EXTENDED REPORT

A polymorphic variant inside the osteopontin gene shows association with disease course in oligoarticular juvenile idiopathic arthritis

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Background: Oligoarticular onset juvenile idiopathic arthritis (JIA) has a variable disease course. In some patients the disease remains confined to a few joints (persistent oligoarticular) while in others it extends to affect more joints (oligoarticular extended). Osteopontin is thought to play a role in the pathogenesis.
Objective: To investigate whether a polymorphic variant in the human osteopontin gene, which is in linkage disequilibrium with recently characterised promoter variants, is associated with the disease course in oligoarticular JIA.
Methods: Genotyping of the two base pair insertion/deletion variant at +245 in the first intron was undertaken by polymerase chain reaction (PCR) amplification of DNA fragments, using a fluorescently labelled primer, followed by allele detection after rapid separation of PCR products on an automated DNA sequencer.
Results: Allele 2 of the polymorphic variant in the osteopontin first intron was significantly associated with the persistent oligoarticular form rather than the extended form of JIA. This was verified at the level of genotype and allele frequencies.
Conclusions: The results suggest that osteopontin gene polymorphism is associated with the disease course in oligoarticular JIA and might therefore represent a useful genetic marker to characterise patients with oligoarticular JIA who are at risk of a worse outcome.

Osteopontin, also named Eta-I or SPP1, is a phosphorylated glycoprotein expressed by different cell types, including osteoblasts, osteoclasts, chondrocytes, smooth muscle cells, epithelial cells, macrophages, and activated T lymphocytes.[1 2] The SPP1 gene is mapped on chromosome 4q21–q25. Functionally, osteopontin interacts with a variety of cell surface receptors. Its binding to these receptors stimulates cell adhesion, migration, and specific signalling functions. Osteopontin has a major role in bone resorption, angiogenesis, and tumorigenesis and has been shown to mediate various inflammatory mechanisms including modulation of macrophage adhesion, migration and phagocytosis, and stimulation of Th1 orientation of adaptive immunity. In view of its critical role in various immunological responses, osteopontin has recently been proposed as a new pro-inflammatory cytokine.

Several studies have suggested an important role for osteopontin in the pathogenesis of inflammatory arthritis. In murine collagen induced arthritis, osteopontin was detected in synovial tissues and at the sites of osteoclast mediated bone resorption.[3] In collagen antibody induced arthritis it has been shown that osteopontin deficient mice have marked attenuation of joint swelling and articular cartilage destruction compared with arthritis in wild type mice.[4] In humans, osteopontin mRNA and protein have been found to be overexpressed in the synovial lining layer, the sublining layer, and the cartilage–pannus junction in patients with rheumatoid arthritis and juvenile idiopathic arthritis (JIA). Moreover, in both conditions, a significantly increased expression of osteopontin has been found in synovial fluid with respect to paired plasma samples.[5–7]

JIA is a heterogeneous condition that comprises all forms of arthritis of unknown origin, persisting for more than six weeks, and with onset before 16 years of age. It is currently classified into different types according to symptoms at onset.[8] Oligoarticular onset JIA is considered the most homogeneous and well defined form.[9] This form, which is typical of childhood and is not observed in adults, is characterised by asymmetrical arthritis, early onset (usually before six years of age), female predilection, a high frequency of positive antinuclear antibodies (ANA), and a high risk of developing chronic anterior uveitis. A strong association with some HLA alleles and in particular with DRB1*0801 (DRw8) confirms that these patients represent a homogeneous entity.[10–14] This presumably homogeneous disease entity has, however, a heterogeneous prognosis which varies according to the number of joints involved. The arthritis may remain confined to a few joints (oligoarticular persistent subgroup) or involve more than four joints after the first six months of disease (oligoarticular extended subgroup), a feature that occurs in up to 50% of cases.[15] The factors responsible for disease extension are unknown, and it is conceivable that genetics are involved. Thus the analysis of polymorphisms within candidate genes represents a useful approach to identifying susceptibility genes for JIA disease course.

Several polymorphisms have been described inside the osteopontin gene, some of which are associated with systemic lupus erythematosus, multiple sclerosis, and autoimmune lymphoproliferative syndrome.[16–18] We have recently identified a polymorphic variant, consisting of a two base pair insertion/deletion (ins/del) in the first intron of the human osteopontin gene[19] at +245, and have functionally

Abbreviations: ILAR, International League of Associations for Rheumatology; ins/del, insertion/deletion; JIA, onset juvenile idiopathic arthritis; TDT, transmission disequilibrium test

Table 1 Clinical characteristics of the patients

	Oligoarticular JIA		
	Persistent	Extended	Total No
No of patients	73 (61.3%)	46 (38.7 %)	119
Male	11 (15.1%)	9 (19.6%)	20
Female	62 (84.9%)	37 (80.4%)	99
Iridocyclitis	20 (27.3%)	13 (28%)	33
ANA positive	70 (95.8%)	42 (91.3%)	112
Onset age (years) (mean (median))	3.58 (3.02)	4.06 (3.52)	3.82 (3.4)
Disease duration (years) (mean (median))	3.49 (3.5)	3.79 (3.5)	3.61 (3.5)

Values are n (%).
ANA, antinuclear antibody; JIA, juvenile idiopathic arthritis.

characterised other promoter polymorphisms in strict linkage disequilibrium with it, which affect promoter activity.[20] A recently published case–control study of osteopontin polymorphisms in rheumatoid arthritis failed to detect a significant correlation[21]; however, there has been no previous investigation involving JIA.

In view of the potential role of osteopontin in the progression of chronic arthritis and the presence of intragenic polymorphisms related to the regulation of gene expression, we have investigated the possible association of the intronic ins/del polymorphism in the occurrence and disease course of oligoarticular JIA. Genotyping of this ins/del polymorphism is particularly convenient as it can be rapidly achieved by separation in a capillary DNA sequencer after a simple polymerase chain reaction (PCR).

METHODS

Patient characterisation

We evaluated consecutively 119 patients (20 male, 99 female) with oligoarticular onset JIA according to the ILAR criteria[8] and followed up for at least two years. Seventy three patients had a persistent oligoarticular form (persistent involvement of four or fewer joints) and 46 an extended oligoarticular form (involvement of more than four joints after the first six months of disease). Polyarticular progression among patients with oligoarticular onset JIA most often occurs during the first two years of disease.[15] The main clinical characteristics of the patients are shown in table 1.

All patients were rheumatic factor (RF) negative. Positive antinuclear antibodies (ANA) were found in 94% of the patients examined. Patients were defined as being ANA positive if they had at least two positive results on indirect immunofluorescence at a titre of $\geqslant$1:160.

A peripheral blood sample was collected at a control visit for DNA genotyping after informed consent had been obtained.

Genotyping

All participants were genotyped for a biallelic ins/del variant, with the two alleles differing for a two nucleotide repetition (TG/TGTG), at +245 in the first intron of the osteopontin gene.[19] Briefly, PCR was done on genomic DNA using a specific pair of primers to amplify the intronic fragment, including the polymorphic variant. A 5′ fluorescence labelled sense primer (5′-6FAM-TGGGTTGTGCATTCAGCTG-3′) and four different antisense primers (R1: 5′-TTAGCATCGGT GGTTTCCG-3′; R2: 5′-TTTTGAGGACCCAGTGGAAG-3′; R3: 5′-CCTGCACAGTCACCCACTG-3′; R4: 5′-CAGTGGCATTATTC AGAAAGGG-3′) were used to analyse the repetition variant on an automatic ABI PRISM 3100 DNA sequencer. The use of four different reverse primers allowed us to analyse four samples per electrophoretic run. PCR was carried out in a 15 µl volume containing 10× PCR Gold Buffer (Applied Biosystems, Foster City, California, USA), 200 µmol of each dNTP/l, 1.5 mmol of $MgCl_2$/l, 0.5 U AmpliTaq Gold Hot Start (Applied Biosystems), and 100 ng of genomic DNA. Primer concentrations were 0.2 µmol/l of each primer. The cycling conditions were 94℃ for seven minutes; 10 cycles of 94℃ for 25 seconds, 55℃ for 20 seconds, 72℃ for 30 seconds, followed by 30 cycles of 90℃ for 25 seconds, 55℃ for 20 seconds, 72℃ for 30 seconds, and a final 72℃ for 10 minutes. The PCR products were separated on an ABI Prism 3100 sequencer with 36 cm capillaries, POP6 and gene scan 500 Rox size standard, after appropriate setting up of the run method. The GeneScan analysis program was used to analyse the results of electrophoresis and attribute the genotype. This method was validated by genotyping 16 samples, using both the electrophoretic procedure and DNA sequencing, and no inconsistencies were found. In addition, no alleles other than TG and TGTG were found in a total population of more than 500 individuals.

Statistical analysis

Descriptive statistics of the characteristics of the patients were first reported in terms of mean, median, and standard deviation for the quantitative variables (age at onset, disease duration at enrolment, and so on) and in terms of frequencies for the qualitative variables. Comparison of allelic and genotype frequency was made by χ^2 based methods. Fisher's exact test was used when the expected value of at least one cell is less than 5. The transmission disequilibrium test (TDT) was used to test the hypothesis of linkage disequilibrium between the marker and the disease; exact probabilities were calculated on the basis of the binomial distribution in order to take into account the small number of expected frequencies. All the tests were two sided and a probability (p) value of less than 0.05 was considered significant.

Table 2 Genotypic and allelic frequencies

Intron 1 +245 TG/TGTG	1/1 N	1/2 N	2/2 N	p Value	Allele 1 freq	Allele 2 freq	p Value
Persistent oligoarticular	26	39	8	0.07	0.62	0.38	0.08
Extended oligoarticular	26	16	4		0.74	0.26	

freq, frequency.

Table 3 Presence versus absence of allele 2

Intron 1 +245 TG/TGTG	2/2 or 1/2	1/1 N	p Value	PLR
Persistent oligoarticular	47(64%)	26(36%)	0.033	1.48
Extended oligoarticular	20(43%)	26(57%)		

PLR, positive likelihood ratio.

RESULTS

Osteopontin polymorphisms and oligoarticular JIA

All patients were genotyped for the biallelic ins/del variant at +245 in the first intron, which we found to be in strict linkage disequilibrium with molecular variants in the osteopontin promoter region.[20]

When we compared oligoarticular JIA patients with a group of 200 control individuals with the same geographical origin for differences in genotype and allele frequencies, we found no significant differences (data not shown).

Genotypes were in Hardy–Weinberg equilibrium in all groups tested.

Osteopontin polymorphisms and disease course

We then assessed possible correlations between osteopontin polymorphism and disease course. Patients with a persistent oligoarticular course were compared with those with an extended oligoarticular course. Naming the TG allele of the intronic osteopontin variant as 1 and the TGTG allele as 2, and comparing the persistent oligoarticular pattern of the disease against the extended form, we observed a modification of genotype and allele frequencies between the two groups, although the difference did not reach statistical significance (table 2A).

We then evaluated presence (2/2 homozygotes plus heterozygotes) or absence of allele 2 (1/1 homozygotes) as a parameter to compare the two different JIA subsets. When we compared the two groups of patients, we found that presence of allele 2 was significantly more common (64%) in the persistent oligoarticular group compared with the extended form (43%) ($p = 0.033$) (table 3), suggesting a dominant effect of allele 2, which was not observed when allele 1 was analysed in the same way (not shown).

The association between alleles of the osteopontin intron 1 polymorphism and JIA disease progression was also evaluated by a family based test, the TDT,[22] in which allele transmission from heterozygous parents to affected child overcomes possible problems caused by population differences in allele frequencies. Results of the TDT test in all available and informative small family groups indicated that allele 2 was preferentially non-transmitted in the group of patients with progression to the extended form, while in the persistent oligoarticular subgoup the result of the TDT test was not significant, as shown in table 4.

Table 4 Results of transmission disequilibrium test

	Non-transmitted	Transmitted
Extended form		
+245 allele 2	8	1 $\chi^2 = 5.44$, $p = 0.02$
Persistent form		
+245 allele 2	8	4 $\chi^2 = 1.33$, $p = 0.25$

DISCUSSION

Oligoarticular onset JIA appears to be a relatively homogeneous form of JIA.[8] Nevertheless, despite sharing many common clinical characteristics (early onset, asymmetrical arthritis, high risk of acute anterior uveitis, female predominance, frequent ANA positivity) and HLA gene association (DRw8), JIA patients have a variable disease course. Some show a persistent oligoarticular involvement, while in others the disease extends to affect more than four joints (extended oligoarticular) and is characterised by a worse prognosis.[8] Reasons for these differences in outcome are unknown.

Several previous studies have suggested an important role for osteopontin in the pathogenesis of inflammatory arthritis,[3–6] in particular the study involving JIA.[7] Our previous work on molecular variants located within the osteopontin gene or an area very close to it in the 5′ flanking region[19 20] has provided a rational basis for correlating allele frequencies with pathophysiological processes. Functional characterisation of these polymorphisms showed that different alleles affected the binding of transcription factors and different allelic combinations in haplotypes caused differential promoter activities. Genotyping the TG/TGTG polymorphism in the first intron of the osteopontin gene has two advantages: first, the technique is very convenient and also suitable for large numbers of samples; second, being in linkage disequilibrium with other functional variants, allele frquencies can be related to functional processes.

In the present study, we investigated whether this polymorphism was associated with the occurrence and progression of oliogoarticular JIA. When we evaluated this association in a case–control study, we did not find significant differences in genotype and allele frequencies between oligoarticular JIA cases and control subjects. This was in accordance with recent data on adult patients with rheumatoid arthritis.[21] However, when we evaluated genotype and allele frequencies in subgroups of patients with oligoarticular JIA in relation to disease course, we observed that the presence of allele 2—that is, 2/2 and 2/1 individuals—was significantly associated with the subgroup of patients in whom the arthritis remained confined to a few joints (persistent oligoarticular). Thus the TGTG allele appears to have a dominant protective effect. This was also confirmed by a family based association analysis, the TDT test.

Genetic factors may be involved in multifactorial disorders such as JIA as either disease susceptibility genes or disease modifying genes, depending on their contribution to the initiation of the disease process or the progression and outcome of the disease after its onset. Our results strongly suggest that the association of oligoarticular JIA with a polymorphic variant in the human osteopontin gene reflects an effect on progression of the disease rather than on its aetiology. Our recent work has shown that allele 2 is in strict linkage disequilibrium with alleles of promoter variants which determine low promoter activity,[20] which is consistent with the hypothesis that a genetic setting associated with low osteopontin expression is also correlated with protection against disease progression. A recent report described an investigation of the expression of osteopontin in mononuclear cells from rheumatoid synovial fluid, correlating it with genetic polymorphism of the osteopontin gene and joint inflammation in adult rheumatoid arthritis.[23] The investigators found that osteopontin overexpression was correlated with inflammation but could not show a significant association with osteopontin genotype. Our results suggest that a study of osteopontin expression in joints in an adequate number of JIA samples, together with genotyping for functional variants such as those located in the promoter sequence, is worth undertaking.

JIA is not a benign condition as many affected individuals develop articular damage and enter adult life with persistently active disease.[24] However, although most children can be categorised into one of the various JIA subsets after the first six months of the disease, this still does not allow the outcome to be predicted reliably because outcome can differ greatly among patients belonging to the same onset type—as occurs in oligoarticular JIA.[24] It is likely to be important to tailor treatment to the risk of disability very early in the disease course, and this might become possible if predictive markers such as genotypes of susceptibility alleles were to become available. Recently, a polymorphism of the macrophage inhibitory factor (MIF) gene has been shown to be a predictor of poor outcome in systemic onset JIA. However, this same polymorphism was not associated with a persistent or extended phenotype in patients with oligoarticular JIA.[25]

In conclusion, our study suggests that the osteopontin gene polymorphism which has been identified is associated with disease outcome in oligoarticular JIA and might therefore be a useful genetic marker for characterising patients with oligoartiarticular onset JIA who are at risk of a worse outcome. Such patients might require more aggressive treatment earlier during the course of the disease.

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REFERENCES

1 **Denhardt DT**, Noda M, O'Regan AW, Pavlin D, Berman JS. Osteopontin as a means to cope with environmental insults: regulation of inflammation, tissue remodeling, and cell survival. *J Clin Invest* 2001;**107**:1055–61.
2 **O'Regan A**, Berman JS. Osteopontin: a key cytokine in cell-mediated and granulomatous inflammation. *Int J Exp Pathol* 2000;**81**:373–90.
3 **Ohshima S**, Kobayashi H, Yamaguchi N, Nishioka K, Umeshita-Sasai M, Mima T, *et al*. Expression of osteopontin at sites of bone erosion in a murine experimental arthritis model of collagen-induced arthritis: possible involvement of osteopontin in bone destruction in arthritis. *Arthritis Rheum* 2002;**4**:1094–101.
4 **Yumoto K**, Ishijima M, Rittling SR, Tsuji K, Tsuchiya Y, Kon S, *et al*. Osteopontin deficiency protects joints against destruction in anti-type II collagen antibody-induced arthritis in mice. *Proc Natl Acad Sci USA* 2002;**99**:4556–61.
5 **Petrow PK**, Hummel KM, Schedel J, Franz JK, Klein CL, Muller-Ladner U, *et al*. Expression of osteopontin messenger RNA and protein in rheumatoid arthritis: effects of osteopontin on the release of collagenase 1 from articular chondrocytes and synovial fibroblasts. *Arthritis Rheum* 2000;**43**:1597–605.
6 **Ohshima S**, Yamaguchi N, Nishioka K, Mima T, Ishii T, Umeshita-Sasai M, *et al*. Enhanced local production of osteopontin in rheumatoid joints. *J Rheumatol* 2002;**29**:2061–7.
7 **Gattorno M**, Gregorio A, Ferlito F, Gerloni V, Parafioriti A, Felici E, *et al*. Synovial expression of osteopontin correlates with angiogenesis in juvenile idiopathic arthritis. *Rheumatology* 2004;**43**:1091–6.
8 **Petty RE**, Southwood TR, Baum J, Bhettay E, Glass DN, Manners P, *et al*. Revision of the proposed classification criteria for juvenile idiopathic arthritis: Durban, 1977. *J Rheumatol* 1998;**25**:1991–4.
9 **Martini A**. Are the number of joints involved or the presence of psoriasis still useful tools to identify homogeneous disease entities in juvenile idiopathic arthritis? *J Rheumatol* 2003;**30**:1900–3.
10 **Fernandez-Vina MA**, Fink CW, Stastny P. HLA antigens in juvenile arthritis. Pauciarticular and polyarticular juvenile arthritis are immunogenetically distinct. *Arthritis Rheum* 1990;**33**:1787–94.
11 **Nepom B**. The immunogenetics of juvenile rheumatoid arthritis. *Rheum Dis Clin North Am* 1991;**17**:825–42.
12 **Barron KS**, Silverman ED, Gonzales JC, Owerbach D, Reveille JD. DNA analysis of HLA-DR, DQ, and DP alleles in children with polyarticular juvenile rheumatoid arthritis. *J Rheumatol* 1992;**19**:1611–16.
13 **Fernandez-Vina M**, Fink CW, Stastny P. HLA associations in juvenile arthritis. *Clin Exp Rheumatol* 1994;**12**:205–14.
14 **Grom AA**, Giannini EH, Glass DN. Juvenile rheumatoid arthritis and the trimolecular complex (HLA, T cell receptor, and antigen). *Arthritis Rheum* 1994;**37**:601–7.
15 **Guillaume S**, Prieur AM, Coste J, Job-Deslandre C. Long-term outcome and prognosis in oligoarticular-onset juvenile idiopathic arthritis. *Arthritis Rheum* 2000;**43**:1858–65.
16 **D'Alfonso S**, Barizzone N, Giordano M, Chiocchetti A, Magnani C, Castelli L, *et al*. Two single-nucleotide polymorphisms in the 5′ and 3′ ends of the osteopontin gene contribute to susceptibility to systemic lupus erythematosus. *Arthritis Rheum* 2005;**52**:539–47.
17 **Caillier S**, Barcellos LF, Baranzini SE, Swerdlin A, Lincoln RR, Steinman L, *et al*. Osteopontin polymorphisms and disease course in multiple sclerosis. *Genes Immun* 2003;**4**:312–15.
18 **Chiocchetti A**, Indelicato M, Bensi T, Mesturini R, Giordano M, Sametti S, *et al*. High levels of osteopontin associated with polymorphisms in its gene are a risk factor for development of autoimmunity/lymphoproliferation. *Blood* 2004;**103**:1376–82.
19 **Giacopelli F**, Rosatto N, Divizia MT, Cusano R, Caridi G, Ravazzolo R. The first intron of the human osteopontin gene contains a C/EBP-beta-responsive enhancer. *Gene Expr* 2003;**11**:95–104.
20 **Giacopelli F**, Marciano R, Pistorio A, Catarsi P, Canini S, Karsenty G, *et al*. Polymorphisms in the osteopontin promoter affect its transcriptional activity. *Physiol Genomics* 2004;**20**:87–96.
21 **Urcelay E**, Martinez A, Mas-Fontao A, Peris-Pertusa A, Pascual-Salcedo D, Balsa A, *et al*. Osteopontin gene polymorphisms in Spanish patients with rheumatoid arthritis. *J Rheumatol* 2005;**32**:405–9.
22 **Allison DB**. Transmission-disequilibrium tests for quantitative traits. *Am J Hum Genet* 1997;**60**:676–90.
23 **Xu G**, Sun W, He D, Wang L, Zheng W, Nie H, *et al*. Overexpression of osteopontin in rheumatoid synovial mononuclear cells is associated with joint inflammation, not with genetic polymorphism. *J Rheumatol* 2005;**32**:410–16.
24 **Ravelli A**, Martini A. Early predictors of outcome in juvenile idiopathic arthritis. *Clin Exp Rheumatol* 2003;**21**(S31):S89–93.
25 **De Benedetti F**, Meazza C, Vivarelli M, Rossi F, Pistorio A, Lamb R, *et al*. Functional and prognostic relevance of the −173 polymorphism of the macrophage migration inhibitory factor gene in systemic-onset juvenile idiopathic arthritis. *Arthritis Rheum* 2003;**48**:1398–407.