Concise Report

Linkage and association studies of discoidin domain receptor 1 (DDR1) single nucleotide polymorphisms (SNPs) in juvenile oligoarthritis


Objectives. Multiple independent juvenile oligoarthritis susceptibility loci have been identified within the major histocompatibility complex (MHC), including HLA-A, HLA-DRB1 and an as yet unlocalized effect in the centromeric class I region. The discoidin domain receptor 1 (DDR1) gene resides within this region and codes for a receptor tyrosine kinase that plays an important role in regulating cell attachment to collagen, chemotaxis, proliferation and matrix metalloproteinase (MMP) production. DDR1 expression in chondrocytes has not been investigated. The objectives of this study were to investigate expression of DDR1 in healthy chondrocytes and to identify linkage and association of this candidate gene with juvenile oligoarthritis.

Methods. A set of 135 simplex juvenile idiopathic arthritis families consisting of one affected child and healthy parent(s) and 199 healthy unrelated individuals were genotyped for six single nucleotide polymorphisms (SNPs) within the DDR1 gene using the primer extension SNaPshot™ method. Single-point and multipoint transmission disequilibrium tests were carried out with the ETDT and TDTPHASE packages. Allele frequency comparisons between cases and controls were carried out with the χ² test. DDR1 expression was investigated in normal articular cartilage by RT-PCR and immunofluorescence methods.

Results. No linkage and association with any of the six SNPs or their haplotypic combinations were observed in the families studied. No significant differences were observed in allele frequencies between patients and controls. DDR1 expression was found in normal articular cartilage by RT-PCR and by immunofluorescence.

Conclusions. The DDR1 SNPs examined are not involved in susceptibility to juvenile oligoarthritis.

Key words: Juvenile oligoarthritis, DDR1, MHC, Linkage, Association, SNP, Haplotype.
this study was based on the chromosomal localization of the DDR1 gene and its putative role in the aetiology of arthritis. The aims of this study were, therefore, to determine the expression of DDR1 in articular tissue and to examine linkage and association of polymorphisms within the DDR1 locus in juvenile oligoarthritis.

Patients and methods

Patients for single nucleotide polymorphism (SNP) analysis

The ARC Epidemiology Unit holds the British Paediatric Rheumatology Group (BPRG) National JIA Repository. This is composed of a collection of samples from UK patients and available parents, as recruited through the BPRG, with the aid of 17 contributory centres. Patients have been classified according to the ILAR (International League of Associations for Rheumatology) criteria [2] and clinical details of individual cases have been collected. One hundred and thirty-five UK Caucasian nuclear families, consisting of an offspring affected with juvenile oligoarthritis (both persistent and extended) and healthy parent(s), were available for study. The sample group consisted of 93 two-parent and 42 one-parent families. DNA from a set of 199 healthy, unrelated and ethnically matched control individuals was additionally available for study. Ethics committee approval was obtained for the study [North-West Multi-Centre Research Ethics Committee (MREC 99/8/84) and the University of Manchester Committee on the Ethics of Research on Human Beings].

SNP genotyping

Six SNPs spanning the DDR1 gene were selected from public databases. The 1597 (5' UTR), 9274 (exon 12, silent mutation), 9795 (intron 12), 10411 (intron 13), 11746 (3' UTR) and 11832 SNPs (3' UTR) (all SNP positions are relative to the first nucleotide in sequence of accession number U48705) were each typed using the SNaPshot™ (PE Biosystems, Foster City, CA, USA) ddNTP primer extension method and capillary electrophoresis. The primer sequences and PCR conditions used to amplify the DDR1 SNPs, as well as the probe sequences used for their identification, are available upon request. Briefly, for each SNP studied, a total of 10 ng genomic DNA was amplified in a 10 μl final PCR reaction volume containing 2.5 pmol of each primer, 1.0 nmol of dNTPs, 15 nmoles of MgCl2, 2.5 μl of deionized formamide and electrophoresed on a Tetrads thermal cycler (MJ Research, Waltham, MA, USA). The PCR product was incubated with 1 unit each of shrimp alkaline phosphatase (Amersham, Amersham, UK) and ExoI (NE Biolabs, Beverly, MA, USA) at 37 °C (1 h), 72 °C (15 min). The extension reactions each used 1 μl of PCR template and 0.25 pmol of probe. Twenty-five cycles of the extension reaction were carried out, each cycle consisting of 96 °C (10 s), 50 °C (5 s) and 60 °C (30 s). Six microlitres of this extension product was incubated with 1 U calf intestine alkaline phosphatase (Amersham). This product (1.2 μl) was pooled with 5 μl of deionized formamide and electrophoresed on a 3100 ABI analyser. The results were analysed by two independent individuals, using Genescan analysis and Genotyper 3.6 software (3100 ABI Analyser, Genescan and Genotyper 3.6 all from ABI, Foster City, CA, USA).

Isolation of chondrocyte mRNA

Normal tissue from adult knee cartilage was obtained from anatomical donations from accident victims 23-47 yr of age (NDRI, Philadelphia, PA, USA). Tissue was obtained and used following institutional review board-approved protocols (Harvard Medical School). Chondrocytes were isolated from the articular cartilage of tibial plateaus and femoral condyles of tissue procured within 6 h of death and processed as previously described [14] and total RNA was extracted using Tri-Reagent (Sigma, St Louis, MO, USA).

RT-PCR of DDR1 from normal cartilage

cDNA was prepared and subsequently PCR for DDR1 was performed using the following primers: forward, 5' GGACATA CGTGCGCGGACT 3' (exon 5/6); reverse, 5' CCTAGGTTGGTGGCCGATGG 3' (exon 7). The cycling conditions were as follows: 94 °C for 2 min, 30 cycles of 94 °C for 15 s, 62 °C for 30 s, 68 °C for 1 min and finally 68 °C for 5 min.

DDR1 immunofluorescence

Articular cartilage was fixed in 4% paraformaldehyde and embedded in paraffin for sectioning using standard procedures. Primary antibodies raised against DDR1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Immunofluorescence was carried out according to standard protocols.

Statistical analysis

Deviation from Hardy-Weinberg equilibrium was tested with the HelixTree® (Golden Helix, Montana, MT, USA) software package. DDR1 SNPs were tested for deviation from random segregation with the extended transmission disequilibrium test (ETDT) [15], which detects linkage in the presence of association. Empirical P values were calculated by running 10 000 Monte Carlo simulations. Haplotypes were inferred through the expectation-maximization (EM) algorithm. TDTPHASE was used to investigate the transmission of multipoint haplotypes [16]. The HelixTree® software package was used to determine pairwise linkage disequilibrium patterns among the SNPs. Allele frequency comparisons between cases and controls were carried out with the χ2 test. Ordering the SNP haplotypes according to their frequency led to the identification of the haplotype-tagging SNPs (htSNPs) for DDR1 [17]. The polymorphic positions that could capture at least 85% of the observed haplotypes were selected as the htSNPs for the locus.

Results

DDR1 SNP analysis reveals no association with juvenile oligoarthritis

The 11832 DDR1 SNP was found to be monomorphic in the UK Caucasian population studied. The 9795 polymorphism deviated significantly from Hardy-Weinberg equilibrium in the patient and control groups (P < 0.05) despite repeat genotyping and confirmation by sequencing and was, therefore, omitted from further analysis. No deviation from Hardy-Weinberg equilibrium was seen for any of the other DDR1 SNPs studied. When deviation from random segregation was examined with the single-point ETDT, none of the DDR1 SNPs were found to be linked and associated with juvenile oligoarthritis (Table 1). The ARC Epidemiology Unit holds the British Paediatric Rheumatology Group National JIA Repository.
markers. HiSNPs were determined for the patient and control haplotype groups separately. In juvenile oligoarthritis, by only considering the 1597 and 10411 DDR1 SNPs, 92.3% of all haplotypes could be successfully captured. In the control group, typing for the 1597 and 9274 DDR1 SNPs could account for 87.3% of all haplotypes. The characterized htSNPs can help by increasing cost-effectiveness in further genetic studies focusing on DDR1 in the Caucasian population.

DDR1 is expressed within articular tissues

Previously, only DDR2 (chromosomal location 1q12-qter) has been detected in articular tissues [18]. In the present study we examined normal articular cartilage to investigate the expression of DDR1 in a non-diseased state, due to the lack of juvenile oligoarthritis tissue samples. Immunofluorescence analysis of normal articular cartilage demonstrated DDR1 expression in the majority of chondrocytes in all samples examined (n = 3). Figure 1A shows the discreet DDR1 immunofluorescence over the normal chondrocytes. This was abolished when the tissue is pretreated with anti-DDR1 antibody (Fig. 1B). In conjunction with the immunofluorescence analysis, we also carried out RT-PCR analysis of DDR1 expression in cells harvested from normal cartilage (Fig. 1C). This analysis confirmed the findings obtained by immunofluorescence.

Discussion

An independent juvenile oligoarthritis susceptibility locus has been localized to the MHC class I region, near microsatellite marker D6S265 [6, 7]. We have focused on the DDR1 gene as it localizes within the implicated interval and codes for a receptor tyrosine kinase that plays an important role in regulating cell attachment to collagen, chemotaxis, proliferation and MMP production. These functions, previously attributed to this receptor in other systems, make it a promising candidate as a mediator in the pathogenesis of juvenile oligoarthritis. Our observation that this receptor is expressed in articular tissues strengthens the possibility that this receptor may play a role in the pathogenesis of JIA. The expression of DDR1 in chondrocytes was not uniform. This may represent the existence of a distinct subpopulation of chondrocytes or differing states within a single population. Preliminary work on rheumatoid arthritis cartilage, in our laboratories, shows that chondrocyte expression of DDR1 is maintained in a diseased state. DDR1 has been documented to play a role in regulation of MMP production [19]. Therefore, DDR1 is a possible candidate for modifying MMP production in articular tissues and for influencing disease progression.

Validated polymorphisms were selected to span the DDR1 gene at a median spacing of 616 bp (average spacing 2 kb), reflecting a SNP map with density equivalent to that of recent high-resolution mapping studies [20]. Family-based linkage and association analyses were initially carried out due to their well-recognized low false positive generation rate. Subsequently, a population-based association study was performed in order to compensate for any loss of power incurred. The observation of no linkage or association with both approaches instills confidence in the results and suggests that they are true negatives. SNP screening of the DDR1 gene was not exhaustive in this study. Therefore, undetected associations of untested markers may exist with juvenile oligoarthritis. However, given the high levels of linkage disequilibrium in the region and the dense spacing of the SNPs examined, involvement of DDR1 in susceptibility to the disease appears to be unlikely. Our study has additionally characterized...
htSNPs within the DDR1 gene. Since not all genetic variation at the locus has been investigated, these htSNPs tag haplotypes of the SNPs examined in this work and can be used in further studies focusing on the Caucasian population. The linkage and association analyses indicate, however, that DDR1 is not implicated in the aetiopathogenesis of juvenile oligoarthritis, although it remains an attractive candidate gene for studies into other arthritic diseases.

**Key messages**

- The DDR1 SNPs studied are not linked or associated with juvenile oligoarthritis.
- DDR1 is expressed in normal articular cartilage.

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**References**