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Complex genetic predisposition in adult and juvenile rheumatoid arthritis

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Abstract

Background: Rheumatoid arthritis (RA) and juvenile rheumatoid arthritis (JRA) are complex multifactorial diseases caused by environmental influences and an unknown number of predisposing genes. The present study was undertaken in order to investigate association of polymorphisms in candidate genes with RA and JRA in German subjects.

Results: Up to 200 unrelated German RA and JRA patients each and 300–400 healthy controls have been genotyped for *HLA-DRB1*, *TNFa*, *TNFA -238a/g*, *TNFA -308a/g*, *TNFA -857c/t*, *TNFR1 -609g/t*, *TNFR1 P12P*, *TNFR2 del 15bp*, *IKBL -332a/g*, *IKBL -132t/a*, *IKBL C224R*, *CTLA4 -318c/t*, *CTLA4 T17A*, *PTPRC P57P*, *MIF -173g/c*, the *MIF* and *IFNG* microsatellites as well as for D17S795, D17S807, D17S1821 by polyacrylamide gel electrophoresis, single-strand conformation polymorphism analysis, restriction fragment length polymorphism analysis or allele specific hybridization. None of the investigated genetic markers is associated with both, RA and JRA, but there are some statistically significant differences between patients and controls that have to be discussed sensibly.

Conclusions: The difficulty in investigating the genetics of complex disorders like RA and JRA may arise from genetic heterogeneity in the clinically defined disease cohorts (and generally limited power of such studies). In addition, several to many genes appear to be involved in the genetic predisposition, each of which exerting only small effects. The number of investigated patients has to be increased to establish the possibility of subdivison of the patients according their clinical symptoms, severity of disease, HLA status and other genetic characteristics.

Background

Rheumatoid arthritis in adult patients (RA) is probably not a single pathogenetic entity leading to a chronic autoimmune disease resulting in the destruction of joints. The subforms cannot yet be distinguished clinically. The worldwide prevalence of RA is about 1% and the precise etiology is still unknown [1]. Multifactorial origins are supposed for RA meaning that environmental factors as well as the contributions of several to many genes appear to play a role in the development of the disease. The genetic component is emphasized by a 3–4 fold higher concordance rate in monozygotic compared to dizygotic

twins [2]. The manifestation of RA is known to be associated with certain *HLA-DRB1* alleles [3]. The *DRB1*04* association has been confirmed for many populations [1]. The associated alleles encode five amino acids at positions 70–74 of the HLA-DRB1 chain. This so-called shared epitope (SE) comprises of the amino acid sequences QKRAA, QRRAA and RRRAA [4]. The functional basis of the SE association has still to be clarified.

Juvenile rheumatoid arthritis (JRA) designates a group of diseases that have in common chronic idiopathic inflammation of one or more joints. According to the clinical spectrum JRA can be divided into at least three subtypes including pauciarticular, polyarticular and systemic onset with extraarticular manifestations as vasculitis, scleritis etc. [5]. The pauciarticular type is characterized by the involvement of one to four joints (monoarticular, oligoarticular), the polyarticular type involves five or more joints. The presence of autoantibodies and T cell clonality primarily seen in the joints points to autoimmune pathogenesis [6]. The prevalence of JRA varies worldwide among different ethnic and geographically distinct populations [7]. *HLA-DRB1*01* and **04* alleles have been repeatedly reported to increase the risk for polyarticular JRA in many populations. *HLA-DRB1*04* is highly associated with IgM rheumatoid factor-positive polyarticular JRA in older children [8]. *DRB1*01* is associated with pauciarticular disease onset in younger patients, changing to a polyarticular course later [9]. Further associated HLA associated alleles are *DRB1*08* and **11* [10].

Since both diseases, RA and JRA, share an autoimmune background as well as other pathogenetic features, the same candidate genes can be considered primarily. These susceptibility genes include an immense number of genes encoding cytokines, their receptors and other immunorelevant genes playing possibly significant roles in the pathogenesis of RA and JRA. A most enigmatic chromosomal region for susceptibility to autoimmune disorders is the HLA region on 6p21. This region harbors also the tumor necrosis factor alpha (*TNFA)* locus, for which several associations with autoimmune diseases have been described [11,12]. In addition, the presence of increased levels of TNF in serum and synovial fluid of RA patients promoting tissue damage and joint destruction necessitate that this genomic region is investigated. Therefore, several polymorphisms in the promotor of the *TNFA* gene on 6p21 as well as in the TNF receptor genes *TNFR1* and *TNFR2* were included. The inhibitor of kappa light chain gene enhancer in B cells-like gene (*IKBL)* is also located in the HLA region and therefore can be considered as a potent candidate gene for RA/JRA [13].

Cytotoxic T lymphocyte-associated protein 4 (CTLA4) is expressed on activated T cells and thus it is critically involved in the immune response [14,15]. Previous studies on multifactorial disorders like RA, insulin-dependent diabetes mellitus, multiple sclerosis (MS) and some other autoimmune diseases investigated in different populations suggested associations with *CTLA4* polymorphisms [16-21].

Protein-tyrosine phosphatase receptor-type C (*PTPRC*, also known as CD45) is expressed on leukocytes and hematopoietic progenitors [22]. This receptor is involved in T and B cell activation and in signal transduction by regulating protein-tyrosine kinases. The protein is found in multiple isoforms, depending on alternative splicing of exons 4 (CD45RA), 5 (CD45RB) and 6 (CD45RC) (CD45RO, exon 4–6 spliced out).

Expression of macrophage migration inhibitory factor (MIF) correlates well with delayed hypersensitivity and cellular immunity in humans. MIF activity can be detected in the synovia of RA patients. The suggested role for MIF includes mediator function in regulating macrophage action in host defense [23,24].

Interferon gamma (IFNγ) is produced by T lymphocytes in response to antigens to which they are sensitized. It displays antiviral activities [25]. In the first intron of the *IFNG* gene a polymorphic $(CA)_n$ microsatellite is located [26]. Microsatellite allele 02 is associated with high levels of IFNγ production *in vitro*, which may be due to its association with a linked SNP within a putative NFκB (nuclear factor kappa B) binding site [27]. Susceptibility to and severity of RA are associated to different alleles of this microsatellite [28-31].

IKBL, *CTLA4*, *PTPRC*, *MIF* and *IFNG* only are selected examples for potent candidate genes for RA association. They all are involved in immunological processes and are different parts of signalling cascades. Another approach to elucidate the genetic background of complex diseases is to draw parallels from animal models providing important information concerning which candidate genes or regions should be studied in humans. This strategy was successful in the investigation of several human autoimmune diseases like MS or type 1 insulin dependent diabetes mellitus [32,33]. A novel RA susceptibility locus on 17q22 homologous to one linked to two rat models of RA was found by linkage and association mapping [34].

To reveal genetic predisposition factors of the complex diseases RA and JRA, in this study many polymorphisms in candidate genes have been genotyped in cohorts of about 200 patients. Since many of the already reported associations have not been replicated in all independent data sets, we compared the allele frequencies in our RA

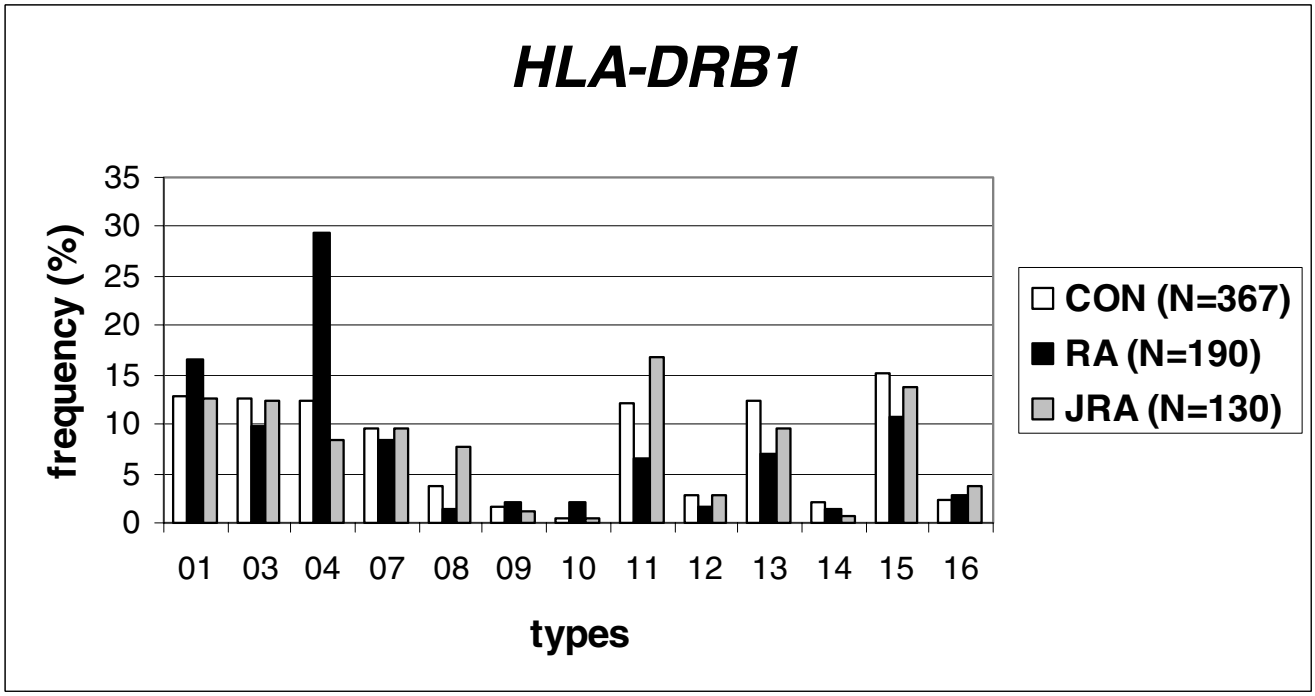

Figure 1
HLA-DRB1 allele frequencies in healthy controls (CON) *vs.* adult rheumatoid arthritis (RA) and juvenile rheumatoid arthritis patients (JRA).

and JRA cohorts with those of controls in order to contribute to define general associations.

Results

The results of the *HLA-DRB1* typing are shown in figure 1. The DNA of 367 healthy controls, 190 RA patients and 130 JRA patients has been analysed. The described association of the *DRB1*04* allele with RA is confirmed in our cohort ($p_c < 0.001$) [35]. In the group of JRA patients the *DRB1** alleles *08* and *11* are highly represented, but the differences are not statistically significant.

Figure 2 shows the distribution of alleles of the TNFa microsatellite. Allele 06 is overrepresented in the group of JRA patients (17.1% *vs.* 11.0% in the control group, $p_c < 0.02$).

In the HLA/TNFA region on chromosome 6p21 we determined allele frequencies of 3 *TNFA* promotor polymorphisms as well as of polymorphisms in the TNFα receptor genes *TNFR1* and *TNFR2*. Additionally, 3 polymorphisms in the *IKBL* gene located also in 6p21 were investigated. In the *TNFA* promotor we analysed the -238a/g, -308a/g and the -857c/t polymorphisms (table 1). No differences of allele frequencies were found in both groups of patients compared to the controls. We analysed the promotor polymorphism -609g/t and a nucleotide exchange a/g at position 36 of the coding sequence of the *TNFR1* gene. The base exchange does not result in an amino acid substitution (P12P). Both polymorphisms are not associated with RA or JRA (table 1). The allele frequency of a 15 bp deletion in the promotor of the *TNFR2* gene has been determined for 428 healthy controls, 190 RA patients and 146 JRA patients. The frequency of the deletion (allele 02)

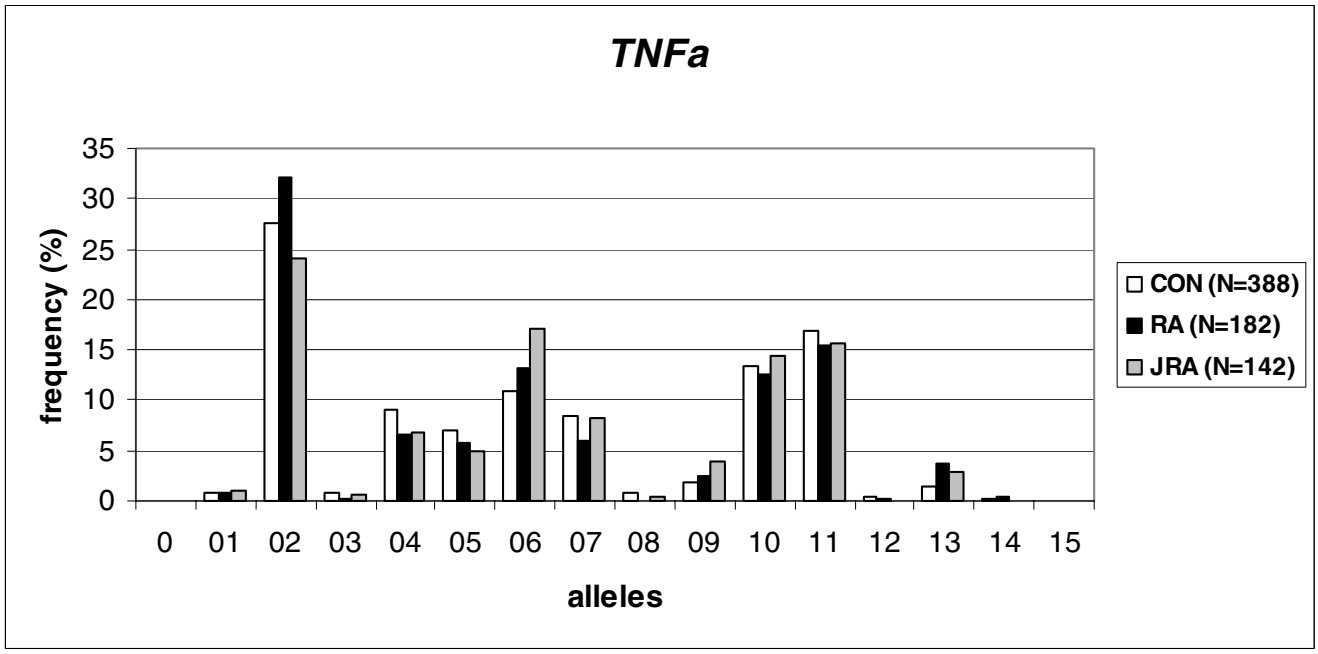

Figure 2
Allele frequencies of the *TNFa* microsatellite in healthy controls (CON) *vs.* adult rheumatoid arthritis (RA) and juvenile rheumatoid arthritis patients (JRA).

is higher in both groups of patients (RA = 32.9%, JRA = 33.3%, controls = 27.5%; $p < 0.05$). Statistical significance, however, is lost after Bonferroni correction of the p value (table 1, figure 3). In the *IKBL* gene we analysed the polymorphisms -332a/g and -132t/a in the promotor and an amino acid exchange C224R in exon 4. The allele frequencies are shown in table 1. The amino acid substitution is probably of functional relevance, since cysteine is a highly reactive and structurally relevant amino acid.

Since the *CTLA4* gene is known to play a crucial role in immunological reactions, we analysed the -318c/t polymorphism as well as the T17A exchange in 182 unrelated RA and 138 JRA patients and 362 healthy controls. The allele and genotype frequencies of the T17A exchange are very similar in the patient and in the control groups. A significant association, however, was observed for the promotor polymorphism, especially for the group of JRA patients ($p_c < 0.001$, table 1).

PTPRC and *MIF* present two other candidate genes for diseases with an immunological background. The c77g (P57P) polymorphism of the *PTPRC* (CD45) gene is not associated with RA or JRA in our cohorts (table 1). DNA of 347 healthy controls, 195 RA patients and 161 JRA patients has been investigated. In the promotor of the *MIF* gene we analysed an already known SNP (-173g/c) for 390 healthy controls, 283 RA patients and 212 JRA patients. Allele 02 is underrepresented in the group of RA patients (16.3% *vs.* 21.0% in the control group, $p_c < 0.05$) (table 1). Furthermore a $(CATT)_n$ tetranucleotide block starting at position -794 was analysed. The $(CATT)_5$ allele appears significantly more frequently in the JRA group (16.5% *vs.* 11.0% in the control group, $p_c < 0.02$) (figure 4). The $(CATT)_7$ allele is slightly overrepresented in the RA group, statistical significance, however, is lost after Bonferroni correction of the p value (figure 4). Evaluation of the genotypes yielded an overrepresentation of 0507 in the JRA group (9.4% *vs.* 4.0% in the control group, $p_c < 0.06$) (figure 4).

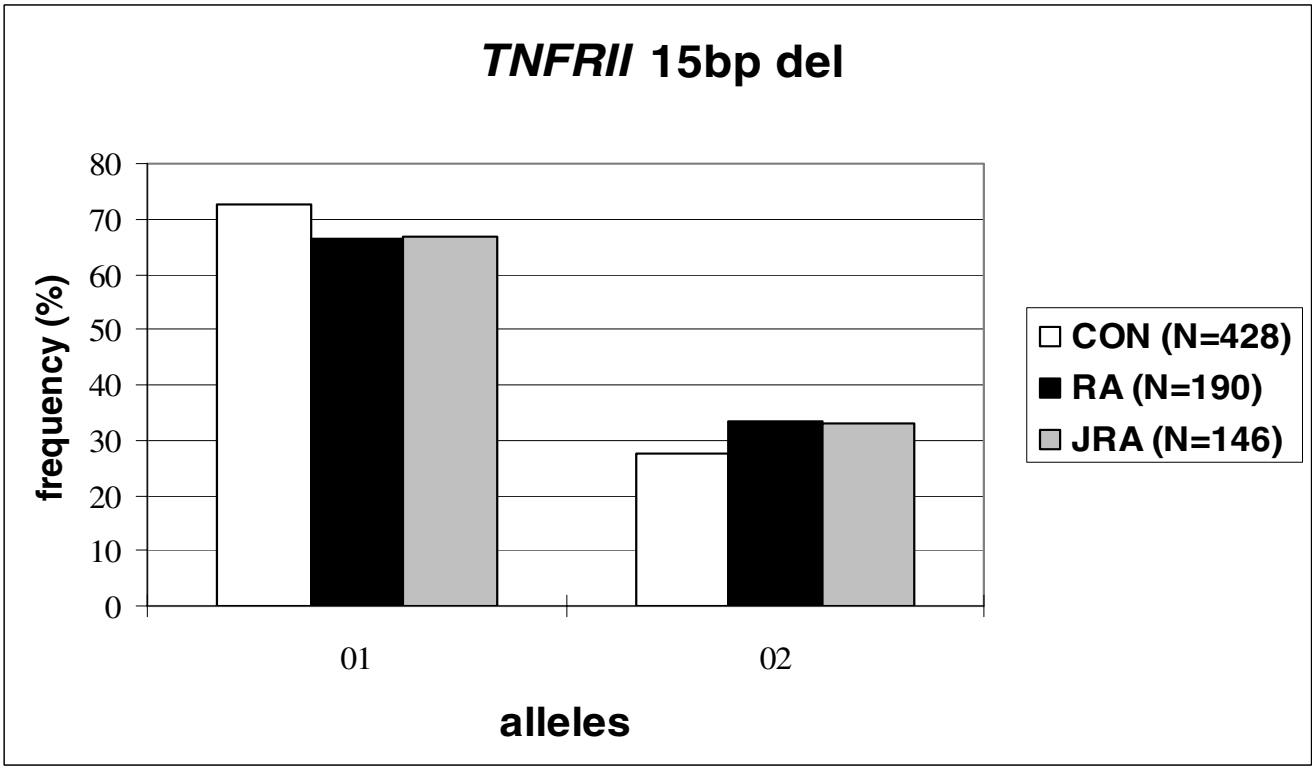

Figure 3
Allele frequencies of the *TNFRII* 15 bp deletion in the promotor in healthy controls (CON) *vs.* adult rheumatoid arthritis (RA) and juvenile rheumatoid arthritis patients (JRA).

Furthermore, we genotyped 4 microsatellites, one in intron 1 of the *IFNG* gene and 3 in a region on chromosome 17 for which an association with RA has been shown in a rat model as well as in a study with human DNA. The allele frequencies of the microsatellite in intron 1 of the *IFNG* gene as well of the three chromosome 17 markers (D17S807, D17S1821, D17S795) do not differ between the patients and the controls (Figures 5, 6).

Discussion

RA and JRA are two distinct disease entities sharing some clinical and pathogenetic factors as well as a genetic background. Both are complex disorders caused by still unknown environmental influences and by probably manifold variations in an unknown number of predisposing genes. To evaluate genetic susceptibility loci several immunorelevant polymorphisms have been genotyped for both cohorts (RA and JRA) and compared to healthy controls.

The manifestation of RA and JRA is associated with certain *HLA-DRB1* alleles [3]. The association of RA with the *DRB1*04* allele was first defined by Stastny et al. [36] Subsequently, this association has been observed in a majority of populations belonging to different ethnic groups [1]. The *HLA-DRB1* typing of our RA cohort confirmed this association. Thus our cohort of RA patients comprises a representative random sample.

The association of *HLA-DRB1*01* and **04* alleles described in the literature is not obvious in the JRA cohort presented here [8,9]. Possible explanations include that the representation of different subclasses in this heterogenous group of patients is not similar to previous collections because of the limited number of participating patients. The *HLA-DRB1*08* and **11* alleles, however, appear more frequently in the JRA group than in the control group corresponding to literature data [10]. Since the HLA region on 6p21 is highly interesting concerning autoimmune diseases and because of the presence of

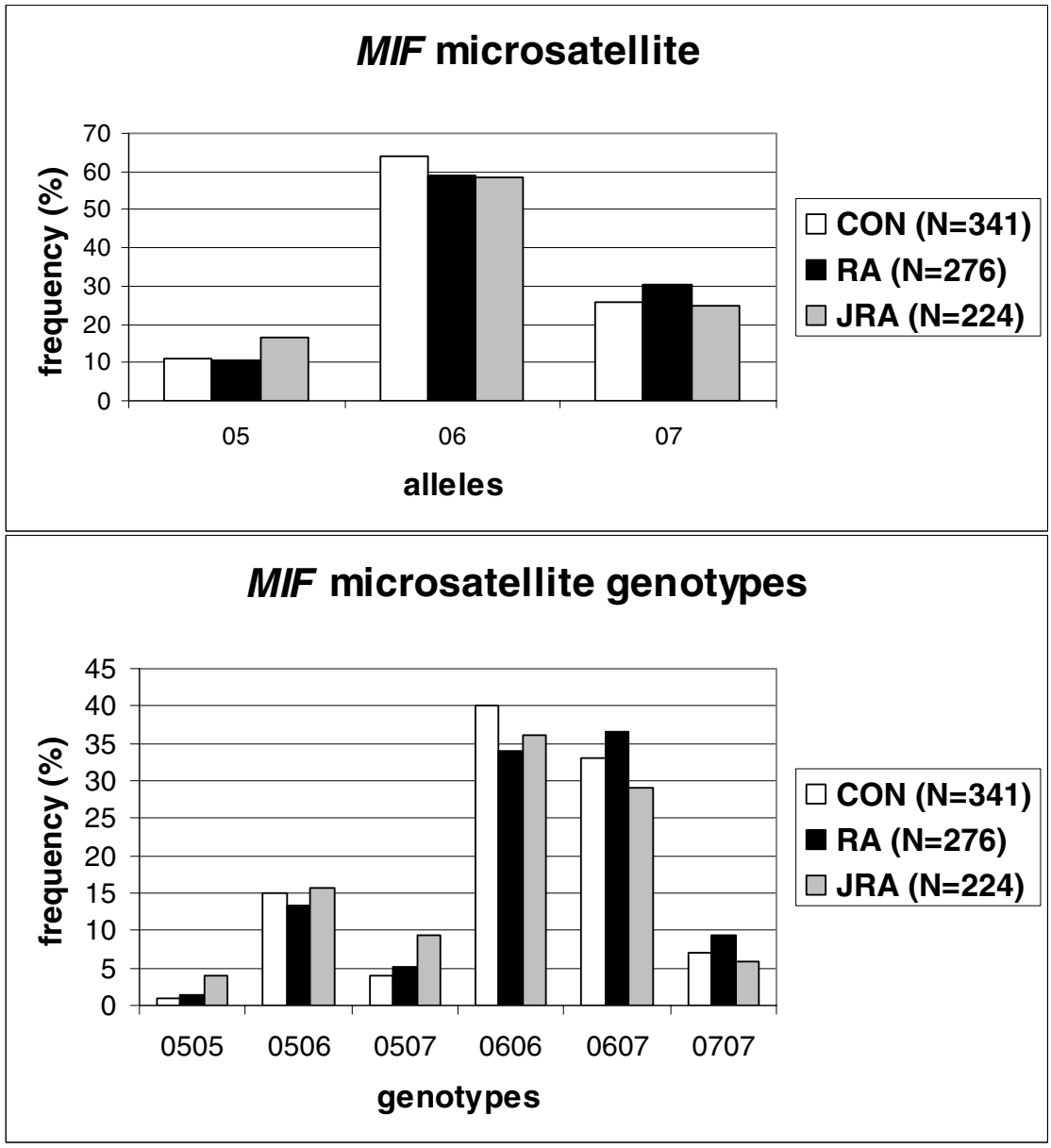

Figure 4
a,b: Allele and genotype frequencies of the *MIF* microsatellite in healthy controls (CON) *vs.* adult rheumatoid arthritis (RA) and juvenile rheumatoid arthritis patients (JRA).

increased levels of TNFα in serum and synovial fluid of RA patients promoting tissue damage and joint destruction, several polymorphisms in the promotor of the *TNFA* gene on 6p21 as well as in the TNF receptor genes *TNFR1* and *TNFR2* were investigated. The *TNFA* locus is located within the HLA class III region. Five microsatellite markers (a-e) have been described [37]. TNFa was analyzed in this study. The allele frequencies are similar to those of other studies [38]. TNFa2 is the most frequent allele in both groups of patients (RA and JRA) as well as in the control group, but a significant difference of the frequency between patients and controls has not been observed.

Table 2: Allele and genotype frequencies

	allele 01 (%)	allele 02 (%)	p_c
***TNFA*-238a/g**			
CON (n = 375)	726 (96.8)	24 (3.2)	
RA (n = 170)	323 (95.0)	17 (5.0)	n.s.
JRA (n = 130)	254 (97.7)	6 (2.3)	n.s.
***TNFA*-308a/g**			
CON (n = 312)	527 (84.5)	97 (15.5)	
RA (n = 151)	257 (85.1)	45 (14.9)	n.s.
JRA (n = 122)	202 (82.8)	42 (17.2)	n.s.
***TNFA*-857c/t**			
CON (n = 415)	738 (88.9)	92 (11.1)	
RA (n = 191)	343 (89.8)	39 (10.2)	n.s.
JRA (n = 170)	299 (87.9)	41 (12.1)	n.s.
***TNFR1*-609g/t**			
CON (n = 324)	384 (59.3)	264 (40.7)	
RA (n = 173)	221 (63.9)	125 (36.1)	n.s.
JRA (n = 136)	166 (61.0)	106 (39.0)	n.s.
***TNFR1* P12P**			
CON (n = 334)	361 (54.0)	307 (46.0)	
RA (n = 166)	167 (50.3)	165 (49.7)	n.s.
JRA (n = 132)	154 (58.3)	110 (41.7)	n.s.
***TNFR2* del15**			
CON (n = 428)	621 (72.5)	235 (27.5)	
RA (n = 190)	255 (67.1)	125 (32.9)	0.1
JRA (n = 146)	195 (66.7)	97 (33.3)	0.1
***IKBL*-332a/g**			
CON (n = 389)	711 (91.4)	67 (8.6)	
RA (n = 184)	333 (90.5)	35 (9.5)	n.s.
JRA (n = 170)	316 (92.9)	24 (7.1)	n.s.
***IKBL*-132t/a**			
CON (n = 389)	555 (71.3)	223 (28.7)	
RA (n = 177)	263 (74.3)	91 (25.7)	n.s.
JRA (n = 170)	232 (68.2)	108 (31.8)	n.s.
***IKBL* C224R**			
CON (n = 379)	701 (92.5)	57 (7.5)	
RA (n = 189)	359 (95.0)	19 (5.0)	n.s.
JRA (n = 169)	320 (94.7)	18 (5.3)	n.s.
***CTLA4*-318c/t**			
CON (n = 362)	674 (93.1)	50 (6.9)	
RA (n = 284)	504 (88.7)	64 (11.3)	**0.01**
JRA (n = 197)	342 (86.8)	52 (13.2)	**0.001**
***CTLA4* T17A**			
CON (n = 362)	455 (62.8)	269 (37.2)	
RA (n = 284)	346 (60.9)	222 (39.1)	n.s.
JRA (n = 197)	255 (64.7)	139 (35.3)	n.s.
***PTPRC* P57P**			
CON (n = 347)	684 (98.6)	10 (1.4)	
RA (n = 195)	382 (97.9)	8 (2.1)	n.s.
JRA (n = 161)	316 (98.1)	6 (1.9)	n.s.
***MIF*-173g/c**			
CON (n = 390)	616 (79.0)	164 (21.0)	
RA (n = 168)	285 (84.8)	51 (15.2)	**0.05**
JRA (n = 150)	227 (75.7)	73 (24.39)	n.s.

CON: healthy controls, RA: adult RA patients, JRA juvenile RA patients

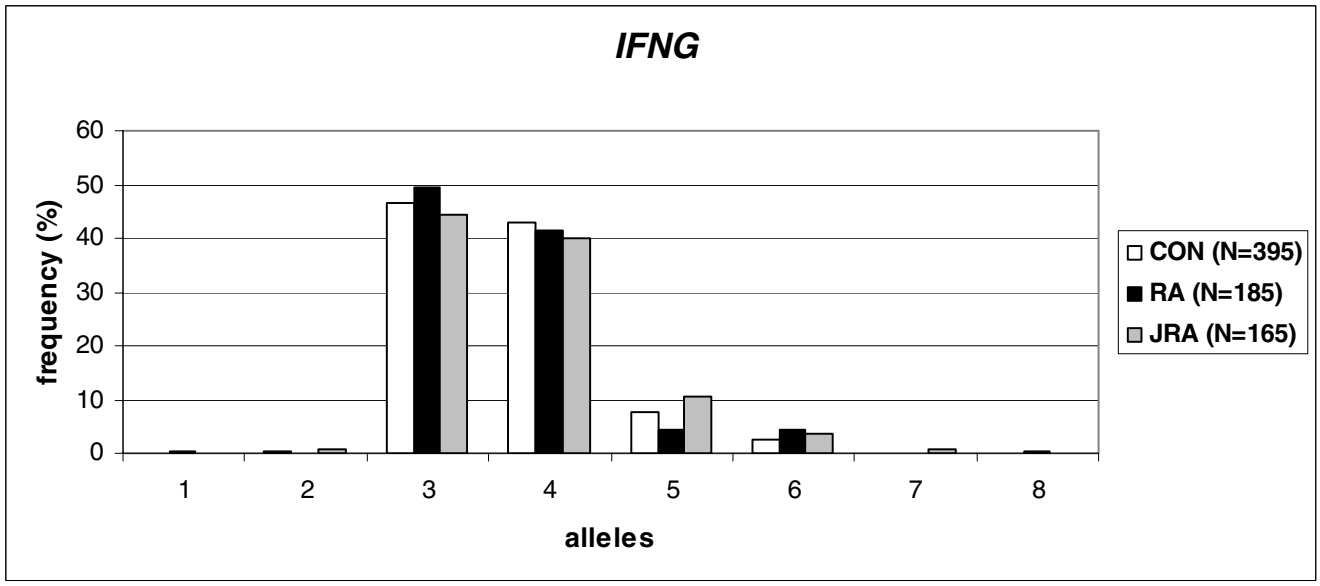

Figure 5
Allele frequencies of the *IFNG* microsatellite in healthy controls (CON) *vs.* adult rheumatoid arthritis (RA) and juvenile rheumatoid arthritis patients (JRA).

TNFa2 was found to be associated with high TNFα production [39]. Zake et al. found evidence for a positive association of this allele with JRA [5]. The contradictory data may result from the different origins of the patients. TNFa6 is significantly overrepresented in the JRA group of this study. A strong association of TNFa6 with RA in *DRB1*04*-negative haplotypes was also shown by Tuokko et al. [40] The mechanisms behind the TNFa6 association with RA has not been clarified yet, but in patients with insulin-dependent diabetes mellitus the TNFa6 allele has been reported to be associated with decreased TNFα secretion, wheras the TNFa2 allele was associated with high TNFα secretion capacity [39]. The coherency of RA-associated TNFa6 and low TNFα secretion is not in accordance with the known involvement of this cytokine in tissue destruction in RA, but the complex pathogenic mechanisms are not understood in detail until now.

In the *TNFA* promotor are described at least 11 polymorphisms and numerous studies have been undertaken to determine whether these polymorphisms have any functional significance [11,12,41]. The results of these studies, however, have been conflicting. Some authors conclude that *TNFA* genotypes influence immunologic diseases while others suggest that *TNFA* promotor polymorphisms exert no functional effects but exist only because they are in linkage disequilibrium with HLA alleles [42,43]. In this study, three promotor polymorphisms of *TNFA* have been chosen to analyse, -857c/t, -308a/g and -238a/g. No association was found for any of those polymorphisms with RA or JRA. The obtained allele frequencies are comparable with the results of other studies on Caucasians [43-45]. In contrast, Seki et al. found that the frequency of the -857t allele in RA patients is significantly higher than in the controls [46]. However, the *DRB1*0405* allele, which is in linkage disequilibrium with the -857t allele, is more strongly associated with disease susceptibility than the -857t allele. Thus the authors concluded that a susceptibility gene to RA is more closely linked to the *HLA-DRB1* locus than to the *TNFA* locus.

Since TNFα acts via binding to cell surface receptors I and II, their genes are interesting candidates for RA. Furthermore animal models have shown that mice deficient in TNFR have a more severe early inflammatory response [47]. The gene of the TNF receptor 1 (*TNFR1*) is localized on chromosome 12p13 and consists of 10 exons [48]. Each of the four protein repeats comprising the extracellular ligand binding domain and characterizing a receptor superfamily is interrupted by an intron [48]. The

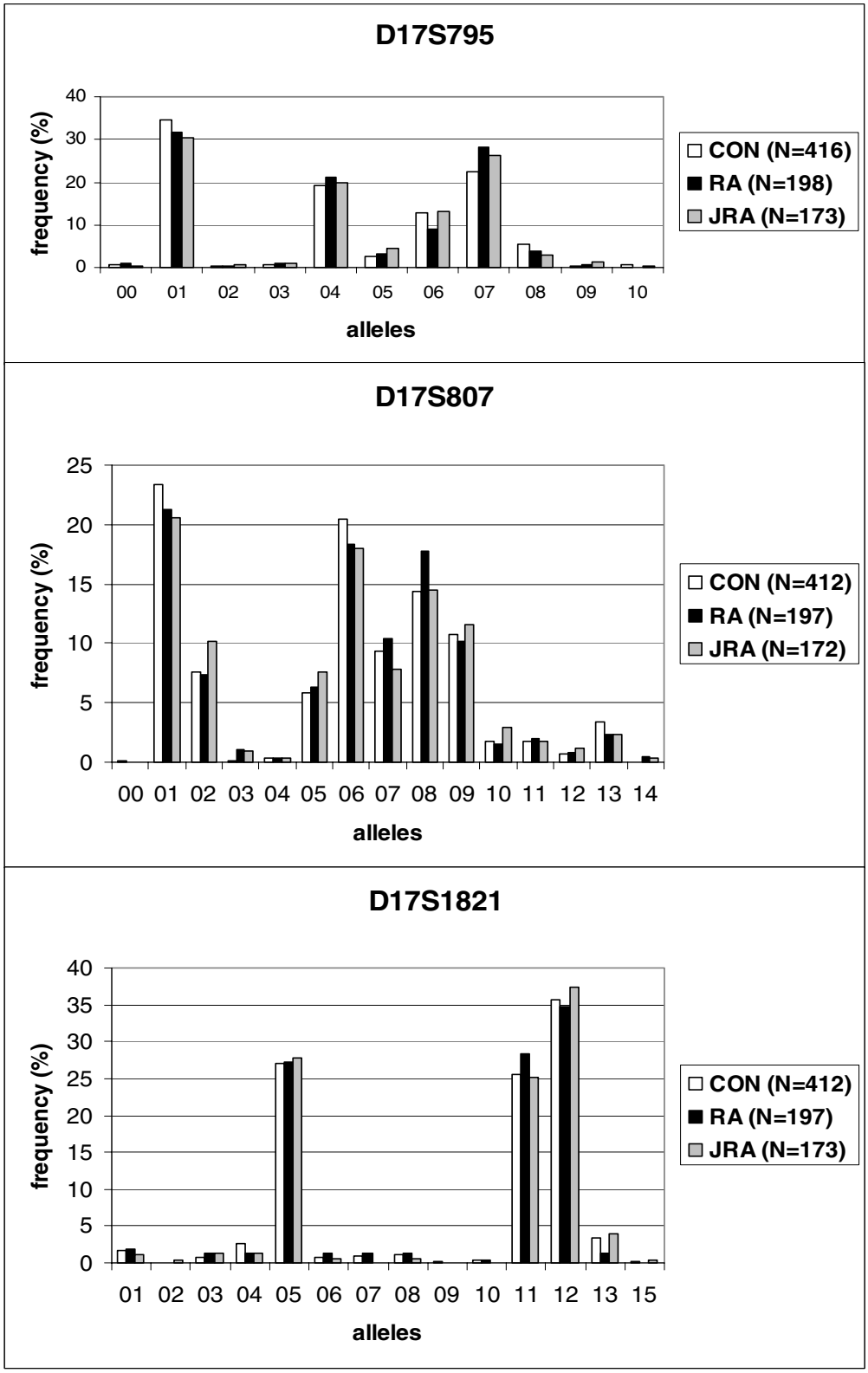

Figure 6
a-c: Allele frequencies of the D17S795, D17S807 and D17S1821 microsatellites in healthy controls (CON) *vs.* adult rheumatoid arthritis (RA) and juvenile rheumatoid arthritis patients (JRA).

promotor polymorphism -609g/t and a nucleotide exchange a/g at position 36 of the coding sequence of the *TNFR1* gene were analysed in this study [48,49]. The base exchange does not result in an amino acid substitution (P12P). Both polymorphisms are not associated with RA or JRA. This result confirms the findings of Barton et al. [50].

The gene of the TNF receptor 2 (*TNFR2*) is localized on chromosome 1p36.2. The 10 exons span 26 kb [51]. In the 5' untranslated region a 15 bp insertion/deletion polymorphism is located between positions -363 to -349 [49]. In this polymorphic region there is similarity to a putative transcription factor binding site. A regulatous function could not be ascribed to this section [52]. Thus the deletion is probably not of functional relevance. The allele frequencies of the deletion are higher in both groups of patients, but statistical significance is lost after Bonferroni correction. Barton et al. [50] and Dieude et al. [53] presented evidence that TNFR2 plays a major role in a subset of families with multiple cases of RA [50,53]. A polymorphism in exon 6 of the *TNFR2* gene has been analysed [50,53]. The cohorts in this study did not encompass a sufficient number of patients with family history to warrant investigation of this specific exonic marker.

Near the TNF cluster at the telomeric end of the MHC on chromosome 6p21.3 the *IKBL* gene is located coding for a 381 amino acid protein and spanning 13.5 kb of genomic DNA [13]. The protein contains ankyrin motifs similar to those of the I-kappa-B (IκB) family of proteins. Since *IKBL* shares homology with other members ot the IκB family, it may interact with NFκB and regulate acute phase proteins such as TNFα [54]. Thus *IKBL* is not only an interesting candidate gene for RA susceptibility because of the position on 6p21.3. In this study two promotor polymorphisms as well as a non-synonymous substitution (C224R) have been analysed [55]. The amino acid exchange lies in a predicted phosphorylation site and it possibly alters the function of the IκBL protein. None of the three polymorphisms is associated with RA or JRA in our cohorts. Further analysis of patients with other autoimmune diseases may elucidate the functional meaning especially of the amino acid substitution.

CTLA4 is expressed on activated T cells binding to the ligands, B7-1 and B7-2 and it is predicted to down-regulate T cell function [56,57]. These observations suggest that the CTLA4 / B7 interaction plays a critical role in regulating self-tolerance and in susceptibility to autoimmune diseases. The *CTLA4* gene comprises 3 exons in addition to the leader sequence and maps to chromosome 2q33 [58]. Three polymorphisms have been described in the *CTLA4* gene: a microsatellite $(AT)_n$ at position 642 of the 3'untranslated region of exon 4 and two SNPs, in exon 1 a a49g base exchange leading to an amino acid substitution T17A in the leader sequence and a c/t transition at position -318 of the promoter sequence [59-61]. Studies on RA as well as on insulin-dependent diabetes mellitus and some other autoimmune diseases suggest an association with these *CTLA4* polymorphisms [16-21]. In this study we analysed the -318c/t polymorphism and the a49g exchange. A remarkable difference of the allele frequency of the promotor polymorphism was found in the group of RA patients and even more clearly in the group of JRA patients. The distribution of alleles of the more interesting amino acid exchange, however, does not show differences between patients and controls. Further investigations are needed to clarify the importance of *CTLA4* as a susceptibility gene for RA and/or JRA.

Furthermore, the *PTPRC* gene has been investigated here [22]. *PTPRC* is localized on chromosome 1q31-q32 and consists of 35 exons. The gene encodes a 180–220 kDa glycoprotein expressed on leukocytes and hematopoietic progenitors [22]. This receptor is involved in T and B cell activation and in signal transduction by regulating protein-tyrosine kinases. Because of this involvement in immunological processes, the gene is another important candidate for RA predisposition. The protein exists in multiple isoforms, depending on alternative splicing of exons 4 (CD45RA), 5 (CD45RB) and 6 (CD45RC) (in CD45RO exons 4–6 are spliced out). The already known c77g (P57P) transition in exon 4 was analysed in our cohorts. This polymorphism does not change the amino acid sequence, but it is probably part of a motif necessary for splicing of CD45RA. The expression of CD45RA is increased in 77c/g heterozygous individuals [62]. The frequency of 77c/g heterozygotes is slightly increased in both groups of RA patients compared to the controls, but the result is not statistically significant. Maybe this gene is more important for predisposition to other autoimmune diseases. The c77g polymorphism has been investigated in several studies with patients suffering from MS. The reported results, however, are controversial. Jacobsen et al. demonstrated an association of this polymorphism with MS in three families whereas 3 studies excluded MS association later on [62-65].

MIF is found in the synovia of RA patients. The expression of MIF at sites of inflammation suggested a role in regulating the function in host defense. Expression of MIF correlates well with delayed hypersensitivity and cellular immunity in humans [23,24]. The *MIF* gene covers only less than 1 kb and has three exons separated by small introns of 189 and 95 bp [66]. So far 4 polymorphisms have been revealed in the *MIF* gene: a tetranucleotide repeat $(CATT)_n$ beginning at nucleotide position -794, a g/c exchange in the promotor at position -173, a t/c substitution in intron 1 and a c/g substitution in intron 2 [67].

The $(CATT)_n$ repeat is functionally relevant and it affects the activity of the *MIF* promotor. 5, 6, 7 and 8 consecutive CATT repeat units have been found. The $(CATT)_5$ allele has the lowest level of basal and stimulated MIF promotor activity *in vitro* [68]. In the JRA cohort presented here, the $(CATT)_5$ allele is overrepresented compared to the controls and the RA group as well as the 0507 genotype. The presence of the low expression-associated $(CATT)_5$ allele correlates with low disease severity in RA patients [68]. Donn et al. demonstrated that the MIF-173g/c variants result in altered expression of MIF in a cell type-specific manner. Serum levels of MIF are also significantly higher in individuals carrying a MIF-173c allele [69]. In this study the -173g/c polymorphism has been analysed. The c allele appears more frequently in the control group and in the group of JRA patients. The frequency of this allele in the RA group is statistically significantly lower.

IFNγ is produced by T lymphocytes in response to antigens. In the first intron of the *IFNG* gene on 12q14 a polymorphic $(CA)_n$ microsatellite is located [26]. Microsatellite allele 02 is associated with high levels of IFNγ production *in vitro*. Khani-Hanjani et al. described susceptibility to and severity of RA are associated to different alleles of this microsatellite [28]. In our study no differences in the distribution of alleles was found between patients and controls. A similar result has been observed by Constantin et al. [70]. Thus the role of *IFNG* in RA susceptibility is not clear today.

Recently, a novel RA susceptibility locus on 17q22 homologous to one linked to two rat models of RA was found by linkage and association mapping [34]. We analysed three highly polymorphic microsatellites in this region, but no one is associated with the disease. *CD79b*, a member of the immunoglobulin superfamily, is located in this region. Owing to the involvement in lymphocyte activation, it is a possible candidate gene for RA predisposition. Another gene located in this region is *ICAM-2*, which participates in the regulation of apoptosis. It is a cell surface adhesion molecule expressed on the endothelium and is involved in leukocyte recruitment into tissues [71]. TNFα and other cytokines are able to down-regulate *ICAM-2* expression on the transcriptional level.

Conclusions

RA and JRA are complex diseases the manifestation of which is depending on the presence of an unknown number of predisposing alleles in a susceptible individuum. A gene of major effect has not been identified, but several statistically significant results have to be interpreted sensibly. Some of the investigated biallelic polymorphisms are not informative because of the low frequency of the second allele. Thus these markers are considered as not suitable for detecting genetic association. Clearly in the cases of 'negative' results, association with the genes tested can formally not be excluded, because other unlinked variations within the gene(s) in question may be pathogenetically relevant (and therefore also associated) in our cohorts. The number of investigated patients has to be increased to establish the possibility of subdivison of the cohorts according their clinical symptoms, severity of disease, HLA status and also with respect to individual genetic factors.

Patients and methods

Patients and controls

More than 200 unrelated RA patients and more than 200 unrelated JRA patients of German origin were attended by the department of rheumatology of the University Hospital (Magdeburg, Germany) and the department of pediatrics of the Karl-Thiem Hospital (Cottbus, Germany). The adult RA patients met the American College of Rheumatology (ACR) criteria for RA [72]. The JRA patients were recruited according the classification of childhood arthritis [73]. The male/female rate was 1:4 (RA) and 1:2 (JRA); the mean age at onset was 48.6 (14–79; RA) and 10.3 (1–22; JRA) years.

The department of transplantation and immunology of the University Hospital Eppendorf (Hamburg, Germany) provided peripheral blood samples as well as informed consent from more than 400 healthy blood donors. The mean age of the participants was 39.3 (20–70) years. The male/female rate was 1.4 [74].

PCR and RFLP analysis

Polymerase chain reactions (PCRs) were carried out in a final volume of 12.5 µl with 50 ng of DNA, 200 µM dNTP, 1 U Taq Polymerase and 0.1 µCi [α^{32}P]-dCTP. Primer sequences, PCR conditions, fragment sizes and restriction enzymes utilized are shown in Additional File 1. PCR cycling started with initial denaturation for 5 minutes at 94°C. The annealing temperature of the first cycle was 6°C, of the second cycle 3°C higher than the remaining 26 cycles to achieve high specificity at the beginning of the reaction and to increase the quantity of PCR products in the following cycles. The annealing time was 1 minute. Extension was performed at 72°C for 1 minute with final extension of 5 minutes. Restriction enzyme digestion of PCR products >200 bp was performed according to the manufacturer's recommendations. PCR products were electrophoresed by loading restricted fragments onto vertical denaturing or non-denaturing 6% polyacrylamide (PAA) gels.

SSCP and sequence analysis

The native PAA gels used for single-strand conformation polymorphism (SSCP) analysis were carried out at 4°C. Each PCR system was tested under two different gel con-

ditions. 5% glycerine was added to the first gel, 2.5% glycerine and 1 M urea to the second. Results were documented by autoradiography. Direct sequencing of PCR products was performed on a 373A sequencing automate using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems).

Allele specific hybridization

Sequences of oligonucleotides used for allele specific hybridization are shown in Additional File 1. These oligonucleotides were designed to locate the discriminating single nucleotide exchange in the middle. PCR fragments were denatured in 0.4 M NaOH and transferred onto nylon membranes, utilizing a robot work station (Biomek 2000, Beckman). Two filters were hybridized in parallel, each with one of the allele-specific oligonucleotides. For control the hybridization probe was stripped and the filters rehybridized with the second oligonucleotide. Results were documented by autoradiography. Oligonucleotide sequences for *HLA-DRB1* genotyping were designed according to published sequences or as described previously [75,76].

Statistics

Comparisons of the frequencies of alleles were performed by Chi-square test in a 2 × 2 contingency table. P_c values are corrected for the number of all alleles tested using Bonferroni correction.

List of abbreviations

CTLA4 cytotoxic T lymphocyte-associated protein 4

IFNγ interferon gamma

IKBL inhibitor of kappa light chain gene enhancer in B cells-like gene

JRA juvenile rheumatoid arthritis

MIF macrophage migration inhibitory factor

MS multiple sclerosis

NFκB nuclear factor kappa B

PAA polyacrylamide

PCR polymerase chain reaction

PTPRC protein-tyrosine phosphatase receptor-type C

RA rheumatoid arthritis

SE shared epitope

SSCP single-strand conformation polymorphism

TNFα tumor necrosis factor alpha

Authors' contributions

BM, WK and JTE carried out the molecular genetic analyses. SD and JK provided the blood samples and clinical investigations of the RA patients. GB and JO provided the blood samples and clinical investigations of the JRA patients. All authors read and approved the final manuscript.

Additional material

Additional File 1

Primer sequences, PCR conditions and restriction endonucleases
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2156-5-2-S1.xls]

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