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FULL PAPER

Extended haplotypes and linkage disequilibrium in the IL1R1–IL1A–IL1B–IL1RN gene cluster: association with knee osteoarthritis

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The interleukin-1 gene cluster is a key regulator in a number of chronic disease processes. We explored the linkage between nine polymorphic loci in the IL1R1 promoter, eight in the IL1A–IL1B–IL1RN gene complex, and their association with osteoarthritis (OA), a common complex disease associated with low-level inflammation. Using 195 healthy controls, we identified eight novel polymorphisms in the IL1R1 exon 1A region. We found limited LD between IL1R1 and the IL1A–IL1B–IL1RN cluster, although LD within these two individual groups was high. To test association with knee OA, we genotyped 141 patients from Bristol (UK) at the 17 loci. IL1R1 promoter haplotypes showed no association with disease. However, within the IL1A–IL1B–IL1RN complex, we identified a common haplotype conferring a four-fold risk of OA ($P = 0.00043$; $P_c = 0.0043$) and one IL1B–IL1RN haplotype conferring a four-fold reduced risk ($P = 0.0036$; $P_c = 0.029$). To replicate these associations, we subsequently examined 163 knee OA patients from London. Here, the effects of the haplotypes were confirmed: the risk IL1A–IL1B–IL1RN haplotype conferred a two-fold risk of OA ($P = 0.02$), and the protective IL1B–IL1RN haplotype conferred a five-fold reduced risk of OA ($P = 0.0000008$). These results may help to explain the genome-wide scan linkage data and functional observations concerning association between IL-1 and OA.

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Introduction

The regulation of the immune system relies on the complex interaction within the cytokine network, where the products of cytokine genes and their respective receptor genes must be finely tuned to maintain an optimal physiological environment. The interleukin-1 (IL-1) system epitomises the highly regulated mechanism required for this to be achieved. Several related gene products interact with each other to adjust the response according to cellular requirements. IL-1 signalling via the interleukin-1 receptor type I (IL-1RI) is an important pathway in the inflammatory process, innate immunity and the immune response.[1] Its effects are various, including the stimulation of fibroblast and keratinocyte proliferation, cartilage breakdown, angiogenesis and increased production of acute phase response proteins by the liver.[2] These physiological effects are mediated at the molecular level by the activation of JNK and p38 MAP kinases, and consequent upregulation of genes via the transcription factors NF-κB, AP-1 and C/EBPβ[3]

The products of the IL-1 gene cluster are thought to play a role in a number of diseases, one of which is osteoarthritis (OA). This disease affects an estimated 5 million people in the UK[4] and over 16 million in the US,[5] and is characterised by focal loss of cartilage matrix in the affected joints. IL-1 is thought to contribute to this loss by stimulating chondrocytes to produce cartilage matrix degrading enzymes. In support of this contention, a strong correlation exists between specific cleavage of the aggrecan core protein at the Glu373–Ala374 bond and the release of aggrecan catabolites in response to IL-1:[6] fragments bearing this epitope are found in synovial fluids of OA patients.[7] In addition, cells from OA synovium produce more IL-1β than normal synovium,[8,9] and similarly, chondrocytes for OA cartilage secrete both

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IL-1α and IL-1β unlike normal chondrocytes.[10,11] It is also known that OA cartilage explants are more susceptible to the effects of IL-1 than similar explants from nonarthritic cartilage[12] and that this susceptibility is related to the expression of IL-1 receptor types 1 and 2 on chondrocytes.[13]

A further line of evidence comes from genetic studies. First-degree relatives of patients with generalised radiologic OA are twice as likely to develop the disease as people in the general population,[14] and monozygotic twins are significantly more concordant for hand and knee osteoarthritis than dizygotic twins.[15] Thus, a genetic predisposition to OA exists. Interestingly, some genome-wide scans for predisposing genetic loci to OA reveal potential linkage with the IL-1 gene cluster on chromosome 2.[16,17] Taken together, these results implicate both IL-1 and its receptors in the progression of OA in at least some patients, and suggest that a search to determine if genes in the IL-1 cluster are associated with OA is justified.

IL-1 acts through its signalling receptor, IL-1 receptor type 1 (IL-1RI), and the degree of expression of this receptor influences the response of cells to IL-1.[18] The cloning and characterisation of the two IL-1 receptors (type 1 and 2)[19] has revealed the IL-1 receptor type 2 to function as an IL-1 binding protein that acts as a competitive decoy, lacking the cytoplasmic residues necessary for intracellular signalling.[20] Three structurally related ligands can bind to the IL-1 receptors: IL-1α and the more abundant IL-1β functioning as agonists, and IL-1Ra as an antagonist that results in no intracellular signalling.

IL-1α, IL-1β, IL-1Ra and the two IL-1 receptors, type 1 (IL-1RI) and type 2 (IL-1RII), are encoded by a cluster of genes (*IL1A*, *IL1B*, *IL1RN*, *IL1R1* and *IL1R2*, respectively) situated on chromosome 2 (Figure 1a). Recently, six new IL-1 family genes have been mapped between *IL1B* and *IL1RN*: *IL1F7*, *IL1F9*, *IL1F6*, *IL1F8*, *IL1F5* and *IL1F10*.[21,22] These genes share the same common ancestor as IL-1 and IL-18, with regions of amino acid and genetic homology, but their function is currently uncharacterised. The *IL1A*, *IL1B* and *IL1RN* genes have been extensively examined for polymorphic nucleotide residues: the literature on these polymorphisms, their effect on gene expression, and their influence on human diseases has been reviewed.[23–25]

The *IL1R1* gene (Figure 1) contains 14 exons and has three distinct promoters, generating three alternative transcripts with differing 5′-untranslated regions, derived from exons 1A, 1B or 1C.[26] Since the levels of transcription of certain cytokine genes are influenced by polymorphic nucleotides in the promoter sequences,[23] and IL-1RI is expressed on a wide variety of cells subjected to different evolutionary pressures (including those from microorganisms), we predicted that the promoter region of *IL1RI* should be highly polymorphic. Indeed, we, and others, have previously reported nucleotide sequence polymorphism in the promoter region spanning introns 1A–1C.[27–31] In the present report,

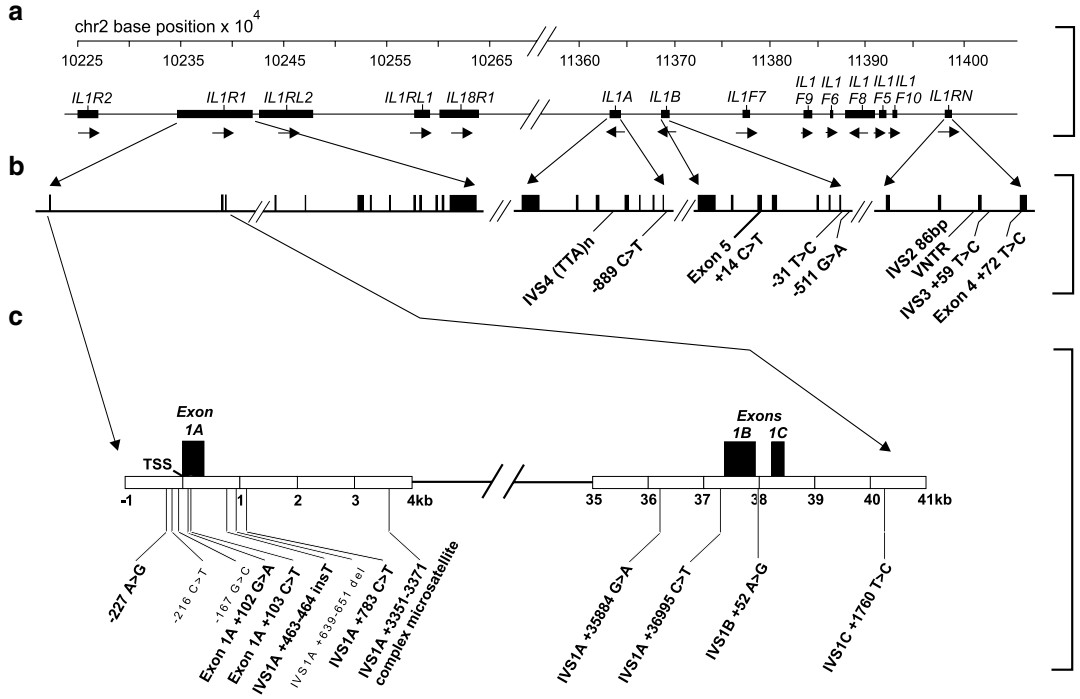

Figure 1 Organisation of the *IL1R1–IL1A–IL1B–IL1RN* gene cluster showing polymorphic nucleotide sequences. (**a**) Map of genes within the region, showing base positions on chromosome 2q according to the UCSC Human Genome Browser July 2003 Assembly (http://genome.cse.ucsc.edu/). Direction of transcription is shown beneath each gene by a horizontal arrow. (**b**) Exon–intron organisation of the *IL1R1*, *IL1A*, *IL1B* and *IL1RN* genes. Exons are shown as vertical lines or boxes. Exon : intron size ratios are shown to scale. For clarity, genes are not shown to scale: genomic sizes of genes are: *IL1R1*, 72.3 kb (14 exons: 1A, 1B, 1C, 2–11); *IL1A*, 10.55 kb (seven exons); *IL1B*, 7.03 kb (seven exons); *IL1RN*, 5.25 kb (four exons). Polymorphic nucleotide sequences examined in the *IL1A*, *IL1B* and *IL1RN* genes are shown. (**c**) Exon–intron organisation of the alternative *IL1R1* promoters. Polymorphic nucleotide sequences are shown and those examined are denoted in bold typeface. TSS, transcription start site for the 1A promoter.

we describe the identification and characterisation of a further eight novel polymorphisms in the *IL1R1* promoter. Recently, a number of reports have indicated that polymorphic IL-1 haplotypes or extended haplotypes, as opposed to single locus polymorphisms, may provide better genetic markers of transcriptional activity and disease association,[32] in particular with the complex nature of the IL-1 agonist/antagonist relationship and the two IL-1 receptors.[33] To begin to address this concept with respect to osteoarthritis, this report examines the extent of linkage disequilibrium and the occurrence of haplotypes and extended haplotypes within the *IL1R1–IL1A–IL1B–IL1RN* region.

Results

Novel polymorphisms in the *IL1R1* exon 1A promoter, exon 1A and intron 1A

Sequencing of amplicons showing PCR-SSCP variation revealed eight novel polymorphisms (rows 1–8, Table 1; Figure 2). We were also able to confirm and further characterise a previously described polymorphism in exon 1A at position +103 C>T (dbSNP, rs1019296). The two contiguous exon polymorphisms at positions +102 G>A and +103 C>T formed only three haplotypes from a possible four, with the genotypic combinations GT, GC and AT at the respective positions. These were considered as a single polymorphism with three alleles for the purposes of haplotype analysis, where GT = allele 1, GC = allele 2 and AT = allele 3 (Table 1). Further details of the polymorphisms identified in the study, and used in the subsequent haplotype analyses, are given in Table 1.

Linkage disequilibrium in the *IL1R1–IL1A–IL1B–IL1RN* region

The patient and control cohorts were genotyped for nine commonly occurring polymorphisms in the *IL1R1* gene (Figure 1c) and a series of eight polymorphisms previously described in *IL1A*, *IL1B* and *IL1RN* genes (Table 1). Pairwise linkage disequilibrium (LD) was determined for all 17 loci in 195 control DNAs (Figure 3). This revealed a high level of LD between the polymorphic loci in *IL1R1*. Similarly, there was considerable intragenic and intergenic LD between polymorphisms in the *IL1A–IL1B–IL1RN* complex, particularly between the *IL1A* IVS4 TTA repeat, the *IL1RN* IVS2 86 bp VNTR and the *IL1RN* IVS3 +59 loci. Although the LD between the *IL1A–IL1B–IL1RN* complex and the *IL1R1* promoter polymorphisms is much reduced, there are loci which show a significant degree of LD, for example three *IL1R1* loci (Exon 1A +102/103 haplotype, IVS1A +463–464 insT and IVS1A +36995) with *IL1B* and *IL1RN*; and two *IL1R1* loci (IVS1A +783 and IVS1A +35884) with *IL1A* IVS4 (TTA)n.

Haplotypes in the *IL1R1* promoter and *IL1A–IL1B–IL1RN* complex and association with OA

Genotypes at the 17 polymorphic loci (Table 1) represented a total of 95 *IL1R1–IL1A–IL1B–IL1RN* haplotypes in the control subjects. In view of the overall weak LD between the *IL1R1* promoter and the *IL1A–IL1B–IL1RN* complex, separate haplotype analyses were conducted. The nine *IL1R1* promoter polymorphisms revealed a total of 33 haplotypes in controls and patients from Bristol and London with knee OA. A total of 299 of the 304 patients were included in the *IL1R1* haplotype study: the five remaining individuals were excluded due to incomplete genotyping data. Table 2 shows details of the five haplotypes with a frequency of 0.05 or more in either controls or patients (haplotype nomenclature is explained in Table 2 footnote). There was no significant difference between any of the *IL1R1* promoter haplotype frequencies in controls or patients.

A parallel analysis of the eight polymorphisms in the *IL1A–IL1B–IL1RN* complex revealed a total of 37 haplotypes in controls and knee OA patients. Table 3 shows details of the nine haplotypes with a frequency of 0.05 or higher, in either controls or patients (haplotype nomenclature is explained in Table 3 footnote).

The Bristol knee OA cohort was examined first. Here, we found one haplotype (2C-CTG-1TT) was significantly more frequent in patients with knee OA than in controls (OR = 3.93, CI = 1.68–9.25, $P = 0.00043$, $P_c = 0.0043$). When sexes were analysed separately, the association remained significant (males, $P = 0.0069$; females, $P = 0.0037$). Further scrutiny of these data indicated that there may be a protective haplotype, masked by the inclusion of the *IL1A* genotypes. We thus conducted a further analysis of the six polymorphisms within the *IL1B–IL1RN* region. This revealed a total of 20 haplotypes in controls and patients. Table 4 shows details of the seven haplotypes with a frequency of 0.05 or higher, in either controls or patients (haplotype nomenclature is explained in Table 4 footnote). One haplotype (CCA-1TT) was significantly less frequent in patients than in controls (OR = 0.37, CI = 0.18–0.77, $P = 0.0036$, $P_c = 0.029$), representing a putative protective factor against knee OA. When sexes were analysed as subgroups, the trends were the same: the significance of the association reduced in males ($P = 0.051$), although the association with females remained significant ($P = 0.014$).

To verify these associations in a different population, we duplicated the analyses in a knee OA patient cohort from London. The putative *risk* haplotype in the *IL1A–IL1B–IL1RN* region (2C-CTG-1TT) was also significantly increased in this OA cohort (OR = 2.32, CI = 1.05–5.08, $P = 0.02$; Table 3). When sexes were analysed separately, the association remained significant (males, $P = 0.00036$; females, $P = 0.038$). A duplicate analysis of the *IL1B–IL1RN* region in the London OA cohort showed that the frequency of the putative *protective* haplotype was also significantly reduced (OR = 0.19, CI = 0.09–0.40, $P = 0.0000008$; Table 4). As observed with the Bristol cohort, the statistical significance was lost in males, but was still highly significant in females ($P = 0.0000008$), although the numbers ($n = 38$) of males in the London cohort was low.

Discussion

IL-1 ligand–receptor signalling is a highly sensitive immune process, where even small changes in gene expression may result in widespread changes to the cell's environment. Indeed, fewer than 10 ligand-occupied IL-1 receptors are sufficient to transduce a strong intracellular signal.[2] IL-1 is a highly proinflammatory cytokine and

Table 1 Specifications and assay conditions for polymorphisms in the *IL1R1, IL1A, IL1B* and *IL1RN* genes

Gene, locus and polymorphism	*Allele frequencies (controls)*	*UCSC reference positions**	*Reference (GenBank No)*	*PCR primers (5′–3′)*	*Ta*	*Assay method or reference*
IL1R1 -227 A>G	A = 0.43; G = 0.57	*chr2:102342131-102342243 (102342188)*	This paper (AF366362)	F = TTGCTCTAAGGATTGGAGGTA R = GGCTGAATCCAGCTCATTATC	57	IHG (Ref. 34)
IL1R1 -216 C>T	T = 0.995; C = 0.005	*chr2:102342107-102342338 (102342200)*	This paper (AF420463)	F = TTGCCTGGGCTGACTCTAGG R = AACACCAACACCCGCCACTGCA	61***	
IL1R1 -167 G>C	G = 0.91; C = 0.09	*chr2:102342107-102342338 (102342249)*	This paper (AF366363)	F = TTGCCTGGGCTGACTCTAGG R = AACACCAACACCCGCCACTGCA	61***	
IL1R1 Exon 1A +102/103 haplotype	1 = 0.58; 2 = 0.31; 3 = 0.11	*chr2:102342450-102342706 (102342516)*	This paper (AF366364)	F = GCTGTGACACAGCCATCTCC R = TTTGGGAATGGCTGGCCTCC	59	RFLP: *Fok*I and *Hha*I
IL1R1 IVS1A +463-464 insT	T = 0.78; delT = 0.22	*chr2:102343114-102343451 (102343194)*	This paper (AF420464)	F = CTGTTGTTACTGTAAGAATTGAG R = GAGATCGAGACCATCCTG	54	RFLP: *Hinc*II
IL1R1 IVS1A +639-651 del	del = 0.005	*chr2:102343114-102343451 (102343371-83)*	This paper (AF420465)	F = CTGTTGTTACTGTAAGAATTGAG R = GAGATCGAGACCATCCTG	54***	
IL1R1 IVS1A +783 C>T	C = 0.43; T = 0.57	*chr2:102343422-102343709 (102343515)*	This paper (AF469754)	F = CACTGTGTTAGCCAGGATG R = GTCTTCTCTATAGCTATAGGTG	54	RFLP: *Nla*III
IL1R1 IVS1A +3351-3371 complex microsatellite	1 = 0.58; 2 = 0.26: 3 = 0.16	*chr2:102345861-102346169 (Allele 1: see Figure 2)*	This paper (AF469755)	F = CTAGCCACCTCACAAGATT	54	PAGE (see text)
		chr2:102345861-102346169 (Allele 3: see Figure 2)	This paper (AF469756)	R = CACACTAAACACTGCTACTG		
IL1R1 IVS1A +35884 G>A	G = 0.73; A = 0.27	*chr2:102378498-102378739 (102378616)*	Ref 28	F = GAGCAGATAGGGATACAGGG R = ACCCGCCCAACACAGAAATG	57	RFLP: *Hinf*I
IL1R1 IVS1A +36995 C>T	C = 0.69; T = 0.31	*chr2:102379635-102379984 (102379727)*	Ref 27	F = TTGGAGGATGGCCCATGAACC R = CTGTTACGCGCCCGGATGAAAAA	60	RFLP: *Pst*I
IL1R1 IVS1B +52 A>G	G = 0.90; A = 0.10	*chr2:102380227-102380481 (102380367)*	Ref 29	F = TCGGGATCTGATGCCCTGGAG R = GAGCAGGCGCGGCTGGACAG	63	RFLP: *Msp*I
IL1R1 IVS1C +1760 T>C	T = 0.60; C = 0.40	*chr2:102382536-102382771 (102382640)*	Ref 28	F = CATAGGATGTCAGCAGTCTCTC R = ACCTCCTAGAGCTCCACATC	57	RFLP: *Alu*I
IL1A IVS4 (TTA)n	1 = 0.70; 2 = 0.30	*chr2:113633425-113633556 (113633473)*	Ref 35	F = GTCTCCATCTCCTCGTGAT R = TTGCTGGTAGTATTCATATAGG	53	PAGE (see text)
IL1A –889 C>T	C = 0.71; T = 0.29	*chr2:113638054-113638157 (113638106)*	Ref 36	F = ACAGGAATTATAAAAGCTGAG R = ATGGAGATTGGGAGAAAGGAA	54	IHG
IL1B Exon 5 +14 C>T ("+3954 C>T")**	C = 0.80; T = 0.20	*chr2:113685471-113685601 (113685536)*	Ref 37	F = GAGGCCTGCCCTTCTGATT R = CGGAGCGTGCAGTTCAGT	56	IHG (Ref 34)

IL1B -31 C>T ("1903 C>T")	C = 0.34; T = 0.66	*chr2:113689485-113689573 (113689533)*	Ref 38	F = CAGAGAAATTTCTCAGCCTCC R = GCCTCGAAGAGGTTTGGTATC	57	IHG (Ref 34)
IL1B –511 G>A****	A = 0.35; G = 0.65	*chr2:113689960-113690054 (113690013)*	Ref 39	F = AATTTTCTCCTCAGAGGCTCC R = CCCTGTCTGTATTGAGGGTGT	58	IHG (Ref 34)
IL1RN IVS2 86 bp VNTR	1 = 0.646; 2 = 0.343; 3 = 0.008; 4 = 0.002	*chr2:113983218-113983629 (113983252-595)*	Ref 40	F = CTCAGCAACACTCCTAT R = TCCTGGTCTGCAGGTAA	51	PAGE (see text)
IL1RN IVS3 +59 T>C ("8006 T>C")	C = 0.30; T = 0.70	*chr2:113982302-113982403 (113982353)*	Ref 38	F = CTGGGATGTTAACCAGAAGAC R = CCTGGCAACCACTCACCTT	57	IHG (Ref 34)
IL1RN Exon 4 +72 T>C ("11100 T>C")	C = 0.24; T = 0.76	*chr2:113985400-113985509 (113985450)*	Ref 38	F = AGCGAGAACAGAAAGCAGGAC R = ATCGCTGTGCAGAGGAACCAA	58	IHG (Ref 34)

*Base range, UCSC July 2003 assembly (http://genome.cse.ucsc.edu/), representing amplicon location (polymorphic nucleotide position shown in parentheses). **Alternative name shown in parentheses. ***Polymorphisms identified but not included in the genotype or haplotype analyses. *Ta*, annealing temperature in PCR (°C). ****Equivalent to rs16944 G > A; polymorphism is C > T on IL1B coding strand.

the levels of signalling are greatly increased in many disorders, including autoimmune diseases. Recent work on IL-1-associated disorders has led researchers to search for predisposing loci within the highly polymorphic *IL1R1–IL1A–IL1B–IL1RN* gene cluster that may help to pinpoint genetic markers of disease susceptibility, severity or outcome. Impetus for examination of these loci has been further provided by results of genome-wide scans for susceptibility loci.[16,17] Much of the research to date has focused on individual polymorphisms or small numbers of polymorphisms in single genes. However, this approach may be oversimplistic due to the complex interaction of the ligands and receptors involved in the transduction and inhibition of IL-1 signalling, and because of linkage disequilibrium within the genes encoding the ligands and receptors. Since the immunopathology of osteoarthritis includes induction of high levels of IL-1 in the synovial fluid, we hypothesised that haplotype or extended haplotype analysis across a region encompassing the *IL1R1*, *IL1A*, *IL1B* and *IL1RN* genes might indicate haplotypic markers of disease susceptibility or severity which individual genotypic markers may fail to reveal.

From analysis of 17 polymorphic loci, we identified 95 extended *IL1R1–IL1A–IL1B–IL1RN* haplotypes. These data also revealed that strong LD exists between polymorphic loci in the *IL1R1* promoter and in the *IL1A–IL1B–IL1RN* complex, but that between these two regions, considerably less LD exists. Allelic or haplotypic polymorphism within the *IL1R1* promoter might involve alteration in transcription factor binding sites and thus exert a regulatory influence on receptor expression, thereby contributing to the aetiology or immunopathology of knee osteoarthritis. However, we found no association between promoter haplotypes in the *IL1R1* gene and knee osteoarthritis. TRANSFAC 6.0 analysis (http://www.gene-regulation.com/pub/databases.html#transfac) of the *IL1R1* promoter polymorphism at position −227 upstream of exon 1A, revealed a potential NF-κB binding site motif. We tested the potential functional significance of this polymorphism using two reporter gene assays. However, no differences were observed in expression or response to IL-1 induction in −227G and −227A clones, either in a pGL3-SV40 luciferase reporter system, or in a lentiviral-GFP vector.

In contrast to the *IL1R1* results, we identified an extended *IL1A–IL1B–IL1RN risk* haplotype (2C-CTG-1TT), which was significantly more frequent in patients with knee osteoarthritis. In an analysis of *IL1B–IL1RN* region haplotypes, one of these (CCA-1TT) was associated with a significant protective effect against knee osteoarthritis. In order to reconcile our extended haplotype approach with potential functional differences, we examined individual polymorphisms within the haplotypes which have been previously associated with regulation of expression. The *IL1B–IL1RN* CCA-1TT protective haplotype differed from the *risk* haplotype in the genotypes at positions −31 and −511 in the *IL1B* gene. These two polymorphisms are in significant LD (see Figure 3). The *IL1B* −31T allele creates a TATA box, which has been shown to bind TATA–binding protein (TBP),[41] whereas the *IL1B* −31C allele does not. It has been suggested that the strongly linked *IL1B* −31T and the *IL1B* −511G alleles may be associated with higher

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                                chr2:102346067      102346081           102346099  102346108
Allele   GenBank      AFL          ↓                   ↓                   ↓          ↓
  2      AC007271     309     GTGTGTGTGTGTGTGT--ATGTATGTGTGTGTGTGT--GGGGGGGGGTGTG…
  1      AF469755     306     GTGTGTGTGTGTGTGT--ATGTATGTGTGTGTGT----GGGGGGGG-TGTG…
  3      AF469756     313     GTGTGTGTGTGTGTGTGTATGTATGTGTGTGTGTGTGTGGGGGGG--TGTG…
                              <---------------->       <------------><-------->
                                 (GT) repeat             (GT) repeat  (G) repeat
                                   region 1                region 2      region
```

Figure 2 Nucleotide sequence alignment of alleles within the complex microsatellite region of *IL1R1* IVS1A. Alleles were designated numerically according to amplified fragment length (AFL) in base pairs: for details of the PCR primers and conditions used see Table 1 and text. Polymorphisms with respect to the reference allele 2 (GenBank AC007271) are: allele 1 = 102346097-8 del GT, 102346108 del G and allele 3 = 102346081 insGT, 102346099 insGT, 102346107-8 del GG. Chromosome 2 locations (top line) correspond to the UCSC July 2003 assembly (http://genome.cse.ucsc.edu/). Allele frequencies are shown in Table 1.

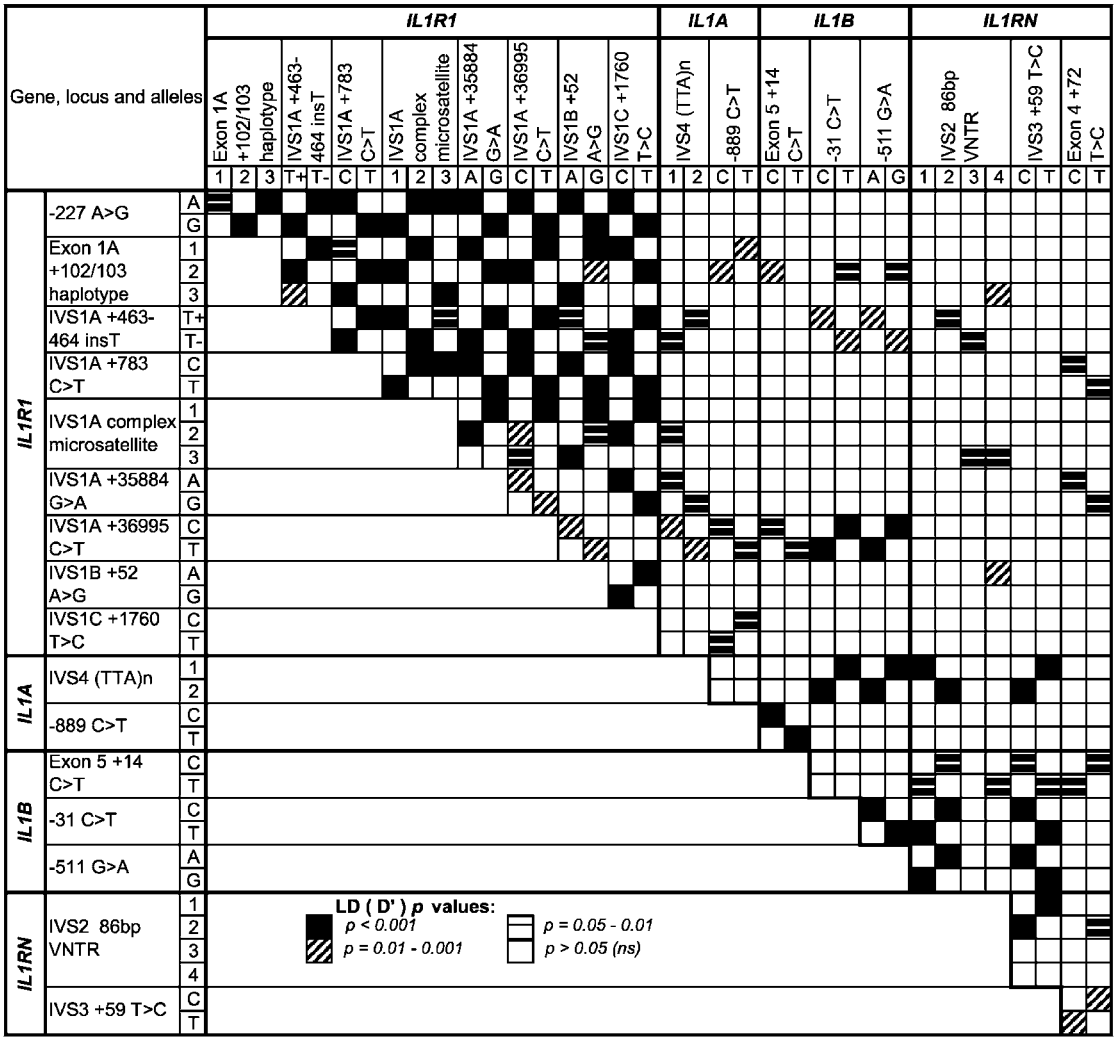

Figure 3 Linkage disequilibrium analysis of polymorphisms in the *IL1R1, IL1A, IL1B* and *IL1RN* genes. *P*-values of the Lewontin's |*D*'| coefficient for each two-way comparison are as shown in the key; ns, not significant.

levels of gene expression than the *IL1B* −31C and *IL1B* −511A alleles:[41,42] we are currently investigating this possibility. This could represent a partial functional explanation for the association of the risk haplotype (2C-CTG-1TT) with knee OA, since this haplotype contains the *IL1B* −31T and *IL1B* −511G genotypes, is TATA-box positive, and may be associated with high-level expression of IL-1β (a potent mediator of inflammation in OA joints). In contrast, the protective haplotype (CCA-1TT) contains the *IL1B* −31C genotype, is TATA-box negative, and is associated with lower levels of IL-1β production.

The importance of examining extended haplotypes of the IL-1 gene cluster is made clear by this study. We identified no association of the individual *IL1B* polymorphisms with osteoarthritis, but statistically significant associations when haplotypic analysis was conducted. The level of IL-1Ra expression will undoubt-

Table 2 *IL1R1* haplotype analysis of controls and OA patients

IL1R1 promoter haplotype	*Controls*	*Bristol knee OA*	*P*	*London knee OA*	*P*
G21T1GCGT	114	96	1.000 (Ref)	98	1.000 (Ref)
A12C2ACGC	75	48	0.234	56	0.529
G11T1GTGT	50	42	0.992	45	0.853
A31C3GCAT	35	22	0.337	19	0.144
G11T1GTGC	29	18	0.535	13	0.068
Others	87	56	0.222	85	0.533
Total	390	282		316	

Haplotype nomenclature (left to right) refers to alleles at each of the nine *IL1R1* loci listed in Table 1, in the following order: -227 A>G, Exon 1A +102/103 haplotype, IVS1A +463-464 insT, IVS1A +783 C>T, IVS1A +3351-3371 complex microsatellite, IVS1A +35884 G>A, IVS1A +36995 C>T, IVS1B +52 A>G, IVS1C +1760 T>C. Numbers represent the total number of haplotypes observed. Ref, reference haplotype (see text). *P*-values are not corrected for multiple comparisons.

Table 3 *IL1A-IL1B-IL1RN* haplotype analysis of controls and OA patients

IL1A-B-RN Haplotype	*Controls*	*Bristol knee OA*	*P*	*London knee OA*	*P*
1C-CTG-1TT	53	21	1.000 (Ref)	38	1.000 (Ref)
1T-TTG-1TT	38	25	0.163	33	0.549
2C-CCA-2CT	38	25	0.163	18	0.234
1C-CTG-2CT	31	27	0.031	27	0.565
1C-CTG-1TC	27	25	0.024	31	0.163
1C-CCA-1TT	24	17	0.153	5	0.016
2C-CCA-1TT	24	6	0.377	15	0.726
1T-TTG-1TC	22	9	0.964	26	0.163
2C-CTG-1TT	18	28	0.00043(a)	30	0.02(b)
Others	155	99	0.007	103	0.377
Total	390	282		326	

Haplotype nomenclature (left to right) refers to alleles at each of the eight *IL1A-IL1B-IL1RN* loci listed in Table 1, in the following order: *IL1A* IVS4 (TTA)n, *IL1A* -889 C>T, *IL1B* Exon 5 +14 C>T, *IL1B* -31 C>T, *IL1B* -511 G>A, *IL1RN* IVS2 86 bp VNTR, *IL1RN* IVS3 +59 T>C, *IL1RN* Exon 4 +72 T>C. Numbers represent the total number of haplotypes observed. Ref, reference haplotype. (a) OR = 3.93; CI = 1.68–9.25; (b) OR = 2.32; CI = 1.05–5.08. *P*-values shown are not corrected for multiple comparisons: see text for corrected values

Table 4 *IL1B-IL1RN* haplotype analysis of controls and OA patients

IL1B-RN Haplotype	Controls	*Bristol knee OA*	*P*	*London knee OA*	*P*
CTG-1TT	80	58	1.000 (Ref)	96	1.000 (Ref)
CCA-1TT	**52**	**14**	**0.0036(a)**	**12**	**0.0000008(b)**
CTG-1TC	48	41	0.549	27	0.007
TTG-1TT	44	36	0.669	36	0.157
CTG-2CT	35	28	0.748	32	0.344
TTG-1TC	26	12	0.244	31	0.983
Others	105	49	0.094	54	0.858
Total	**390**	**282**		**326**	

Haplotype nomenclature (left to right) refers to alleles at each of the six *IL1B-IL1RN* loci listed in Table 1, in the following order: *IL1B* Exon 5 +14 C>T, *IL1B* -31 C>T, *IL1B* -511 G>A, *IL1RN* IVS2 86 bp VNTR, *IL1RN* IVS3 +59 T>C, *IL1RN* Exon 4 +72 T>C. Numbers represent the total number of haplotypes observed. Ref, reference haplotype. (a) OR = 0.37; CI = 0.18–0.77; (b) OR = 0.19; CI = 0.09–0.40. *P*-values are not corrected for multiple comparisons: see text for corrected values.

edly have a major impact on a cell's responsiveness to IL-1α and IL-1β. Both the identified *risk* and *protective* extended haplotypes possess the *IL1RN* '1TT' haplotype, which includes the VNTR allele 1, implicated to express IL-1Ra at lower levels than VNTR allele 2.[43] It is believed, however, that IL-1Ra expression is also influenced by *IL1B* genotype: previous research has identified association between the *IL1B* −511, *IL1B* exon 5 +14 and *IL1RN* VNTR genotypes and their combined influence on the level of IL-1Ra plasma levels.[33] One logical explanation for our findings is that in the *risk* haplotype population, the *IL1A* and *IL1B* alleles may not only lead to high IL-1β

production, but may also confer a lower level of IL-1Ra expression: for the *protective* haplotype, the opposite might be the case. This might explain the observation in some individuals, of both higher levels of IL-1β found in OA synovium, and the increased susceptibility of OA cartilage to IL-1β, due to the concurrent reduction of IL-1Ra expression. Our results indicate potential *IL1A–IL1B–IL1RN* extended haplotypes associated with susceptibility to OA. This could help to explain previous reports of linkage from some groups between markers in this region and OA in genome-wide scans.[16,17] However, it should be stressed that the disease is a multigenic disorder and our data identifies only one of the susceptibility loci involved, each of which represent an as yet unknown proportion of the overall genetic influence.

Methods

Patients and controls

Osteoarthritis patients ($n = 304$) were studied from two UK centres: Bristol ($n = 141$, 65 male and 76 female Caucasians: mean age = 62; median age = 63); and London ($n = 163$, 38 male and 125 female Caucasians: mean age = 71; median age = 71). All patients had osteoarthritis as defined by the ACR criteria with at least grade 2 radiological change. Control subjects were 195 (97 male and 98 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 procedure.[44]

Identification of novel polymorphisms in the *IL1R1* exon 1A region

PCR-SSCP analysis[45] was used to determine the extent of nucleotide sequence polymorphism around the first untranslated exon of the human *IL1R1* exon, using a large series of overlapping PCR primers derived from a published *IL1R1* sequence (GenBank AC007271), designed to produce amplicons of approximately 250 bp, overlapping each other by 20–50 bp. DNAs from 195 healthy controls (see above) were screened for polymorphism, within the region 2 kb upstream to 4 kb downstream of the exon 1A transcription start site (Figure 1c). PCR conditions were essentially as described below. Following amplification, PCR-SSCP analysis was performed using polyacrylamide gel electrophoresis (PAGE) as described[45] in 12% nondenaturing polyacrylamide gels (Protogel, National Diagnostics, Atlanta, GA, USA) containing 1 × TBE buffer. Electrophoresis was carried out at 300 V for 140 min on a triple-wide minigel system (CBS Scientific Company Inc., Del Mar, USA). DNA was visualised by staining for 10 min with ethidium bromide (0.5 μg/ml in 1 × TBE buffer) and gels were imaged using a Kodak EDAS 120 digital camera. Nucleotide sequencing of SSCP variants was performed by automated nucleotide sequencing on an ABI 310 sequencer (Perkin-Elmer, Norwalk, USA).

SNP and VNTR genotyping

For each polymorphic locus to be analysed, PCR amplifications were set up in a total volume of 20 μl containing 200 ng genomic DNA, 0.1 U *Taq* polymerase (Abgene, Epsom, Surrey, UK), 0.05 μM of each PCR primer (Table 1), 2 μl of 10 × reaction buffer (160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl (pH 8.8 at 25°C), 15 mM $MgCl_2$, 0.1% v/v Tween-20 (Ameresco Chemicals, Anachem, Luton, UK)), 0.8 μl 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 temp (see Table 1) (40 s) and 72°C (30 s); with a final extension for 5 min at 72°C.

RFLP analysis was carried out for the *IL1R1* SNPs where appropriate (Table 1). The restriction endonucleases, *Msp*I, *Pst*I, *Hinf*I, *Hinc*II, *Alu*I, *Fok*I, *Nla*III and *Hha*I (New England Biolabs Inc., MD, USA), were used according to the manufacturer's instructions. Briefly, 4 U of enzyme were used per 10 μl amplified DNA, and incubated at 37°C for 6 h. Following digestion, DNA samples were electrophoresed in 15% nondenaturing polyacrylamide gels for 60 min and imaged on a UV transilluminator using a Kodak EDAS 120 digital camera. Analysis of repeat sequences was carried out by PAGE using 12% Accugel (National Diagnostics, Atlanta, GA, USA) for 50 min (*IL1RN* IVS2 86 bp VNTR, *IL1A* IVS4 TTA repeat, and 100 min (*IL1R1* IVS1A complex microsatellite) (see Table 1). For the *IL1R1* –227 A > G SNP, induced heteroduplex generator (IHG) analysis was used, incorporating an [AAAA] insertion adjacent to the polymorphic site.[34] The same method was used to identify the polymorphisms *IL1A* –889 C > T, *IL1B* Exon 5 +14 C > T, *IL1B* –31 C > T, *IL1B* –511 G > A, *IL1RN* IVS3 +59 T > C and *IL1RN* Exon 4 +72 T > C (Table 1).

Linkage disequilibrium, haplotype frequency 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*. For the control population Arlequin version 2.000[46] was used to determine pairwise linkage disequilibrium (LD), expressed as Lewontin's $|D'|$ coefficient—statistical significance of the *P*-value was defined at the 5% level.

Haplotypes were constructed from genotypic data using the PHASE 2.0 software platform.[47,48] Haplotypes with an observed frequency of less than 0.05 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). For all analyses, the reference haplotype was that which occurred most frequently.[49] 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.

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