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ORIGINAL ARTICLE

Strong effect of SNP rs4988300 of the LRP5 gene on bone phenotype of Caucasian postmenopausal women

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Abstract The purpose of this study was to identify relationships between single nucleotide polymorphisms (SNPs) in the genes of the Wnt pathway and bone mineral density (BMD) of postmenopausal women. We chose this pathway due to its importance in bone metabolism that was underlined in several studies. DNA samples of 932 Hungarian postmenopausal women were studied. First, their BMD values at different sites (spine, total hip) were measured, using a Lunar Prodigy DXA scanner. Thereafter, T-score values and the patients' body mass indices (BMIs) were calculated, while information about the fracture history of the sample population was also collected. We genotyped nine SNPs of the following three genes: *LRP5, GPR177,* and *SP7*, using a Sequenom MassARRAY Analyzer 4 instrument. The genomic DNA samples used for genotyping were extracted from the buccal mucosa of the subjects. Statistical analyses were carried out using the SPSS 21 and R package. The results of this analysis showed a significant association between SNP rs4988300 of the *LRP5* gene and total hip BMD values. We could not reveal any associations between the markers of GPR177, SP7, and bone phenotypes. We found no effect of these genotypes on fracture risk. We could demonstrate a significant gene–gene interaction between two SNPs of *LRP5* (rs4988300 and rs634008, $p = 0.009$) which was lost after Bonferroni correction. We could firmly demonstrate a significant association between rs4988300 of the *LRP5* gene and bone density of the hip on the largest homogeneous postmenopausal study group analyzed to date. Our finding corroborates the relationship between *LRP5* genotype and bone phenotype in postmenopausal women, however, the complete mechanism of this relationship requires further investigations.

Keywords Osteoporosis · Wnt signaling · Bone metabolism · Genetic epidemiology

Introduction

Osteoporosis is a complex disease with a strong genetic background [1]. The genetic effects are mediated through a wide variety of genes [2]. Genetic effects were observed in all phases of bone metabolism, in bone formation as well as in bone resorption. The receptor activator of nuclear factor kappa-B (RANK)/receptor activator of the nuclear factor kappa-B ligand (RANKL) pathway is a crucial element in bone resorption [3]. Genome-wide association studies (GWAS) studies showed that single nucleotide polymorphisms (SNPs) in the genes of the RANK/RANKL pathway were associated with bone mass and fracture risks [4, 5].

The Wnt pathway plays an important role in bone metabolism, especially bone formation, and its alterations are associated with osteoporosis [6–11]. Members of this pathway are in close relationship with bone development and bone density. The most investigated factor of the Wnt pathway in osteoblast differentiation is the *LRP5/6* [12]. The gain of function mutations of *LRP5/6* result in high bone mass or osteopetrosis, and the loss of function

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mutations lead to osteoporosis-pseudoglioma syndrome [13–15]. So far, all GWAS studies concentrating on *LRP5/6* gene have found a relationship between its genotypes and osteoporosis [16, 17].

GPR177 is a 7-transmembrane protein with an effect on the secretion of Wnt proteins; it facilitates the excretion of Wnts from the Golgi to the extracellular space [18]. Loss of *GPR177* leads to disturbed axial differentiation in mice. Therefore, *GPR177-null* mice show severe impairment in skeletal development, which suggests that *GPR177* plays an important role in the Wnt pathway [19]. Furthermore, a recent GWAS study showed that *GPR177* affects bone mass in humans [20].

SP7 (also known as osterix) is a zinc-finger containing a transcription factor of bone formation and osteoblast differentiation. The SP7 and Hypoxia Inducible Factor-1alpha (HIF1A) have been demonstrated to synergistically inhibit the Wnt pathway [21]. TheSP7 is important in shifting mesodermal cells away from the cartilage cell line towards osteoblast lineage [22]. The *SP7* gene expression in circulating mesenchymal stem cells was decreased in osteoporotic patients compared to healthy individuals [23]. In the Sister Study, the SNPs of the *SP7* gene appeared to be associated with bone mineral density (BMD) in African–American women [16].

Based on these previous studies, it seemed likely that the polymorphisms of these genes in the WNT pathway might have an effect on bone metabolism. Therefore, we tested how the functioning SNPs of LRP5, *GPR177*, and *SP7* genes affected BMD and fracture risk in Caucasian postmenopausal women.

Materials and methods

Patient and phenotypic information

The population in our study consisted of 932 postmenopausal, non-related women referred to Hungarian osteoporosis centers. Patients were considered postmenopausal if they were older than 40 and there was no menstruation period within 1 year prior to our study. Secondary osteoporosis was excluded. We collected data on patients' age, height, and body weight to calculate BMI. Patient data can be seen in Table 1. After measuring femoral and spinal BMD using a Lunar Prodigy DXA scanner (GE Medical Systems, Madison, Wisconsin, USA), we calculated femoral and spinal T-scores based on the Hungarian reference range. We sorted subjects into different diagnostic subgroups (osteoporosis, osteopenia, and normal bone status) based on the WHO criteria. Patients with a T-score less than −2.5 at any site were considered osteoporotic, patients with a T-score value between −2.5 and −1.0 at any site are osteopeniac, and subjects with T-score values higher than −1.0 have normal bone status. The fracture history of a subpopulation of 385 patients, obtained from their medical history, was also utilized. Non-vertebral osteoporotic fracture was defined as a low-trauma fracture after the age of 40 years excluding the fractures of the face, skull, fingers, toes, and spine. Vertebral compression fractures were not investigated in this study. Written informed consent was obtained from all participants. The study was approved by the Science and Research Ethics Committee of the Medical Science Council, Hungary.

SNP selection

We used publicly available online databases (http://genome.ucsc.edu/, http://www.genome.gov/gwastudies/, and http://www.ncbi.nlm.nih.gov/omim) to select SNPs of the following genes: LRP5, *GPR177, SP7.*

Nine SNPs were chosen based on their function or previous appearance in GWAS studies. On the basis of the first criteria, missense variations were chosen which cause a change in the amino acid sequence of the encoded protein. Regarding the second criteria, we chose SNPs that have shown a p value less than or equal to 5×10^{-8}.

Genotyping

We collected genomic DNA from the patients' buccal mucosa, brushing off the superficial layer of cells lining the oral cavity. DNA was extracted using a High Pure PCR Template Purification kit (Roche Diagnostics, GmbH, Mannheim, Germany), and genotyping was performed on a Sequenom MassARRAY Analyzer 4 (Sequenom, San Diego, CA, USA).

Statistical analysis

Genotyping data and fracture risk

To determine the relationship between genotypes and fracture risk, we created contingency tables containing the genotypes and fracture history. To test the statistical significance of the results, we applied Pearson's chi-squared test.

Table 1 Characteristics of our study population

$N = 889$	Mean	SD
Age (year)	61.05579	9.951067
BMI (kg/m^2)	28.06399	4.800989
Spine BMD (g/cm^2)	0.823829	0.326662
Spine T-score	−1.60151	1.173225
Total hip BMD (g/cm^2)	0.755713	0.121174
Total hip T-score	−1.83702	1.102675

Genotyping data and bone parameters

The subjects were assigned to different groups based on the genotyping results, meaning that three groups (homozygote recessive, homozygote dominant, and heterozygote) were created for each SNP. The genotype groups were tested for normality using Shapiro–Wilk's test and homogeneity of variances by Levene's test. We utilized analysis of covariance (ANCOVA) to test the different genotypes against each other, and the Bonferroni method was used to adjust for multiple testing. We adjusted BMD values for age and BMI. All tests were performed using SPSS 21 (SPSS Inc., Chicago, IL, USA). Linkage disequilibrium plots based on our own data were created using HaploView 4.0 (Broad Institute, Cambridge, MA, USA). Interactions of different genes on phenotype were calculated with SNPassoc [24], haplotype analyses were carried out using haplo.stats. Both of these are R packages (R Foundation for Statistical Computing, Vienna, Austria). We chose an alpha value of 0.05.

Table 2 Patient characteristics by osteoporosis status

	Mean	SD
Normal $N = 90$		
Spine BMD	1.08	0.31
Spine T-score	−0.13	0.62
Femur BMD	0.96	0.086
Femur T-score	−0.18	0.64
Osteopenia $N = 455$		
Spine BMD	0.86	0.33
Spine T-score	−1.29	0.82
Femur BMD	0.79	0.12
Femur T-score	−1.56	0.65
Osteoporosis $N = 344$		
Spine BMD	0.71	0.27
Spine T-score	−2.35	1.14
Femur BMD	0.67	0.11
Femur T-score	−2.61	0.99

SD Standard deviation

Results

Basic statistics of subject group and pre-analysis

Basic statistical characteristics of the sample group are detailed in Table 1. Forty-three samples of our initial sample size of 932 did not contain enough DNA for genotyping, and they were excluded from further analysis. Published results here (Table 2) are based on the remaining 889 women. Table 2 contains the bone parameters of our subjects in the different study groups.

Genotyping results

General data about genotyping are presented in Table 3. All of the genotyped SNPs followed the Hardy–Weinberg equilibrium. Call rate was at least 98.54 % for all SNPs. One SNP turned out to be monomorphic in our population (SP7 rs191240606), even though it was polymorphic in all populations of the 1000 genomes project. The SNP is not present in the HapMap database. Genotyped SNPs in *GPR177* formed two haplotype blocks, while in *LRP5* we could create one haplotype block (Fig. 1).

Table 3 Genotyping results of the examined SNPs

Gene	SNP rsID	SNP position	SNP site	Callrate [%]	MAF*	Allele	HWE-Test**	Effect on BMD (p value)†	Effect on T-score (p value)††
GPR177	rs1430742	chr1:68635075	intron	98.54	0.209	G/A	0.2991	0.379	0.310
	rs2566755	chr1:68635390	intron	99.78	0.21	A/G	0.3822	0.260	0.169
	rs2566785	chr1:68604094	non coding RNA	99.78	0.39	A/G	0.4963	0.881	0.399
	rs983034	chr1:686035086	exon	99.44	0.389	C/T	0.5216	0.456	0.550
LRP5	rs4988300	chr11:68088831	intron	99.78	0.463	G/T	0.4399	0.004	0.079
	rs599083	chr11:68192346	intron	98.65	0.313	G/T	0.7046	0.615	0.040
	rs634008	chr11:68094741	intron	98.99	0.443	C/T	0.6447	0.017	0.034
SP7	rs191240606	chr12:53722883	exon	99.89	0.00	C/A	1.00	–	–
	rs2016266	chr12:53727955	intron	99.78	0.315	A/G	0.5534	0.231	0.096

* *MAF* Minimum allele frequency

** Hardy–Weinberg equilibrium test. HWE test was carried out with Haploview 4.0

† BMD values were adjusted for age and BMI

†† *T* score values were adjusted for BMI

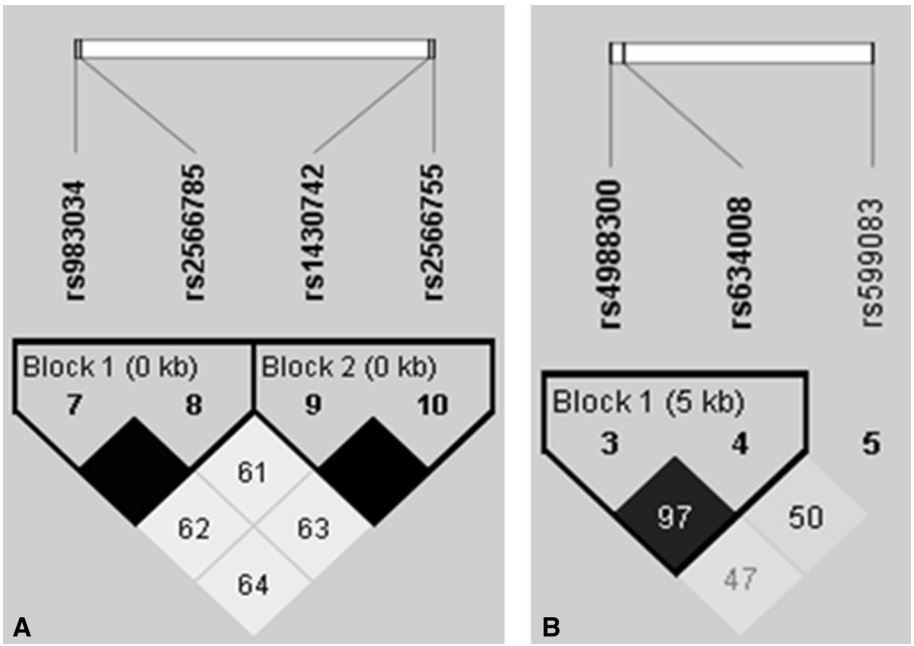

Fig. 1 *Haplotype blocks* in the studied polymorphisms. *Haplotype blocks* were built using Haploview 4.0. *Darker colors indicate* stronger LD. **a** contains SNPs from *GPR177,* **b** contains SNPs from *LRP5*

Influence of *LRP5* SNPs on bone mass and fracture risk

We found that rs4988300 and rs634008 had an association with total hip BMD. The association between rs4988300 and total hip BMD remained significant after Bonferroni correction ($p = 0.004$). Heterozygotes of this SNP have a significantly higher total hip BMD than homozygotes. Our data showed an association between rs599083, rs634008, and total hip T-score, respectively ($p_1 = 0.040$, $p_2 = 0.034$), but the significance disappeared after Bonferroni correction. We found no correlation between spine BMD and the tested genotypes. Furthermore, we observed no interaction between the tested genotypes and the incidence of osteoporotic fractures at any sites.

Haplotype analysis and gene–gene interactions

A gene–gene interaction plot was drawn using SNPassoc (Fig. 2). The gene–gene interaction plot suggested an interaction between two SNPs of *LRP5* (rs4988300 and rs634008, $p = 0.009$), which was lost after Bonferroni correction. No other interactions were found between the studied SNPs. Using the haplo.score function of haplo.stats, we found no significant change in total hip T-score or total hip BMD. Additional information about the haplotype analysis can be found in Table 4.

Influence of *GPR177*and *SP7* SNPs on bone mass and fracture risk

We were unable to find any associations between SNPs of these two genes and BMD of our subjects. We observed no association between the tested genotypes and the incidence of osteoporotic fractures at any site. *(No data shown).*

A table with the adjusted BMD and T-score values for all genotypes of the selected SNPs is provided as supplementary material.

Discussion

In this study, we investigated the relationship between the candidate genetic variations of the Wnt pathway and bone density/fracture rate. We could firmly demonstrate a significant association between rs4988300 of the *LRP5* gene and bone density of the hip on the largest homogeneous postmenopausal study group analyzed to date.

LRP5 is a co-receptor of the Wnt pathway; several loci of this gene were described as potential factors in osteoporosis by GWAS studies [25]. Loss of function mutations in this gene lead to osteoporosis-pseudoglioma syndrome [26, 27]. Gain of function mutations result in high bone density [28]. A strong effect of *LRP5* gene SNPs on BMD and fracture risk has been established [13–15, 29, 30]. A report by Xiong et al. [31] concluded that four genes (LRP5, DBP, CYP17, and RANK) showed highly suggestive associations with spine BMD. They found that rs4988300 showed an association with spine osteoporosis (OP) and rs634008 was associated with spine hip and ultradistal radius OP. However, these findings lost significance after correction for the number of haplotypes in the haplotype-based association test. Rs4988300 showed a statistically significant association with total hip BMD in our samples even after Bonferroni correction. This finding is in accordance with

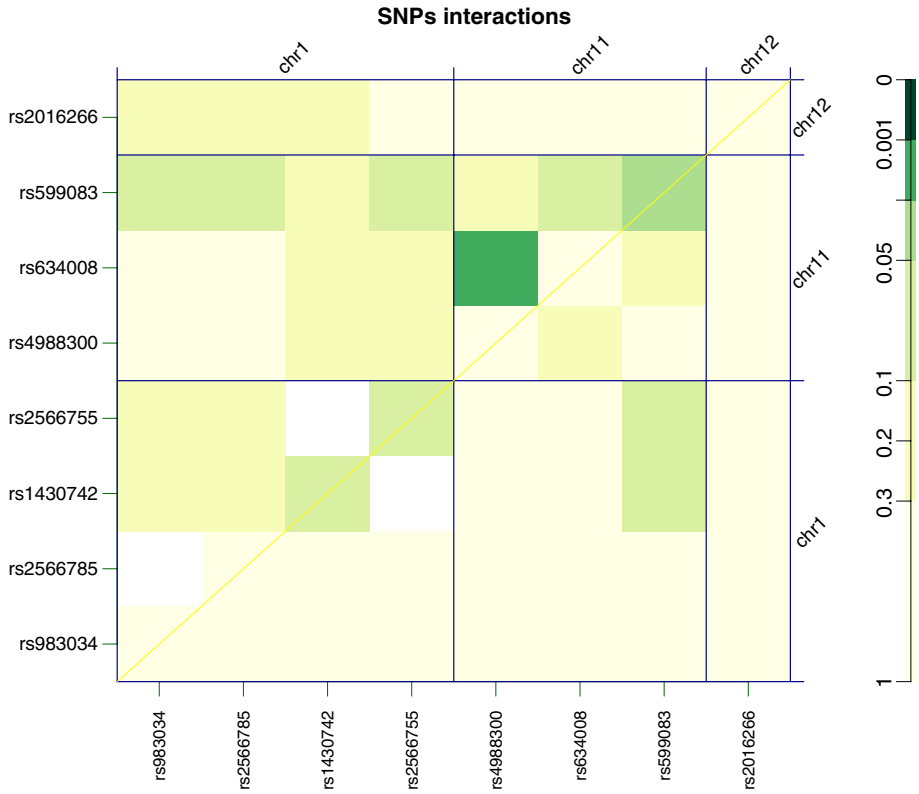

Fig. 2 SNP interactions in the studied genes. The plot suggests an interaction between rs4988300 and rs634008, both SNPs of *LRP5* ($p = 0.009$). SNPs interaction plot was drawn using SNPassoc extension of R

Table 4 Haplotype frequencies and their association to total hip *T* score

			Hap-freq	Hap-score	*p* value	Sim *p* value
LRP5	rs4988300	rs634008				
HT1	G	C	0.00523	−1.77528	0.07585	0.068
HT2	T	T	0.02507	−0.10453	0.91675	0.916
HT3	G	T	0.52595	0.26365	0.79205	0.807
HT4	T	C	0.43128	0.3294	0.74185	0.744
GPR177	rs1430742	rs2566755				
HT1	A	A	0.77721	−0.95755	0.33829	0.345
HT2	G	G	0.20578	0.33422	0.73821	0.746
	rs2566785	rs983034				
HT1	G	T	0.38435	−1.13311	0.25717	0.284
HT2	A	C	0.60658	1.29942	0.1938	0.211

HT1 of LRP5 is close to statistical significance. As the haplotype frequency of HT1 is low, increasing the sample size could clarify this finding. Haplotype frequencies and haplotype scores were computed using haplo.stats extension of R

the previous results of Kiel et al. [32]. Our study cohort was stratified for gender, this way we could eliminate the difficulties caused by the different pathogenesis in men and women. Our study group was bigger for osteoporotic women than that of Kiel et al. They studied 646 postmenopausal women; we had genotyping information on 889 subjects. We also had a subgroup of women with detailed fracture history. In the study of Kiel et al., genotypes of rs4988300 were associated with femur shaft section modulus in males. Based on this data, we tested if the genotypes influenced fracture risk. We found no association between these traits; however, there are other factors besides BMD that influence fracture risk. These include cortical thickness of bone and bone microarchitecture [33]. Differences in these bone parameters might explain why the genotypes did not associate with fracture history.

We could not find evidence for the role of the other two factors of the Wnt pathway, *SP7* and *GPR177*. This does not rule out the association with BMD; however, the effect seems to be too small to be identifiable in our study population of osteoporotic women. GPR177 is a glycoprotein, which mediates secretion signals for the Wnt pathway [34]. In rodents GPR177 is expressed even in adulthood, its presence is essential for healthy organogenesis [35]. It is also

essential for adequate antero-posterior development. Fu et al. [36] hypothesized a reciprocal interaction between GPR177 and Wnt to regulate the levels of both factors. GPR177 level elevates as it is a target of Wnt, as a result GPR177 increases Wnt signaling by promoting Wnt excretion from the Golgi. Kumar et al. [6] found no association of BMD with the SNPs (rs983034, rs3748705, rs2566755, and rs2820475) of *GPR177* gene on groups of postmenopausal and young women. For the same SNPs (rs983034 and rs2566755), we could not find an association with BMD in our study population either. In contrast, Roshandel et al. [37] showed a significant association between spinal BMD of men and *GPR177* gene SNP rs1430742 in 2,359 participants of the European Male Ageing Study (EMAS). We also included this SNP in our study; however, we found no statistically significant association between this trait and BMD in our study group. The reason for this difference might be explained by the different genders included in the two studies. Rs983034 and rs3748704 code a missense variation; however, rs983034 did not cause a detectable change of bone status in our study group. Even though previous GWAS studies (studying rs2566755 and rs1430742) could demonstrate a slight effect on BMD, our results could not corroborate this effect.

SP7 has a known effect on bone development; it is involved in embryogenesis, and has a role in bone reparation too [38]. *SP7* has a decreased expression in the circulating mesenchymal stem cells of osteoporotic patients [23]. Another study showed no association between *SP7* genotypes and BMD [39]. In our study, we could not find evidence for any relationship between rs2016266 and BMD or fracture history. Rs191240606 turned out to be monomorphic despite the fact that this SNP is not described as monomorphic in any population of the 1000 genomes project (http://www.ncbi.nlm.nih.gov/variation/tools/1000genomes/). There is no data collected from Eastern European populations in the 1000 genomes project, thus, a putative difference between the Hungarian population and CEU (US residents of northern and western European ancestry) population might explain this finding.

As with every study, ours has its strengths and limitations. The advantages are that we had a relatively large, genetically homogenous study group. The main disadvantages include a low number of cases in the fracture history subgroup, a low number of SNPs per gene, a small cohort size for haplotype analyses, and we did not examine the biological background associated with the genotypes. Despite these pitfalls, our study further strengthened the close relationship of LRP5 genotypes and bone density as well as provided more insights into the cause of the so far often contradictory results by clarifying them in a rather confounding factors-free study environment.

Conflict of interest All authors declare no conflict of interest.

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