypes with a frequency $>5\%$ and their effect on covariate-adjusted spine BMD are represented. ^aPermuted P value of global association was 0.022. ^b'GT' haplotype was entered into the GLM as reference haplotype, and P values of the GLM sign the significance of t -statistic of regression variables characterized by the estimated regression coefficients and their standard errors; ^c'TTC' haplotype was entered into the GLM as reference haplotype, and P values of the GLM sign the significance of t -statistic of regression variables characterized by the estimated regression coefficients and their standard errors; ^dpermuted P value of global association was 0.065.

under dominant model). The common 'CCT' haplotype was related to low BMD either in score test or in GLM, while the other common haplotype 'TTC' correlated with increased BMD at the spine. The global association between the TNFRSF11B haplotypes and study phenotypes was not significant, but two haplotypes have shown significant individual effect on hip BMD (Table 4). The most common 'GT' haplotype was significantly associated with high hip BMD, while a rare haplotype 'AC' was related to low hip BMD in the haplotype analysis. We did not find significant association with nonvertebral fractures.

Gene expression assay

The quantitative real-time PCR was successfully performed in 16 bone samples, and mRNA was degraded in one sample. The relative expression of TNFSF11-specific mRNA seemed to be dependent on the number of 'CCT' haplotype (Fig. 1) (permuted P value of global association was 0.007). In subjects with one or two copies of 'CCT' haplotype, TNFSF11 expression was significantly 2.25-fold increased compared to those with no 'CCT' haplotype (mean $RQ \pm$ s.d. values were 0.126 ± 0.050 and 0.056 ± 0.026 in 'CCT' carriers and noncarriers respectively, $P = 0.025$). No correlation was found between gene-specific mRNA expression and TNFRSF11B genotypes.

We performed 'in silico' sequence analysis to identify whether changes in mRNA levels according to haplotypes could be associated with the different transcription factor (TF) binding profile of the TNFSF11 promoter. A putative binding site of seven different TFs was identified in the promoter region of 'CCT' haplotype, while only two haplotype-specific TF binding sites were seen in the other common haplotype, 'TTC'.

Figure 1 Quantitative expression of RANKL depending on RANKL haplotypes. Bars represent the mean $\pm$ S.E.M. of the relative expression (RQ) of the *RANKL* gene to GAPDH in the three haplotype groups. Number of carriers in each group and permuted P value of the global association are also represented.

Discussion

In this study, we have investigated SNPs and haplotypes in the *TNFRSF11B*/*TNFSF11* genes exhibiting a significant correlation with BMD at the spine and hip. Furthermore, we have shown that increased tissue mRNA expression in subjects carrying 'CCT' *TNFSF11* haplotype was associated with reduced BMD.

Bone resorption process is influenced by the *TNFSF11*/*TNFRSF11B* ratio. Increase in this ratio is mainly due to the increase in *TNFSF11* gene expression (24). The *TNFSF11* gene is a member of the TNF superfamily localized at 13q14, with eight exons. Mutations in the gene lead to an osteoclast-poor form of osteopetrosis in humans (32). Promoter SNPs of the *TNFSF11* gene are good candidates for the genetic regulation of BMD. While studying rs9533156 and rs9525641 SNPs in the promoter region of *TNFSF11* gene, Mencej *et al.* (24) found both polymorphisms to be associated with hip BMD, and later (25) with lumbar spine BMD. We could confirm their results, and in addition, we have firstly identified a third SNP in an intronic region of *TNFSF11* gene showing an association with spine BMD as well as a strong linkage with the other two SNPs. The latter fact provides a rationale for examining the relationship between the haplotypes of these SNPs and BMD.

Mencej *et al.* (24) studied a haplotype consisting of three SNP alleles where the third SNP (rs9533155) was also located in the promoter of the *TNFSF11* gene. Subjects with the 'CCG' haplotype had decreased spine BMD. They also transfected human embryonic kidney cells (HEK 293) with amplified fragments of different haplotypes. Increased *TNFSF11* expression could be seen in 'CCG' haplotype by this method. Using rs3742257 SNP as part of the new haplotype examined in our study, we demonstrated for the first time in the literature that elevated *TNFSF11* expression in human bone samples is associated with 'CCT' haplotype. In this case, the new element of the haplotype was coming from an intronic SNP being in strong linkage with the promoter region SNPs. The same subjects with 'CCT' haplotype exhibited reduced BMD, which was possibly a consequence of increased *TNFSF11* expression resulting in stimulated osteoclast activity. The presumable role of the intronic SNP was shown in haplotype analyses where only 'CCT' haplotype and not 'CCC' haplotype was associated with lower BMD. We also performed the *TNFSF11* haplotype analysis using the two common SNPs (rs9533156 and rs9525641) without the third one (rs3742257) representing the effect of the promoter haplotypes of *TNFSF11* on study phenotypes (data not shown). 'TT' haplotypes of the two SNPs were significantly associated with higher BMD ($P = 0.003$), while global association was also significant in the score test ($P_{\text{global}} = 0.010$). These results show – according to Mencej *et al.* – those promoter variants of the *TNFSF11* that have a significant effect on BMD. However, in our

three-SNP haplotype analyses, the 'CC' promoter haplotype resulted in a significantly lower spine BMD only in combination with the 'T' allele of the third genotyped SNP (rs3742257), suggesting that besides the promoter, the other region tagged by the rs3742257 could play a potential role in the association between the genetic variants of the TNFSF11 and BMD.

TNFSF11 expression is controlled by various transcription factors, cytokines, and hormones (33). The promoter region SNPs could influence the binding of these regulators, and thus, gene expression. Our 'in silico' search for binding sites in the promoter region of *TNFSF11* gene resulted in a difference according to haplotypes. 'CCT' haplotype could potentially bind seven haplotype-specific transcriptional factors in contrast to 'TTC' that could bind only two. This difference might be reflected in the altered expressional activity of the different haplotypes.

The association of rs3102735 in *TNFRSF11B* gene with bone metabolism is very contradictory. It was identified in the promoter region by Kusk (34). In their study, postmenopausal women carrying the 'G' allele had lower bone mass than women homozygotic for the 'A' allele. Langdahl *et al.* (14) have shown the 'G' allele to be more common in osteoporotic patients. Their regression analyses revealed that this polymorphism predicts bone mass and tends to predict osteoporotic fractures independently of BMD. Jorgensen *et al.* (13) have found a significant influence of this mutation on bone mass and fracture status independently of serum TNFRSF11B level. Our results confirm these positive findings. We found an association between the rs3102735 polymorphism and BMD, but did not find any effect on the fracture risk; however, the present study was underpowered to show small effects. Other authors (16, 17, 35) have reported no effect of this genetic variation on BMD. There are several possible explanations for these discrepancies. BMD and fracture risk are complex traits, and the contribution of a single gene is expected to be relatively small and is influenced by other genes and environmental factors. Inconsistency in results could therefore arise from differences in the ethnic background, age, calcium intake, and sites of BMD measurements. In our study, no detectable influence of this promoter SNP on *in vivo* gene expression of TNFRSF11B was observed, and this SNP appeared not to affect any recognition sites for known transcription factors based on 'in silico' sequence analysis. Our results suggest that this SNP could be a marker of the altered gene function rather than a direct contributor.

The rs1564858 SNP, located in the second intron of the *TNFRSF11B* gene, was significantly associated with hip BMD; however, it is not in linkage disequilibrium with the rs3102735 polymorphism. This fact raises the possible genetic importance of both SNPs in bone metabolism. No effect on the TNFRSF11B expression was seen depending on TNFRSF11B genotypes. This could be explained by the fact that rs1564858

lies in an intronic region, and 'in silico' data indicate that it is located in a splice site of the gene, suggesting a possible indirect role of this SNP in the regulation of gene function. The heterozygote genotype of this SNP was significantly associated with the lowest BMD. This could be explained by the phenomenon of molecular heterosis. Molecular heterosis occurs when subjects for a polymorphism show a significantly greater or lesser effect for a trait than homozygotes. Regarding this TNFRSF11B polymorphism, molecular heterosis could be a result of the post-transcriptomic changes in the TNFRSF11B-specific mRNA that might be caused by this SNP. Further studies need to clarify the exact role of this region. Common haplotype constructed by the two SNPs of the *TNFRSF11B* gene seemed to be related to higher hip BMD, while a less common one was associated with lower BMD, which also underline the importance of the genetic variations of TNFRSF11B in OP. Nevertheless, this result should be handled with some caution since the global association of TNFRSF11B haplotypes and hip BMD was not significant in our model. This apparent controversy can be explained by the possibility that these two SNPs did not construct a haploblock inside the gene. In general, haplotype estimation processes are more reliable when minor genotype frequency is more than 10%.

Considering the well-known biological relation of the two molecules, we studied the gene–gene interaction between the SNPs of the *TNFRSF11B* and *TNFSF11* genes. We did not find any significant interaction among the genotyped polymorphisms. There are two possible explanations for this result. The biological interaction of the two molecules could arise from a post-transcriptomic modification, and/or the real genetic interaction might occur between other genomic regions than we studied here.

There are several potential limitations to this study. First, the study population comprised women who visited a university hospital and they may not have been representative of the general population residing in a community, thus possibly resulting in selection bias. Secondly, we investigated only five SNPs and did not screen two genes for the biologically active loci. Thirdly, power of the study for fracture risk was low (< 50%) due to the sample size, and we did not study the vertebral compression fracture. Lastly, similar to any other candidate gene studies on complex diseases/traits, this work only reveals part of the whole genetic architecture underlying the etiology of OP. Our study also has a few strengths. Our subjects were ethnically homogenous. We applied statistical procedures with permutation tests to reduce population-based bias. Also, this is the first study to evaluate the TNFSF11/TNFRSF11B signaling system haplotype effects on gene expression in human bone samples.

In summary, we have found a significant correlation between TNFSF11 and TNFRSF11B genotypes and BMD. Also, we are the first to show a new TNFSF11

haplotype (CCT) that is associated with increased *in vivo* TNFSF11 expression in human bone tissue as well as with reduced BMD in Hungarian postmenopausal women. The importance of these findings deserves further investigations.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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