MOLECULAR GENETIC STUDIES IN SERONEGATIVE SPONDYLOARTHRITIDES

SUMMARY OF THE PHD THESIS

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Keywords: Spondyloarthritis, ankylosing spondylitis, psoriatic arthritis, HLA-B27, TNF-alpha, genetic association, haplotype
PART 1. THEORETICAL ASPECTS

1. Introduction

The spectrum of rheumatic diseases is wide and includes conditions with diverse pathology, although most have in common a heritable risk with a complex genetic basis. Intense efforts have been undertaken to understand the contribution of genotype to the expression of the rheumatic disease in terms of both basic pathogenesis and clinical characteristics.

2. Spondyloarthritides – general aspects

Spondyloarthritides (SpA) are a group of rheumatic diseases characterized by a few commune features that include: inflammation of the axial skeleton, more specifically the spine and sacroiliac joints and enthesitis or peripheral arthritis of the large joints of the lower limbs.

The disorders included in this group are: ankylosing spondylitis (AS), psoriatic arthritis (PsA), inflammatory bowel disease-associated arthritis, reactive arthritis (ReA), juvenile SpA and undifferentiated spondyloarthritis (USpA) (Khan 2002).

3. Ankylosing spondylitis

Ankylosing spondylitis (AS) is the prototype of spondyloarthritides. Genetic factors are the primary determinants not only of the risk of developing ankylosing spondylitis, but also of its severity (Madsen et al. 2010).

The disease is strongly associated with the gene HLA-B27, 60-95% of the patients being B27-positives, however, only 1 to 5% of B27-positive individuals from the general population develop AS, and there is increasing evidence to suggest that other genes must also be involved (Brown 2009).
Tumor necrosis factor-alpha (TNF-α) plays a key proinflammatory role in AS. Accordingly, inhibition of TNF-α was found to substantially improve signs and symptoms of AS patients (Braun et al. 2008, Davis et al. 2005). TNF-alpha promoter polymorphisms have been reported to be associated with AS susceptibility, but the results of these studies have not been replicated in other populations.

Several genome-wide association studies on AS patients confirmed the known strong association with the major histocompatibility complex on chromosome 6p and identified other genetic associations with regions from chromosomes 1 and 5 for example (Maksymowych et al. 2009).

4. Psoriatic arthritis

Psoriatic arthritis (PsA) is a systemic rheumatic disease characterized by inflammation of the skin (psoriasis) and joints (arthritis).

Polymorphisms in the genes coded in the major histocompatibility complex (MHC) region on chromosome 6p21.3 have been shown to be associated with psoriatic arthritis.

5. Reactive arthritis

Reactive arthritis (ReA) is defined as an asymmetrical inflammatory oligoarthritis or monoarthritis triggered by infection, most often in the gut or in the urogenital tract by various Gram-negative bacteria (Vahamiko et al. 2005).

Different studies suggested that reactive arthritis occurs in a genetically predisposed individual, but this condition is less frequent and less investigated for genetic associations.
6. Undifferentiated spondyloarthritis

The term undifferentiated spondyloarthritis (USpA) refers to patients with clinical and roentgenographic features suggestive of spondyloarthritides (SpA), but not fulfilling the diagnostic or classification criteria for any of the currently established disease categories (Zeidler et al. 1992).

Although undifferentiated spondyloarthritis is one of the most common subtypes of spondyloarthritides, there are limited data regarding genetic markers related to susceptibility to this disease. As with all ‘seronegative’ spondyloarthritides, there is a high incidence of HLA B27 positivity in USpA.

PART 2. PERSONAL RESEARCH AND CONTRIBUTIONS

7. Aims

This study has focused on the molecular genetics of several genes in relation with diseases from the group of spondyloarthritides: ankylosing spondylitis, psoriatic arthritis, reactive arthritis and undifferentiated spondyloarthritis.

Currently available data show that this group of rheumatic inter-related disorders has never been studied from a genetic point of view in the Romanian population.

Six genetic loci were investigated for possible genetic associations with spondyloarthritides: HLA-B27, HLA-C, TNF-alpha, HSP70-1 and HSP70-2 (heat shock protein) and E-selectin.
8. The establishment of spondyloarthritides database and DNA collection

**Spondyloarthritides database**

The total number of subjects included in the study was 551, from whom 340 patients and 211 healthy controls (control group C1).

The samples were collected from consecutive spondyloarthritides patients attending the Division of Rheumatology, Galati County Hospital, “St. Maria” Hospital and “I.C. Cantacuzino” Hospital, Bucharest, Romania in the period 2006-2010.

The control subjects were obtained from collaboration with “Prof. Dr. C. T. Nicolau” National Institute of Blood Transfusion, Bucharest, Romania.

**DNA collection**

**DNA extraction**

DNA extraction was performed from 200-400µl blood with commercial kits (mi-Blood Genomic DNA Isolation Kit - Metabion, Germany and QIAamp DNA Blood Mini Kit Qiagen, Germany) according to manufacturer protocol.

Table 1. Demographic and clinical characteristics of spondyloarthritides patients.

<table>
<thead>
<tr>
<th>Ankylosing spondylitis n=158</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age/range (years)</td>
<td>40.2 (18-74)</td>
</tr>
<tr>
<td>Female/male</td>
<td>33/125 (1:4)</td>
</tr>
<tr>
<td>Axial pattern</td>
<td>64%</td>
</tr>
<tr>
<td>Mixed pattern (axial and peripheric symptoms)</td>
<td>36%</td>
</tr>
<tr>
<td>Psoriatic arthritis n=81</td>
<td></td>
</tr>
<tr>
<td>Mean age/range (years)</td>
<td>51.5 (24-86)</td>
</tr>
<tr>
<td>Female/male</td>
<td>38/43 (1:1)</td>
</tr>
<tr>
<td>Oligoarthritis</td>
<td>18 subjects (22%)</td>
</tr>
<tr>
<td>Polyarthritis</td>
<td>37 subjects (46%)</td>
</tr>
<tr>
<td>Axial pattern (spondylitis)</td>
<td>26 subjects (32%)</td>
</tr>
<tr>
<td>Arthritis onset age (years)</td>
<td>45.7</td>
</tr>
<tr>
<td>Psoriasis onset age (years)</td>
<td>39.7</td>
</tr>
<tr>
<td>Rheumatoid factor</td>
<td>0%</td>
</tr>
<tr>
<td>Reactive arthritis n=22</td>
<td></td>
</tr>
</tbody>
</table>


Mean age/range (years) | 29 (18-44)  
Female/male | 12/10 (~1:1)  
Enteric infections | 70%  
Other | 30%  

Undifferentiated spondyloarthritis n=79  
Mean age/range (years) | 37.5 (16-59)  
Female/male | 49/30 (3:2)  
Rheumatoid factor | 0%  

Quantification of DNA concentration and purity
The DNA concentrations and purity were measured on a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA). For the DNA samples included in this study the concentrations ranged between 20ng/µl and 921ng/µl. The samples included in this study had a high purity and were successfully genotyped not only by regular PCR technics, but also by PCR-SSP (PCR with sequence specific primers) which requires a high quality DNA template.

9. The study of HLA-B27 in Romanian spondyloarthritides patient

Materials and methods
HLA-B27 genotyping. From the total group with spondyloarthritides, 310 patients (158 AS, 60 PsA, 22 ReA and 70 USpA) and control group C1 (n=211) were genotyped for HLA-B*27 allele by polymerase chain reaction with sequence-specific primers (PCR-SSP) using the HLA-B27-SSP low resolution kit (Olerup, Sweden).

Statistical analysis. For this study, the data were analyzed using the OpenEpi Collection of Epidemiologic Calculators Version 2.3. The 95% confidence limits (CL) for the HLA-B27 proportions in the analyzed populations were estimated using the Mid-P exact test. The significance of the association was determined using the Mid-P exact test. The odds ratios (OR) were calculated as conditional maximum likelihood estimates and the corresponding 95%
confidence intervals were calculated by the Mid-P exact method (Martin and Austin 1991). The statistical power (alpha=0.05, two tailed) was calculated based on normal approximation. The comparison between populations was performed with a two-tailed t-test.

Results and discussion

From the group of spondyloarthritides, 161 of 310 patients were found to be B27-positive (51.9%). The highest frequency of HLA-B27 (72.1%) was observed in the ankylosing spondylitis patients, while in the undifferentiated spondyloarthritis group, HLA-B27 was present in 37.1% of cases. Psoriatic arthritis and reactive arthritis patients revealed the lowest frequencies of the investigated marker, 25% and 27.2% respectively. Among the psoriatic arthritis group, 26 patients exhibiting the axial pattern of arthritis (spondylitis) were investigated and 8 of them were B27 positive (30.7%). In the control group of healthy Romanian subjects, the HLA-B27 marker was detected in 22 of 211 individuals (10.4%).

The statistical analysis confirmed the positive association of HLA-B27 with susceptibility to each of the investigated SpA disease groups and to SpA as a whole (Table 4). The study reports adequate levels of statistical power ranging from 78.7% for PsA to 100% for AS and SpA group as a whole, and a lower statistical power for the ReA disease (60.3%).

Table 2. Association between HLA-B27 and susceptibility to spondyloarthritides in Romania. Original, published in Int J Immunogenet 2010.

<table>
<thead>
<tr>
<th>Disease</th>
<th>HLA-B27 positive % (95% CL)</th>
<th>p-value, Mid-P exact test</th>
<th>OR</th>
<th>CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spondyloarthritides (n=310)</td>
<td>51.9 (46.4-57.5)</td>
<td>&lt;0.0000001</td>
<td>9.24</td>
<td>5.7 - 15.5</td>
</tr>
<tr>
<td>Ankylosing spondylitis (n=158)</td>
<td>72.1 (64.8-78.7)</td>
<td>&lt;0.0000001</td>
<td>22.00</td>
<td>12.7 - 39.3</td>
</tr>
<tr>
<td>Psoriatic arthritis (n=60)</td>
<td>25 (15.3-37.1)</td>
<td>&lt;0.01</td>
<td>2.85</td>
<td>1.3 - 5.9</td>
</tr>
<tr>
<td>Reactive arthritis (n=22)</td>
<td>27.2 (11.9-48.3)</td>
<td>0.02</td>
<td>3.20</td>
<td>1.1 - 8.9</td>
</tr>
<tr>
<td>Undifferentiated spondyloarthritis (n=70)</td>
<td>37.1 (26.5-48.9)</td>
<td>&lt;0.000001</td>
<td>5.04</td>
<td>2.6 - 9.8</td>
</tr>
<tr>
<td>Controls (n=211)</td>
<td>10.4 (6.8-15.1)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

p-value for the disease association with HLA-B27; OR, odds ratio; CI, confidence interval for OR
We compared the frequency of the HLA-B27 marker in our Romanian population samples with the frequency of the same marker in several other European populations. In the general population, the HLA-B27 frequency in Romania was significantly higher than that reported from Italy (5%, p=0.03) (Ferri et al. 1982), but not different from those reported from countries in the surrounding geographic area - Greece (6%) (Alamanos et al. 2004), Bulgaria (10.8%) (Minev et al. 1979), Serbia (12.3%) (Jajić 1979), Hungary (12.8%) (Gömör et al. 1977) and also from Spain (9.3%) (Fernandez-Sueiro et al. 2004), United Kingdom (9.5%) (Brown et al. 1996), Norway (15.9%) (Gran et al. 1984) and Finland (10.4%) (Jaakkola et al. 2006).

In the AS group, the HLA-B27 frequency in our Romanian sample was significantly lower than that reported from different European countries (Table 5) in which this marker has a high frequency among ankylosing spondylitis patients. In contrast, the HLA-B27 frequency in Romania was not significantly different from that found in several regions in the Mediterranean area: Italy (68-76%) (Ferri et al. 1982; Paladini et al. 2005), Greece (80.5%) (Alamanos et al. 2004), Turkey (70%) (Gunal et al. 2008), Tunisia (62%) (Kchir et al. 2010).

<table>
<thead>
<tr>
<th>Country</th>
<th>HLA-B27 positive (%)</th>
<th>p-value</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Romania</td>
<td>72.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bulgaria</td>
<td>88</td>
<td>p=0.0005</td>
<td>Minev et al., 1979</td>
</tr>
<tr>
<td>Hungary</td>
<td>92.7</td>
<td>p=0.0001</td>
<td>Gomor et al., 1977</td>
</tr>
<tr>
<td>Spain</td>
<td>84.14</td>
<td>p=0.0018</td>
<td>Collantes et al., 2007</td>
</tr>
<tr>
<td>Spain-Galicia</td>
<td>94.3</td>
<td>p&lt;0.0001</td>
<td>Fernández-Sueiro et al., 2004</td>
</tr>
<tr>
<td>Germany</td>
<td>82.2</td>
<td>p=0.029</td>
<td>Rudwaleit et al., 2009</td>
</tr>
<tr>
<td>Norway (north)</td>
<td>93</td>
<td>p&lt;0.0001</td>
<td>Bakland et al., 2005</td>
</tr>
<tr>
<td>United Kingdom</td>
<td>90.2-94</td>
<td>p&lt;0.0002</td>
<td>Brown et al., 1996, Freeston et al., 2007</td>
</tr>
<tr>
<td>Finland</td>
<td>93</td>
<td>p&lt;0.0001</td>
<td>Jaakkola et al., 2006</td>
</tr>
</tbody>
</table>
10. The study of HLA-C locus in Romanian psoriatic arthritis patients

Materials and methods

**HLA-C genotyping.** All psoriatic arthritis patients (n=81) were genotyped for HLA-C locus by PCR-SSP using HLA-C-SSP low resolution kit (Olerup, Sweden). All the typing has been performed in the Laboratory of Immunogenomics and Immunoproteomics, Department of Immunology, Palacky University, Olomouc, Czech Republic. The data for 124 healthy subjects (control group C2) from "Prof. Dr. C. T. Nicolau" National Institute of Blood Transfusion, Bucharest, Romania were used for the statistical analysis.

**Statistical analysis.** The software package OpenEpi Collection of Epidemiologic Calculators Version 2.3 was used for the analysis of HLA-C locus in psoriatic arthritis patients. The significance of the association (p<0.05) was determined using the Mid-P exact test (Martin and Austin 1991). The Bonferroni correction was used to control for multiple comparison using a factor of 13 (the number of HLA-C allelic groups found in the population).

Results and discussion

The carriers of at least one HLA-C*06 allele appeared more frequently among PsA patients (22/81, 27.1%) than within the control group (18/124, 14.5%, p=0.014, OR 2.1, 95% CI 1.08-4.46). This association was not maintained when Bonferroni correction for the number of compared markers was applied. The frequency of other HLA-C allelic groups did not differ between the patients and control population.

In the subsequent analysis of the PsA clinical phenotypes, the patients with psoriasis onset age before 40 years - type I psoriasis (n=33) showed an increased frequency of HLA-C*06 compared with controls (39.3% versus 14.5%, p=0.001, OR 3.7, 95% CI 1.58-9). This association remains significant after Bonferroni correction (p_corr=0.013). The mean age at the onset of psoriasis was significantly lower in C*06-positive patients compared with C*06-negative ones (33.2/41.9 years,
p=0.04). No significant differences were observed for the distribution of HLA-C allelic groups in patients with psoriasis onset age after 40 years or in relation with the onset age of joint symptoms.

Table 4. Distribution of HLA-C allelic groups in Romanian patients with PsA and healthy controls. Original.

<table>
<thead>
<tr>
<th>HLA-C</th>
<th>PsA (n=81)</th>
<th>Spondylitis (n=26)</th>
<th>Polyarthritis (n=37)</th>
<th>Ps type I (n=33)</th>
<th>Controls (n=124)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C*06</td>
<td>27.1%</td>
<td>38.4%</td>
<td>21.6%</td>
<td>39.3%</td>
<td>14.5%</td>
</tr>
<tr>
<td>C*07</td>
<td>35.8%</td>
<td>38.4%</td>
<td>24.3%</td>
<td>44%</td>
<td>46%</td>
</tr>
</tbody>
</table>

(1) p=0.014, pcorr=ns, OR 2.1, 95%CI 1.08-4.46;
(2) p=0.004, pcorr=ns, OR 3.6, 95%CI 1.3-9.3;
(3) p=0.009, pcorr=ns, OR 0.3 95%CI 0.1-0.8.
(4) p=0.001, pcorr=0.013, OR 3.7, 95% CI 1.58-9
p corr corrected by Bonferroni method for 13 detected alleles

The analysis of HLA-C locus association with the pattern of arthritis revealed that HLA-C*06 was found with increased frequency among patients with axial form of arthritis (spondylitis) (p=0.004, OR 3.6, 95% CI 1.3-9.3). By contrast, we observed a lower phenotype frequency, (protective effect) of HLA-C*07 in polyarthritis group (24.3% patients versus 46% controls, p=0.009, OR 0.3, 95%CI 0.1-0.8). Both these associations did not remain significant after correction.

Figure 1. Genotyping of HLA-C with HLA-C-SSP low resolution kit (Olerup, Sweden) for a psoriatic arthritis patient. Original.
Recent findings jointly suggest that HLA-C*06 influences the disease onset age (PsA with type I psoriasis), rather than confers susceptibility to PsA per se (Ho et al. 2008). Our study confirmed the association of HLA-C*06 with type I psoriasis in Romanian PsA patients and also the fact that HLA-C*06-positive patients have an earlier psoriasis onset.

11. The investigation of tumor necrosis factor alpha gene polymorphisms in Romanian spondyloarthritides patients

**Materials and methods**

**Subjects.** The spondyloarthritides groups included in the study (324 subjects in total) were: ankylosing spondylitis (n=143), psoriatic arthritis (n=80), reactive arthritis (ReA, n=22) and undifferentiated spondyloarthritis (n=79). 147 subjects from control group C1 were genotyped as control population.

**Genotyping of TNF-alpha SNPs by Real-Time PCR.** The Real-time PCR method (Popa et al. 2009) was used in this study to genotype three single nucleotide polymorphisms of TNF-α gene on a 7300 Real Time PCR System (Applied Biosystems, USA): -238G/A (rs 361525), -308G/A (rs 1800629) and -857C/T (rs 1799724). The samples were genotyped using TaqMan SNP Genotyping Assays C-7514879-10, C-2215707-10 and C-11918223-10, respectively, according to manufacturer protocol with minor changes (Applied Biosystems, USA). Positive controls obtained from other laboratories were used for -238 and -308 SNPs.

**Genotyping of TNF-alpha SNPs by PCR-RFLP.** For TNF-alpha -857C/T polymorphism, a number of 70 samples, randomly selected, were tested by a second method, semi-nested PCR-RFLP (restriction fragment length polymorphism) as described by Zhu et al., 2007 and concordant results were obtained in each case. This second genotyping method was selected for this polymorphism because no positive controls were available.
Statistical analysis. Chi-square analysis was used in order to test for any deviation of genotype distribution from Hardy-Weinberg equilibrium (HWE) for every single nucleotide polymorphism studied, for both control and patients groups.

Association tests for each polymorphism and haplotype frequency estimations were performed with the software package PLINK v 1.07 and p values ≤0.05 were considered statistically significant.

Figure 2. Genotyping of TNF-α -308G/A SNP by Real-Time PCR TaqMan allelic discrimination assay. Blue – homozygous GG, green – heterozygous GA, red – homozygous AA, black – no template control. Original.

Results and discussion

The test for Hardy-Weinberg equilibrium suggested that there was no deviation from Hardy-Weinberg equilibrium for controls and the four groups of patients, although no homozygous genotype was observed for the 238 SNP minor allele.
The data regarding the frequency of the investigated SNPs in the general Romanian population were submitted to an international database – www.allelefrequencies.net - and represent the first set of data for TNF-α SNPs frequencies for Romania.

**Figure 3. RFLP with HpaI restriction enzyme of TNF-α -857C/T polymorphism of AS patients. Lanes 1, 6, 8, 9 homozygous TT; lanes 3, 5 heterozygous CT; lanes 2, 4, 7, 11, 12, 13, 14, 15 homozygous CC; lane 10 molecular weight maker 100bp DNA ladder. Original.**

**TNF-α polymorphisms in the Romanian ankylosing spondylitis group**

AS patients exhibited a decreased frequency of the A allele at position -308 (8%) when compared with controls (14%), suggesting that this could be a protective factor for disease susceptibility. In addition, the -308GA/AA genotypes were less frequent in patients (15.8%) than in controls (24.6%, p=0.049).

The distribution of the other two investigated polymorphisms was not significantly different between patients and controls, suggesting a possible lack of association with the susceptibility to ankylosing spondylitis.

An analysis of AS patients with a high grade of severity of the disease versus AS patients with mild forms was conducted regarding the distribution of the three TNF-α polymorphisms. A significant association was observed for -308G/A SNP. The 308*A allele was completely missing in the group with severe symptoms (0% vs. 9.3%, p=0.02). This allele could be a protective factor not only for the susceptibility for the disease, but also for its severity.
The study of HLA-B27 positive AS patients compared with controls revealed that the frequency of 308*A allele was significantly lower in patients (7% vs 14%, p=0.02). This allele might have a protective effect in this AS subgroup.

The frequency of -857*T allele was higher in patients than in controls, but this difference was not statistically significant (24% vs. 19%, p=0.2). The distribution of -238G/A polymorphism was not different in the HLA-B27 positive patients compared to controls.

A haplotype estimation analysis was conducted for SNPs -238, -308 and -857 in the whole group of ankylosing spondylitis and in HLA-B27 positive AS subgroup. The most notable results concerned the frequencies of haplotype 857C/308A/238G in AS patients and controls (8% vs 14%, p=0.04) and haplotype 857C/308A/238G in AS B27+ patients and controls (7% vs 14%, p=0.03). These results show that this haplotype is associated with ankylosing spondylitis in our population.

**TNF-α polymorphisms in the Romanian psoriatic arthritis group**

The 857*T allele was more prevalent within psoriatic arthritis group compared to controls (p=0.01, OR=1.77, 95% CI 1.12-2.78). The percentage of subjects bearing homo- or heterozygous T genotypes was significantly higher in PsA patients than controls (48.8 vs. 33%, p=0.01, OR=1.91, 95% CI 1.05-3.49) using Fisher’s exact test.

The distribution of the -238G/A and -308G/A polymorphisms was not significantly different between patients and controls.

Among psoriatic arthritis patients, 22 subjects had peripheral arthritis with less than 5 joints affected (oligoarthritis), 34 individuals had more than 5 joints affected (polyarthritis) and 24 subjects presented arthritis with axial involvement (spondylitis). The analysis of the subgroups of PsA patients revealed similar distribution of the investigated polymorphisms in oligoarthritis and polyarthritis patients. In contrast, the distribution of -857C/T polymorphism differed between spondylitis PsA subjects and controls with the 857*T variant occurring at frequencies of 33.3% in patients versus 19% in controls (p=0.03, OR=2.13, 95%CI 1.09-4.15).
These results show that 857*T allele of TNF-α may represent a risk factor for psoriatic arthritis and for the axial pattern of this condition in Romanian population. The significant association found between -857C/T polymorphism and psoriatic arthritis is similar with data reported previously for Canadian population (Balding et al. 2003) and German population (Reich et al. 2007).

The frequency of 857T/308G/238G and 857C/308G/238G haplotypes tends to increase in PsA patients with respect to healthy controls (p=0.009 and 0.04 respectively). These two haplotypes show an association with the disease, a marginal effect for 857C/308G/238G haplotype (p=0.04) and a strong association for 857T/308G/238G haplotype (p=0.009) which contains also the 857*T allele.

For the group of undifferentiated spondyloarthritis patients, no association was found with the three investigated TNF-α polymorphisms. No significant difference in TNF-α genotypes combinations in USpA patients was observed when compared to control subjects.

In the group of reactive arthritis, only two TNF-alpha SNPs were studied, -308G/A and -238G/A. The allele 238*A was not found among the patients genotypes. The distribution of the -238G/A and -308G/A polymorphisms was not significantly different between patients and controls. These results have to be interpreted with caution because of the small size of the patient group.

12. The investigation of HSP70 genes polymorphisms in Romanian spondyloarthritides patients

Materials and methods

Subjects. Ankylosing spondylitis (n=111), psoriatic arthritis (n=73) and undifferentiated spondyloarthritis (n=79) patients and 101 healthy subjects (from control group C1) were genotyped for two HSP70 genes polymorphisms.

HSP70 genes SNPs genotyping. The analysis of HSP70-1 +190 G/C polymorphism (rs1043618) was performed by allelic discriminating TaqMan Real-
Time PCR on a 7300 Real Time PCR System (Applied Biosystems, USA) with TaqMan SNP Genotyping Assay C-11917510-10.

The HSP70-2 +1267A/G polymorphism (rs1061581) was genotyped by PCR-RFLP with PstI restriction enzyme as described by Vargas-Alarcon et al., 2002.

Figure 4. Genotyping of HSP70-1 +190C/G SNP by Real-Time PCR TaqMan allelic discrimination assay. Blue – homozygous GG, green – heterozygous CG, red – homozygous CC, black – no template control. Original.

**Statistical analysis.** Hardy-Weinberg equilibrium was tested using the Chi-square test. Association tests for each polymorphism and haplotype frequency estimations were performed with software package PLINK v 1.07 and p values ≤0.05 were considered statistically significant.
Results and discussion

All groups were in Hardy-Weinberg equilibrium for the two polymorphisms, except for AS group for 1267 SNP that presented a small deviation (p=0.034).

**HSP70 genes polymorphisms in the Romanian patients with ankylosing spondylitis**

Alleles or genotypes frequencies in the Romanian AS group did not differ significantly between cases and controls for any of the investigated polymorphisms.

In the group of HLA-B27 positive patients, the frequency of the minor allele 190*C was 30.2% versus 32.4% in controls (p=0.78, OR 0.89). The frequency of 1267*G allele was also very similar with the control group (31.2% vs. 37.5%, p=0.45, OR 0.75).

The results show that these polymorphisms are not associated with susceptibility to AS in our population.

**HSP70 genes polymorphisms in the Romanian psoriatic arthritis group**

Similar frequencies of genotypes and alleles were observed for HSP70-1 polymorphism in the PsA group and control population. No association was found between the investigated SNPs of the two HSP70 genes and susceptibility to psoriatic arthritis in Romanian population.

The study of heat shock protein genes polymorphisms in relation with psoriatic arthritis was not performed before in any population. Our data do not suggest a possible implication of these molecules in the pathogenic process of psoriatic arthritis, but given that PsA is a very heterogenous disease, a larger investigation on different subgroups of patients and with more than two HSP polymorphisms could bring new interesting informations.
HSP70 genes polymorphisms in the Romanian undifferentiated spondyloarthritis patients

Alleles or genotypes frequencies in the studied subjects did not differ significantly between cases and controls for any of the investigated polymorphisms. The results show that these polymorphisms are not associated with susceptibility to USpA in our population.

13. The study of TNF-α-HSP70 haplotypes in association with spondyloarthritides in Romania

Introduction

This is the first study of tumor necrosis factor alpha and heat shock protein 70 genes extended haplotypes in Romanian spondyloarthritides patients. No other study was found on any population regarding the investigation of these haplotypes in SpA patients.

TNF-alpha and HSP70 genes are both located in the same chromosomal region, the short arm of human chromosome 6, in the class III of the major histocompatibility complex. The chromosomal region surrounding TNF is abundant in genes of immunologic relevance. In order to identify true susceptibility genes, the genetic variation of the region must be studied, and extended haplotypes must be constructed and analyzed.

Materials and methods

Subjects. The individual genotypes for five SNPs for ankylosing spondylitis and psoriatic arthritis patients and healthy controls were analyzed. The SNPs were: -238G/A, -308G/A and -857C/T from TNF-α gene, 190C/G from HSP70-1 gene and 1267A/G from HSP70-2 gene. The genotyping methods for each polymorphism as well as the frequency of the genotypes were described before (Section 11 and 12).
**Statistical analysis.** A combined TNF-alpha-HSP70 haplotype analysis was conducted for ankylosing spondylitis and psoriatic arthritis compared with healthy controls with the software PLINK v 1.07.

In order to evaluate the possibility of haplotypes reconstruction between the investigated SNPs, the level of linkage disequilibrium (LD) was studied by appropriate statistics for each pair of single nucleotide polymorphisms.

**Results and discussion**

The SNPs list was pruned in order to keep the polymorphisms that are in approximate linkage equilibrium with each other. As such, the polymorphism +190G/C of HSP70-1 gene was excluded from the further haplotype association analysis because it exhibited a strong linkage disequilibrium with 1267 SNP of HSP70-2 gene ($r^2=0.79$ for AS).

A strong association ($p=0.02$) was observed for the haplotype 857T/308G/238G/1267A which was also present with a high prevalence in both patients and controls (22% and 14%, respectively). A weak association was obtained for 857C/308A/238G/1267G ($p=0.04$), but this haplotype has a very low frequency in the analyzed population (under 5% in patients), so the association is not significant.

In the analysis of AS HLA-B27 positive patients, 857T/308G/238G/1267A haplotype was also more frequent in patients than in controls (22% versus 14%), but the association was weaker ($p=0.03$). These data sustain the hypothesis that this haplotype contains a susceptibility factor for ankylosing spondylitis independent from HLA-B27 marker.

For psoriatic arthritis group, none of the 7 haplotypes was associated with the disease. Like for the AS patients, the polymorphism +190G/C of HSP70-1 gene was excluded because of linkage disequilibrium with 1267 SNP of HSP70-2 gene ($r^2=0.73$ for PsA). A marginally positive association was observed for 857T/308G/238G/1267A haplotype (19% in patients versus 12% in controls, $p=0.05$).

Although polymorphisms of TNF are likely to contribute to spondyloarthritides, the complex pattern of associations that has been revealed
could also be attributable to LD with another susceptibility locus in the vicinity of the TNF-alpha-HSP70 genes. Future studies including more polymorphisms and large cohorts of patients could bring more information about MHC class III association with spondyloarthritides.

14. E-selectin A561C polymorphism in Romanian patients with ankylosing spondylitis

Introduction

The gene for the adhesion molecule E-selectin is located on chromosome 1q23-25. The best characterized polymorphism in E-selectin molecule is A561C, which codes for Ser128Arg (S128R).

Materials and methods

Subjects. A group of 97 ankylosing spondylitis patients and 117 healthy subjects (from control group C1) were genotyped for E-selectin A561C polymorphism.

Genotyping of E-Selectin S128R polymorphisms. E-selectin gene S128R (561A/C) polymorphism was genotyped by PCR-RFLP technique.

Statistical analysis. Allele and genotype frequencies were compared between patients and control groups. Chi-square analysis was used in order to test for Hardy-Weinberg equilibrium for both control and patients groups.

Results and discussion

The frequency of AA genotype was higher in the control subjects (72.7%) as compared to patients group (69.1%). However, there was no statistically significant difference between the two groups (p>0.05).

These results show that A561C polymorphism of E-selectin gene is not a susceptibility factor for ankylosing spondylitis in Romanian population.
Despite this lack of association for E-selectin gene polymorphism with susceptibility to ankylosing spondylitis in Romanian population, this gene remains an interesting and attractive candidate for studies in spondyloarthritides patients. A pilot study on psoriatic arthritis patients treated with anti-TNF agents showed that there was a significant decrease in E-selectin level in the skin lesions of patients after successful treatment (Cordiali-Fei et al. 2006).
15. Conclusions

Ankylosing spondylitis

1. The positive association of HLA-B27 with susceptibility to ankylosing spondylitis (AS) and to SpA as a whole was confirmed for Romanian patients.

In the AS group, the HLA-B27 frequency in our Romanian sample was significantly lower than that reported from different European countries (Bulgaria, Spain, Finland, Germany, United Kingdom, Norway) in which this marker has a high frequency among ankylosing spondylitis patients. In contrast, the HLA-B27 frequency in Romania was not significantly different from that found in several regions in the Mediterranean area: Italy, Greece, Turkey, Tunisia.

2. Our results suggest a weak protective effect of TNF-α 308*A allele for this condition. The other two investigated polymorphisms of TNF-α gene had a similar distribution in AS cases and controls.

The 308*A allele was completely missing in the group of ankylosing spondylitis patients with severe symptoms. This allele could be a protective factor not only for the susceptibility for the disease, but also for its severity. TNF-alpha appears to be a modulatory factor for ankylosing spondylitis and not a causative one in Romanian population.

The haplotype analysis shows that the frequency of 857C/308A/238G haplotype in AS patients and in AS B27+ patients was significantly lower than in controls. These results show that this haplotype is associated with ankylosing spondylitis in our population.

3. The two investigated polymorphisms of HSP70-1 and HSP70-2 genes were not individually associated with susceptibility to AS in the Romanian population.

4. A strong association was observed between ankylosing spondylitis and the TNF-α-HSP70 extended haplotype 857T/308G/238G/1267A which was present with a high prevalence in both patients and controls (22% and 14%, respectively).
respectively). A weak association was obtained for 857C/308A/238G/1267G, but this haplotype has a very low frequency in the analyzed population (under 5% in patients), so the association is not significant.

In the analysis of AS HLA-B27 positive patients, 857T/308G/238G/1267A haplotype was also more frequent in patients than in controls, but the association was weaker. These data sustain the hypothesis that this haplotype contains a susceptibility factor for ankylosing spondylitis independent from HLA-B27 marker.

5. The A561C polymorphism of E-selectin gene is not a susceptibility factor for ankylosing spondylitis in Romanian population. Despite this lack of association this gene remains an interesting and attractive candidate for studies in spondyloarthritides patients.

**Psoriatic arthritis**

1. In the studied group, a positive association was found between HLA-B27 and susceptibility to psoriatic arthritis (PsA). In our PsA patients, HLA-B27 is associated with the axial pattern of arthritis, similar with the data reported for Spanish population.

In this group, the frequency of the HLA-B27 marker in the investigated Romanian sample was significantly higher than that reported from continental Italy and Serbia, but not different from the frequencies reported from United Kingdom and Spain. In Italy, there are two other studies reporting frequencies of HLA-B27 that are not statistically different from our study.

2. Positive associations were found between HLA-C*06 and susceptibility to PsA, between HLA-C*06 and axial pattern of arthritis and between HLA-C*07 and polyarthritis subgroup, but these associations did not remain significant after correction for multiple testing.

This study confirmed the association of HLA-C*06 with type I psoriasis (psoriasis onset before 40 years) in Romanian psoriatic arthritis patients and also the fact that HLA-C*06-positive patients have an earlier psoriasis onset. These data are similar with those previously reported for British, Czech, Spanish and Polish populations.
3. The TNF-alpha polymorphism -857C/T was associated with susceptibility to psoriatic arthritis similar with data reported for German and Canadian populations and with the axial pattern of the disease. The other two investigated TNF-α polymorphisms (-238 and -308) were not individually associated with this condition.

These results show that 857*T allele of TNF-α may represent a risk factor for psoriatic arthritis and for the axial pattern of this condition in the Romanian population.

The haplotypes 857T/308G/238G and 857C/308G/238G show an association with the disease, a marginal effect for 857C/308G/238G haplotype and a strong association for 857T/308G/238G haplotype which contains also the 857*T allele.

4. The investigated polymorphisms of HSP70 genes were not found to be associated with psoriatic arthritis.

5. For psoriatic arthritis group a marginally positive association was observed for the TNF-α-HSP70 extended haplotype 857T/308G/238G/1267A.

**Reactive arthritis**

1. For reactive arthritis, the HLA-B27 frequency in our group was lower than in other studies. HLA-B27 is associated also in the Romanian population with the susceptibility to this disorder.

2. The distribution of the -238G/A and -308G/A polymorphisms was not significantly different between patients and controls. These results have to be interpreted with caution because of the small size of the patient group.

**Undifferentiated spondyloarthritis**

1. For undifferentiated spondyloarthritis (USpA) patients, HLA-B27 was associated with the susceptibility of this condition, but the frequency in our Romanian USpA sample was significantly lower comparing with data reported in the literature. The HLA-B27 frequency in USpA is influenced by the prospective course of the disease, because at least some of the patients will develop ankylosing spondylitis.
2. No association was found with the three investigated TNF-\(\alpha\) polymorphisms. The association of -308*A allele with this condition in Mexican population can reflect not only the differences in the genetic background of the population, but also a future evolution of the investigated USpA patients to ankylosing spondylitis known as being associated with this polymorphism in different populations.

3. The results of this study show that the two investigated polymorphisms in the HSP70 genes do not contribute to the risk of undifferentiated spondyloarthritis in the Romanian population.

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