**IL10** promoter polymorphisms are associated with systemic onset juvenile idiopathic arthritis (SoJIA)

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**Abstract**

**Objective**

Juvenile idiopathic arthritis (JIA) is a rare, but severe cause of childhood disability. Systemic onset JIA (SoJIA) accounts for approximately 5.8% of all JIA cases and is associated with cytokine dysregulation, including interleukin (IL-)1, IL-6 and tumour necrosis factor (TNF-)α. IL-10 is an immuno-regulatory cytokine, which in part regulates inflammation by controlling inflammatory cytokine expression. Dysregulation in IL-10 expression and certain single nucleotide polymorphisms (SNPs) in the IL-10 promoter were shown to be associated with autoimmune and infectious diseases.

**Methods**

Genomic DNA-samples from SoJIA patients from two German Paediatric Rheumatology centres, and healthy controls were analysed for three well defined IL-10 promoter SNPs (-1082G>A, -819C>T, and -592C>A). These SNPs are in tight linkage disequilibrium, and result in three predominant (or "classical") haplotypes: ATA, ACC, and GCC. ATA and ACC are associated with low and medium, GCC is associated with high IL-10 expression.

**Results**

Here, we show a strong association of IL-10 promoter polymorphisms with SoJIA. We demonstrate a significantly increased frequency of low IL-10 expressing -1082A/A alleles, the medium IL-10 expressing ACC haplotype (p=0.01), and an enrichment of the rare GTC haplotype (p<0.001) in patients with SoJIA. Heterozygous -1082G/A alleles (p<0.001), and the GCC haplotype (p<0.001) on one allele protect from developing SoJIA.

**Conclusion**

This suggests a central role of the immuno-regulatory cytokine IL-10 in the pathogenesis of SoJIA.

**Key words**

IL-10, systemic onset JIA, Still’s disease, inflammation, polymorphism, SNP, cytokine
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**Introduction**

Systemic onset juvenile idiopathic arthritis (SoJIA), or “Still’s disease”, is a rare but severe cause of childhood disability (1-3), and accounts for approximately 5.8% of all JIA cases in Germany (Data from German JIA registry, 2007, personal conversation with M. Niewerth, Deutsches Rheuma-Forschungszentrum, Berlin, Germany). Other European countries and the USA report higher incidences of SoJIA up to 10% of JIA cases (1, 4, 5). Among the seven (sub-)groups of JIA, SoJIA is the most severe, and usually most difficult to treat. SoJIA is characterised by fevers, evanescent rash, generalised lymphadenopathy, hepatosplenomegaly, and/or serositis in the absence of high titer autoantibodies (3). Previously, polymorphisms in other cytokine genes have been associated with SoJIA, including the interleukin 6 (*IL6*) (chromosome 7p21) and Macrophage migration inhibitory factor (*MIF*) (chromosome 22q11.2) genes (1, 7, 8).

**Materials and methods**

**Patient and control samples**

Seventy-four patient samples were collected by members of the Department of Paediatric and Adolescent Rheumatology, North-Western German Centre for Rheumatology, St. Josef-Stift Sendenhorst, and the Paediatric Rheumatology and Immunology Section, University Children’s Hospital Dresden, University of Technology Dresden. Ethical approval for the study was obtained from the ethics commission of the University of Technology Dresden, and parents gave informed consent. An ethnically-matched healthy control population (Caucasian, of German origin) from first time blood donors (249 individuals) was used as controls. Prior to the main study, 120 healthy controls from Münster/Sendelboch, North Rhine-Westphalia and 129 healthy controls from Dresden, Saxony were compared to screen for local differences in allele frequencies.

**Genotyping**

DNA from 74 SoJIA patients and 249 healthy controls was collected and purified, using EZ DNA II isolation kits (Zymo research). All samples were genotyped for three previously described SNPs: -1082G>A (rs1800896), -819C>T (rs1800871), and -592C>A (rs1800872).
Polymerase chain reaction (PCR) sequencing was used for genotyping. The IL10 5’ SNP block was amplified, using the following primers: forward 5’ GACAACACTAAGGCTTC 3’, and reverse 5’ GCTAACTTAGGGAGTCACT 3’. The cycling parameters consisted of an initial denaturation for 10 minutes at 94°C, followed by 35 cycles of 30sec denaturation at 94°C, 45sec annealing at 61°C and 45sec extension step of 72°C for 5 minutes. PCR products were purified, using rAPid Alkaline Phosphatase cleaning (Roche-applied-science), and used as template for PCR sequencing.

The BigDyeTM Terminator cycle sequencing reaction was purchased from Applied Biosystems to determine polymorphisms in the IL10 gene promoter according to the protocol provided. Briefly, after mixing 2μL of the above PCR products (approximately 50ng DNA) with 0.5 μL primer (5ng), 2μL BigDye terminator ready reaction mix (containing Mg2+, pyrophosphatase, and DNA polymerase) and 2μL BigDye Terminator 10x Sequencing buffer and ddH2O to a final reaction volume of 20μL, the following cycle conditions were applied: 25 cycles of 96°C for 30 sec, 15 sec at annealing temperature adapted to the primer pairs (54–62°C), and 60°C for 4 min (Trio Thermoblock, Biometra, Göttingen, Germany). The sequence was determined, using the ABI 310 automatic seqencer (Applied Biosystems).

The promoter sequence of the IL10 gene was determined in each patient and control sample. The DNA sequence of each patient was compared with the IL10 promoter sequence of 249 healthy controls and the published sequence, using the ABI 310 sequence navigator software, version 1.0.1 (Applied Biosystems).

**Association analysis**

Rare haplotypes with frequencies <5% were excluded from statistical analysis. Statistical analysis was performed, using PASW Statistics 17.0 software (IBM). Statistical analysis was performed to compare single marker and haplotype frequencies in both groups, using Fisher’s exact test. P-values <0.05 were considered to be significant.

**Results**

*Comparison of IL10 promoter polymorphisms in healthy controls from Munster and Dresden*

One hundred and twenty healthy controls from Müster and Dresden, Saxony, were compared in order to screen for possible local differences in allele frequencies. No significant differences in allele distribution between the two groups were detected (Supplement 1). Thus, DNA samples from 129 Dresden and 120 Müster 1st time blood donors were combined to one group and used as control population for the main study (249 controls).

*Comparison of IL10 promoter haplotypes in SoJIA patients and healthy controls*

IL10 promoter haplotypes in 249 control individuals were compared to 74 SoJIA patients from the Department of Paediatric and Adolescent Rheumatology, North-Western German Centre for Rheumatology, St. Josef-Stift Sendenhorst, and the Paediatric Rheumatology and Immunology Section, University...
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As a first step, single marker analyses of the -1082 A>G and -592A>C polymorphisms were performed. A strong association of -1082A/A alleles (42/74 (0.56) vs. 74/249 (0.29), p<0.001) with SoJIA was observed. Healthy controls showed a higher frequency of -1082A/G (3/74 (0.04) vs. 107/249 (0.46), p<0.001) (Fig. 2A) alleles. At position -592, a higher frequency of -592 A/C alleles was detected in healthy controls, compared to SoJIA patients (67/249 (0.27) vs. 5/74 (0.06), p<0.001) (Fig. 2B).

Using a single allele based approach of the investigated haplotypes, that result from -1082G>A, -819C>T, and -592C>A polymorphisms, a strong association of ACC and GTC haplotypes with SoJIA was observed. Conversely, the 148 examined alleles of 74 SoJIA patients showed a higher frequency of ACC (63/148 (0.42) vs. 150/498 (0.31), p=0.01) and GTC (20/148 (0.13) vs. 10/498 (0.02), p<0.001) haplotypes, compared with controls. The high IL-10 expressing haplotype GCC showed a significantly higher frequency in the control group (32/148 (0.21) vs. 225/498 (0.45), p<0.001) (Fig. 3).

Comparing the paired allele distribution in all homozygous and heterozygous individuals, a higher frequency of heterozygous alleles was found in controls (9/74 (0.12)), compared to SoJIA patients (156/249 (0.62)). A strong association of the homozygous ACC haplotype (29/74 (0.39) vs. 33/249 (0.13), p<0.001) with SoJIA was identified. In the healthy control group, a significantly higher frequency of heterozygous ACC/GCC (2/74 (0.02) vs. 51/249 (0.2), p<0.001), ATA/GCC (0/74 (0) vs. 29/249 (0.12), p<0.001) and ATC/GCC (0/74 (0) vs. 19/249 (0.08), p=0.01) alleles was seen. The frequency of homozygous GCC alleles was comparable in SoJIA patients and healthy controls (15/74 (0.20) vs. 56/249 (0.22), p=0.751) (Fig. 4).

Discussion

JIA is a group of heterogeneous diseases, with a wide range of symptoms and severity. Chronic inflammation and the elevation of (pro-) inflammatory
cytokines is a common feature to all groups of JIA, including SoJIA. Several studies have identified an imbalance of cytokines with any form of JIA (1, 17-20). The direct involvement of IL-1, IL-6, and TNF-α in systemic inflammation was shown by several groups, measuring serum and synovial fluid levels of SoJIA patients (4, 18, 21, 22, 23). Since IL-10 has a direct influence on IL-6 and TNF-α expression and has previously been shown to play a role in severe extended oligoarthritis (21), we investigated the association of IL10 promoter polymorphisms with SoJIA. In this study, we show an association of IL10 promoter polymorphisms with SoJIA. The three IL10 promoter variants -1082G>A (rs1800896), -819C>T (rs1800871), and -592C>A (rs1800872) form three predominant haplotypes (ATA, ACC and GCC). Next to these “classical” haplotypes, some rare haplotypes, including GTC, have been reported (24, 25). Here, we demonstrate an association of the low IL10 expressing -1082G>A IL-10 promoter SNP with SoJIA. This suggests an involvement of IL10 in the dysregulation of (pro-)inflammatory cytokines, and CD4+ T cells, which were suggested to play a central role in SoJIA (1, 4). This is in agreement with Fife et al.’s (1) finding, that SoJIA is associated with -1082A/A alleles, but not with -592A/A. In this study, the authors performed single marker analysis within the IL10 promoter and additionally screened for SNPs in and around IL19 and IL20. Fife and colleagues did not investigate IL10 promoter haplotype blocks, which complicates a detailed comparison with our study. Other than Fife et al., we investigated sequence variants at positions -1082, -819, and -592, and were able to associate -1082A/A SNPs with possible ATA and ACC haplotypes. Interestingly, we found a strong association of SoJIA with the medium IL10 expressing ACC haplotype, but not the low producing ATA haplotype. These findings are in agreement with results of Fife et al.’s study, since the authors found an association of SoJIA with -1082A/A, but not -592A/A alleles. GTC is a rare haplotype, with frequencies usually <3% in healthy controls (24, 25) (Fig. 3). The GTC haplotype has been reported to protect from developing nephropathy in type-2 diabetes (25). In this study, the GTC haplotype is highly enriched in the SoJIA population (13% of SoJIA patients have homozygous GCC haplotypes, Fig. 4). These findings suggest a role of the rare GTC haplotype in the development of SoJIA. In a further study in a Caucasian British JIA population, a relatively small cohort of SoJIA patients was included (26). In contrast to our findings, the authors did not see an association with the rare GTC haplotype. Possible explanations could be the small patient number of the studies (50 and 74 SoJIA patients), but also differences in the haplotype distribution of British and German SoJIA populations. Normal controls in the two studies did not show significant differences in the haplotype distribution, including the rare GTC haplotype (<3%). Thus, our findings could be an indication of an enrichment of the rare GTC haplotype in a German SoJIA cohort. Differences in haplotype distribution in different regions of the world have been reported (27) and could explain different disease associated haplotype frequencies in SoJIA patients in Great Britain and Germany. As, to our knowledge, no functional data is available for the GTC haplotype, further studies are necessary to investigate the association of this haplotype with high or low level IL-10 expression. Furthermore, GCC haplotypes are significantly more frequent in healthy controls. This suggests a dominant recessive and protective effect of GCC alleles, since heterozygous GCC haplotypes are significantly more common in the healthy control population. The “high expressing” GCC haplotype on one allele might lead to a reconstitution of an impaired IL-10 expression by the second allele. Donn et al. (26) reported comparable frequencies of heterozygous GCC haplotypes in their control population. Heterozygous GCC was more frequent in the SoJIA cohort, which further suggests regional differences in the haplotype distribution in SoJIA patients in Great Britain and Germany. Nonetheless, results need to be interpreted with caution, since patient numbers were relatively small in all studies, secondary to the rarity of the disease.

The pathophysiological effect of this finding is still not entirely clear, but the relevance of our findings is suggested by the role of IL-10 in immune regulation. Multiple autoimmune and infectious diseases were shown to be associated with dysregulation of IL-10 expression. Shin et al. reported that certain IL10 alleles were predictive for HIV progression rates (28) and showed that the SNPs in this haplotype block display unique DNA-protein-binding patterns. The authors reported reduced protein binding to homozygous -592AA alleles, compared to heterozygous -592A/C alleles, to SP-1 binding sequences. SP-1 is a transcription factor that strongly promotes IL-10 expression by macrophages and monocytes and binds to GC rich regions within the proximal promoter of the IL-10 gene (29). In the present study, healthy controls showed a significantly higher frequency of -592A/C alleles (23/100 (0.23)), compared to SoJIA patients (5/74 (0.06)). This might lead to
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alternate IL-10 expression and strongly suggests that allelic variants may influence protein recruitment to regulatory regions and gene expression.

It is important to note, that individual IL10 promoter SNPs could be markers of larger haplotypes that may extend several hundred kilobases over the entire IL10 gene cluster (11, 12, 30) rather than directly influencing binding of regulatory proteins. Indeed, a recent study was unable to find a link between IL10 promoter SNPs and IBD, but did find a strong association between a SNP 3’ to the IL10 gene and ulcerative colitis (31). An association of the IL10 -1082G/A polymorphism and IL20 -468C>T polymorphisms with SoJIA has been shown (1), but warrants further study and more extensive analysis of the IL10 gene cluster. Jones and Flavell (28) identified distal enhancer elements in the mouse IL10 cluster, and we previously reported the presence of activation dependent intergenic transcripts between the IL10 and II19 genes in murine macrophages (9). These findings suggest the presence of tissue specific regulatory elements in the IL10 cluster. Polymorphisms in and around these regions could alter cytokine expression and influence the course of (auto-) inflammatory diseases, including SoJIA.

SNPs with putative function within the entire IL10 cluster may be in linkage disequilibrium or a part of extended haplotypes. This highlights the necessity to characterise the molecular and genomic requirements for appropriate cell type-specific IL-10 expression in health and disease.

Apart from genetic control mechanisms and promoter polymorphisms, which were addressed in this study, other mechanisms, such as chromatin structure and DNA methylation were shown to play a role in the regulation of different cytokine genes, including IL10 (9). These mechanisms could influence the different clinical courses and outcomes of patients with identical or similar promoter haplotypes (9).

Conclusion
In the present study, we demonstrate a strong association of homozygous -1082A/A (rs1800896) IL10 alleles, and the ACC and GCC haplotypes with SoJIA. Heterozygous -1082G/A (rs1800896) IL10 promoter alleles and GCC haplotypes seem to protect from developing SoJIA. This suggests a central role of the immuno-regulatory cytokine IL-10 in the pathogenesis of SoJIA. To our knowledge, this is the first study that shows an enrichment of the rare GCC haplotype in patients with autoimmune disease. Further studies are needed to investigate the functional relevance of the GCC haplotype on IL-10 expression.

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References
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