

Haplotypes of the low-density lipoprotein receptor-related protein 5 (*LRP5*) gene: Are they a risk factor in osteoarthritis?

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Summary

Objective: Several genome-wide scans have revealed an osteoarthritis (OA)-susceptibility locus on chromosome 11q in close proximity to the low-density lipoprotein receptor-related protein 5 (*LRP5*) gene. The regulation of bone mass is under the control of *LRP5* and since increased bone mass is thought to play a role in the pathology of OA we examined *LRP5* polymorphisms and haplotypes to determine if variants of this locus may predispose to OA.

Methods: A UK control population of 187 individuals was examined for five commonly occurring polymorphisms against a cohort of 158 DNAs from patients with knee OA. An additional UK cohort was also examined to confirm the findings of the first study; this second group consisted of 110 knee OA patients. Haplotype analysis was also performed on patient and control DNAs.

Results: A study of individual polymorphisms revealed no association with disease. However, haplotype analysis of the initial two populations revealed a common haplotype (C-G-C-C-A) that provided a 1.6-fold increased risk of OA ($P_c = 0.021$). The data obtained from the second cohort confirmed the initial findings, with a 1.6-fold increased risk observed within this cohort for the risk haplotype ($P = 0.012$).

Conclusions: A closer investigation of *LRP5* and associated Wnt signalling molecules in OA will help determine disease aetiology and the development of novel treatment strategies that specifically target the bone compartment.

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Introduction

Osteoarthritis (OA) affects an estimated 5 million people in the UK¹ and represents the most common form of joint disease². The condition is characterised by subchondral and cancellous bone sclerosis, osteophyte growth, cartilage loss and joint space narrowing. The widely held belief that OA is a disease of cartilage has, particularly in recent years, been a subject for debate^{3,4}, and several researchers have postulated that bone changes may account for subsequent joint deterioration and development of OA^{5–10}. However, whether bone matrix composition and metabolism are actually altered in OA has only emerged from more recent

investigations, for example the studies on OA osteoblasts^{11–15} and OA bone matrix^{16–19}. Collectively these recent works add further support of a role for bone in OA aetiology; primary OA osteoblasts behave differently to healthy controls, e.g., marked basal and vitamin D induced expression of osteocalcin¹¹. In addition, bone matrix turnover is very high and the collagen hypomineralised^{16,17}, findings that may explain some of the changes observed for the biomechanical properties of OA bone^{18,19}.

In addition to the changes observed for bone matrix composition and metabolism, individuals with OA exhibit striking increases in bone mass for both affected sites, such as the hip or knee, as well as non-synovial sites, such as the lumbar spine²⁰; the literature supports a systemic increase in bone mass in patients who have OA. Indeed the general, but still controversial opinion, is that higher bone mass increases the risk of developing OA^{20–22}. This concept is strengthened by the increased incidence of OA in subjects who present with osteopetrosis²³, a condition characterised by greater bone mass. The possibility that

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greater bone mass could lead to cartilage loss, joint deterioration and progression to OA was first put forward by Radin and colleagues^{8,9}. They proposed that stiffening of the subchondral bone would result in increased shear stresses at the bone–cartilage interface, culminating in cartilage splitting and erosion. This mechanism was serendipitously supported by the replacement of subchondral bone matrix with much stiffer polymethylmethacrylate resin in a canine model of hip arthroplasty²⁴; modified femoral heads displayed the classic signs of cartilage loss consistent with advanced OA, whereas contralateral control hips remained unaffected. The authors concluded that joint deterioration was probably a consequence of changing the stiffness of the subchondral material.

Furthermore, in OA bone there is an overall increase in its metabolism^{25,16,17} and an increased expression of osteogenic factors such as transforming growth factor- β and insulin-like growth factors^{11,17}. These local growth factors also show increased expression in those regions, for example, the iliac crest²⁶, which are deemed not to be “OA sites”. The study of spontaneous OA in animal models such as the Macaque²⁷ and guinea pig^{28,29} have provided compelling evidence for bone thickening prior to cartilage tissue loss. Collectively, increased bone mass and/or metabolism, particularly at synovial joints may predispose subjects to OA.

It is well established that OA is a disease with a large multigenic component. Interestingly, evidence has emerged that supports OA-susceptibility loci to chromosome 11q12-13^{30,31}, a region well known to harbour genes controlling bone mass^{32,33}. One such gene encodes for the low-density lipoprotein receptor-related protein 5 (*LRP5*), a co-receptor for Wnt/ β -catenin signalling³⁴. It is now known that *LRP5* is required for osteoblast proliferation^{35,36} and possibly the prevention of osteoblast apoptosis³⁷. In a recent review of the literature³⁸, it is becoming clear that *LRP5*/Wnt signalling is central for post natal bone development and adult bone accrual making *LRP5* an exciting candidate for further study in the context of musculoskeletal diseases, including OA.

We propose that *LRP5* may represent a potential candidate gene in the predisposition of OA due to its strong association with bone mineral density (BMD)³⁸. Loss-of-function mutations in *LRP5* result in osteoporosis–pseudoglioma syndrome (OPPG), an autosomal recessive disorder³⁵. Conversely a gain-in-function mutation, for example a G171V point mutation, results in elevated bone mass, enhanced alkaline phosphatase activity and raised active osteoblast number³⁷. Furthermore, mutation analysis of families and patients has revealed at least 19 *LRP5* sequence variants of which six are thought to result in a high bone mass phenotype³⁹. Given the compelling association of high bone mass and OA, *LRP5* certainly warrants closer investigation into this debilitating disease.

Methods

PATIENTS AND CONTROLS

OA Patients ($n = 268$) were studied from two UK centres: Bristol ($n = 110$, 45 male and 65 female Caucasians: mean age = 62; median age = 63); and London ($n = 158$, 41 male and 117 female Caucasians: mean age = 71; median age = 71). All patients had knee OA as defined by the American College of Rheumatology (ACR) criteria with at least grade 2 radiological change. Radiographic diagnoses were performed by the same consultant rheumatologist in all cases. Control subjects were 187 (94 male and 93 female) unrelated healthy Caucasian volunteer blood donors from South West England. Informed consent was obtained with appropriate local ethics committee approval. Genomic DNA was isolated from peripheral blood leucocytes using a salting out procedure⁴⁰.

SINGLE NUCLEOTIDE POLYMORPHISMS (SNPs) GENOTYPING

Five *LRP5* SNPs from LocusLink (<http://www.ncbi.nlm.nih.gov/LocusLink/>) were selected for the study: rs491347, rs583545, rs667126, rs314751, and rs689179. These were chosen to represent the 3′-end of the *LRP5* gene, including two haplotype tag (ht) SNPs represented in the International HapMap project (rs491347, htSNP 34, block 4; and rs583545, htSNP 38, block 5), see <http://www.hapmap.org>. In view of the relatively small number of patients, three other appropriate 3′-SNPs were chosen with minor allele frequencies above 0.25 to generate an appropriate distribution of haplotypes to provide sufficient statistical power for the association study. For each polymorphic locus to be analysed, polymerase chain reaction (PCR) amplifications were set up in a total volume of 20 μ l containing 200 ng genomic DNA, 0.1 Unit *Taq* polymerase (Abgene, Epsom, Surrey, UK), 0.05 μ M of each PCR primer (Table I), 2 μ l of 10 \times reaction buffer [160 mM (NH₄)₂SO₄, 670 mM Tris–HCl (pH 8.8 at 25°C), 15 mM MgCl₂, 0.1% v/v Tween-20 (Ameresco Chemicals, Anachem, Luton, UK)], 0.8 μ l DNA polymerisation mix (Abgene). Amplification was carried out in a PTC-100 thermal cycler (MJ Research Inc., Waltham, MA, USA) using cycle parameters of: 95°C for 5 min (initial denaturation), then 35 cycles of: 95°C (30 s), annealing temperature (*Ta*) (see Table I) (40 s) and 72°C (30 s); with a final extension for 5 min at 72°C.

PCR-single strand conformational polymorphism was employed for the purpose of genotyping using polyacrylamide gel electrophoresis as described⁴¹ in 12% nondenaturing polyacrylamide gels (Protogel, National Diagnostics, Atlanta, GA, USA) containing 1 \times Tris–borate–ethylenediaminetetraacetic acid (TBE) buffer. Electrophoresis was carried out at 300 V for 150 min on a triple-wide minigel system (CBS Scientific Company Inc., Del Mar, USA). DNA was visualised by staining for 10 min

Table I
LRP5 polymorphisms analysed in this study

dbSNP rs# cluster I.D.	Gene position	Allele frequencies	PCR primers (5′–3′)	Ta
rs491347	<i>LRP5</i> intron 8	C = 0.31, T = 0.69	F = AACACCTGAACCAACGGAG R = CGCTTTTCTGGTAAGCTGGA	60
rs667126	<i>LRP5</i> intron 11	A = 0.68, G = 0.32	F = GTGCGTCTTGTGTTCACC R = CCAGCACTGTGCCTGATACA	62
rs583545	<i>LRP5</i> intron 11	C = 0.27, T = 0.73	F = ACCCCTGCCACACACATATC R = GCTTGTGTCCATCCTGGTTT	61
rs689179	<i>LRP5</i> intron 12	C = 0.26, T = 0.74	F = CATGATCGAGTCTGCCAACA R = GGACAACCTCCACAGCAGAG	62
rs314751	<i>LRP5</i> intron 12	A = 0.32, G = 0.68	F = GTTCAGGATCCCCAGAGAT R = CCTGTTGCTGTGTGACTTGG	61

The dbSNP rs reference number is provided with the location of each SNPs within *LRP5* and allele frequencies from the control population. PCR primer sequences are provided with the Ta for PCR indicated.

with ethidium bromide (0.5 µg/ml in 1× TBE buffer) and gels were imaged using a Kodak EDAS 120 digital camera.

HAPLOTYPE AND DISEASE ASSOCIATION ANALYSIS

Allele frequencies were estimated by gene counting and potential deviation in Hardy–Weinberg equilibrium was tested using the χ^2 test with 1 df. Haplotypes were constructed from genotypic data using the PHASE 2.0 software platform^{42,43}. Haplotypes with an observed frequency of less than 0.02 were analysed as a combined group. To identify potential risk or protective haplotypes, haplotype frequency comparisons between control and OA patients were performed using haplotypic odds ratios (OR) with Cornfield 95% confidence intervals (CI). Pearson's χ^2 test or Fisher's exact test were used to determine uncorrected *P*-values and corrections were made for multiple comparisons (*P*_c) where appropriate.

Results

ANALYSIS OF *LRP5* GENE POLYMORPHISMS IN CONTROL DNAs

Genotyping of 187 controls DNAs for the five common *LRP5* polymorphisms: rs491347, rs583545, rs667126, rs314751, and rs689179 were carried out (see Table I for allele frequencies). Examination of the data using PHASE 2.0 analysis resulted in a total of 13 haplotypes, although 90% of controls comprised three predominant haplotypes due to the high levels of linkage disequilibrium (LD) in the region examined (Table II).

POLYMORPHISM OF THE *LRP5* GENE AND ASSOCIATION WITH OA

One hundred and fifty eight DNAs derived from a London-based centre of patients with knee OA (at least grade 2 on the ACR scale) were genotyped for the five polymorphisms examined in the control population. The genotype frequencies of the two populations were analysed using Pearson's χ^2 test and revealed no association between each polymorphism and OA. All genotype results were in Hardy–Weinberg equilibrium. Haplotype analysis was carried out as with the control DNAs, revealing the presence of eight haplotypes within the London OA cohort. All PHASE-generated posterior probabilities for reconstructed haplotypes were >0.9. Comparison of haplotypes between the control and OA cohorts identified a common haplotype (C-G-C-C-A) with a significantly increased frequency in the OA population (OR = 1.6, CI = 1.12–2.29; *P* = 0.0069; *P*_c = 0.021; Table II). Upon stratification for gender, the association remained within the female OA group (OR = 1.72, CI = 1.06–2.78; *P* = 0.019), although was

lost within the male group, possibly due to low numbers of this population (*n* = 41).

To verify these results in a different population, an additional study was carried out in a cohort of 110 DNAs from a Bristol-based knee OA population. As with the London OA cohort, there was no association between single genotypes and OA, however, when the PHASE haplotypic data were examined, the correlation that was observed previously was confirmed in this cohort. The C-G-C-C-A haplotype was present at a significantly higher frequency than observed in the control population (OR = 1.62, CI = 1.09–2.40; *P* = 0.012).

Discussion

Osteoarthritic subjects often present with a systemic increase in bone mass, suggesting that increased production of bone may predispose to OA, particularly at synovial sites, where increased bone content could be sufficient to precipitate the changes in cartilage that ultimately contribute to joint failure. A potential candidate that may help explain the increased bone mass in OA is *LRP5*, a single span transmembrane protein belonging to the low-density lipoprotein receptor family³⁴.

LRP5 contains 23 exons and spans 160 kb of the genomic sequence on chromosome 11q13.4⁴⁴. Such a large region inevitably contains a significant level of polymorphism, and genetic association studies of this nature require the use of haplotypic analysis. Indeed, a study by Twells *et al.*⁴⁵ has examined the level of LD between *LRP5* polymorphisms, characterising the presence of common haplotypes. They identified three conserved regions of LD throughout the gene, separated by recombination hot spots: intron 1, intron 3 to intron 5, and intron 5 to intron 7.

To ascertain whether the *LRP5* gene acts as a susceptibility locus for OA, we examined a population of 158 knee OA patients from London for five common polymorphisms within the *LRP5* gene; each contained within the largest haplotype block (exon 7 to exon 23). Single polymorphisms provided no correlation with disease status, but when analysed as a group, a common haplotype (C-G-C-C-A) was evident, which provided a 1.6-fold increased risk of OA. A further study using a 110 knee OA cohort from Bristol confirmed these findings, again with a 1.6-fold increased risk with the susceptibility haplotype. Given the relatively small patient cohort sizes, the findings presented provide a preliminary indication of an association between this common *LRP5* haplotype and OA. Further studies will be required in order to confirm the data in larger cohorts and from other centres. Although we do not have these data it will be pertinent to obtain information concerning body mass index in view of the strong association of knee OA in overweight populations. Because *LRP5* not only regulates adult bone accrual but also lipid metabolism³⁴ a similar

Table II
LRP5 haplotypes in control and OA populations

Haplotype	Controls	London knee OA	Uncorrected <i>P</i> -value	Bristol knee OA	Uncorrected <i>P</i> -value
T-A-T-T-G	238	193	0.488	129	0.226
C-G-C-C-A	81	97	0.0069	68	0.012
C-G-T-T-A	16	5	0.04	5	0.958
Others	39	21		18	
Total	374	316		220	

Uncorrected *P*-values are provided in the table (see text for corrected *P*-values).

haplotype frequency for non-obese OA subjects will help strengthen a role of LRP5 in skeletal rather than lipid metabolism *per se*.

Although it is generally agreed that OA is multifactorial, the data presented support further studies into *LRP5* in the context of OA aetiology since it may be linked to compromises in bone accrual and/or turnover. Bone represents the major component of joint tissues, and the tightly co-ordinated process of bone remodelling is crucial for the maintenance of healthy joint competence and architecture. If the process of bone metabolism and turnover are disrupted, skeletal disorders including osteoporosis (OP) and osteopetrosis can occur. It is becoming clearer that both bone architecture and metabolism are profoundly altered in OA, and the subchondral bone compartment, in particular, is involved in the pathogenesis of OA^{16,17,4}.

There is strong evidence that the pathology of OA is related to bone density^{20–23}. Indeed, the inverse relationship between the development of OP, a disease of low bone density, and OA, is well documented²⁶. Previous research has implicated the vitamin D receptor (*VDR*) alleles in predicting BMD⁴⁵, and several research groups have analysed polymorphic markers in this gene for OA association but with varied results. Indeed, the extent of the role of *VDR* polymorphisms in bone density has now been brought into question^{46,47}. Genome-wide scans amongst OA probands have suggested a link close to chromosome 11q12-13^{30,31}, and proposed that this finding may be related to the location of a high bone mass gene. Interestingly, this correlates with the location of the *LRP5* gene, situated on chromosome 11q13.4. In light of recent developments into OA aetiology we sought to focus our attention on *LRP5* because of its strong association with bone accrual; loss-of-function mutations in *LRP5* result in OPPG³⁵, whereas gain-in-function mutations result in elevated bone mass, enhanced alkaline phosphatase activity and raised active osteoblast number³⁷. Mutation analysis of families and patients has revealed at least 19 *LRP5* sequence variants of which six are thought to result in a high bone mass phenotype³⁹. It is now established that LRP5 is involved with osteoblast proliferation and may have a role in the regulation of osteoblast apoptosis^{35–37}. In osteoblasts, LRP5 acts as a co-receptor for Wnt/ β -catenin signalling and it is now known that Wnt signalling is of critical importance in skeletal differentiation⁴⁸. During the course of our investigation Loughlin and colleagues⁴⁹ identified functional variants within the secreted frizzled-related protein 3 (*sFRP3*) gene in women with hip OA. These particular findings are of enormous significance since they provide further evidence of a role for Wnt signalling in the development of OA; *sFRP3*, by binding to Wnt5a blocks the noncanonical Wnt pathway⁵⁰. The *sFRP3* variant (Arg324Gly) identified by Loughlin *et al.* is unable to antagonise Wnt signalling, the result of which is postulated to predispose subjects to getting OA. Although *sFRP3* is restricted to cartilaginous tissue, the study by Loughlin *et al.*, together with our findings, strengthen the need to examine the Wnt signalling pathways in situations of musculoskeletal abnormality.

As with most signalling systems there are many adaptor and substrate molecules that serve to regulate and/or integrate multiple cellular signalling networks. Such complexities are true of osteoblast Wnt signalling and the associative changes with bone accrual and disease⁵¹. It will be interesting to see if some of the LRP5 adaptor/substrate molecules and/or interactions with other proteins are

affected in subjects predisposed to getting OA. For example, Dickkopf 1 (DKK1), the endogenous antagonist of LRP5 canonical signalling, is unable to antagonise the G171V LRP5 mutant, a finding that may help explain the high bone mass phenotype in subjects with this mutation⁵². To date no studies have been undertaken to examine whether DKK1 is expressed during bone development or whether the levels of DKK1 are altered in subjects with OA. Wise, by interacting with LRP5, antagonises Wnt/ β -catenin signalling and is also capable of antagonising bone morphogenetic protein signalling³⁴. Of further significance is the striking finding that ablation of the Wnt antagonist, *sFRP1*, results in prolonged and enhanced trabecular bone accumulation in adult mice but with no effect on other tissues⁵³. Clearly there are several key branches off a single signalling cascade that are central to skeletal development and turnover that could be the subject of further study in OA. Some of these components, e.g., Wise, DKK1 and *sFRP1* could be investigated in OA given the importance of bone in the development of this disease. Collectively these studies should help disentangle the mechanism of disease initiation and progression and shed further light into the role played by bone in OA aetiology.

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