Research Article

Genetic Polymorphisms of Toll-like receptors 2 and 9 as Susceptibility Factors for the Development of Ankylosing Spondylitis and Psoriatic Arthritis

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1. Introduction

Spondyloarthritis (SpA) is a group of rheumatic diseases (RD) with immunological origin that presents chronic inflammatory and autoimmune conditions, and SpA shares clinical, serological, and genetic features, besides presenting a complex pathogenesis [1–3]. This group of diseases includes ankylosing spondylitis (AS), psoriatic arthritis (PsA), reactive arthritis (ReA), arthritis associated with inflammatory bowel disease (IBD), and undifferentiated arthritis (USpA) [1]. AS is the most prevalent clinical form of SpA. In Brazil, 65.1% of the cases of SpA are classified as AS while PsA represents 18.3% [4, 5]. A high number of SpA patients are HLA-B27 positive [1], a molecular marker already associated with AS.

1. Introduction

Spondyloarthritis (SpA) is a group of rheumatic diseases (RD) with immunological origin that presents chronic inflammatory and autoimmune conditions, and SpA shares clinical, serological, and genetic features, besides presenting a complex pathogenesis [1–3]. This group of diseases includes ankylosing spondylitis (AS), psoriatic arthritis (PsA), reactive arthritis (ReA), arthritis associated with inflammatory bowel disease (IBD), and undifferentiated arthritis (USpA) [1]. AS is the most prevalent clinical form of SpA. In Brazil, 65.1% of the cases of SpA are classified as AS while PsA represents 18.3% [4, 5]. A high number of SpA patients are HLA-B27 positive [1], a molecular marker already associated with AS.
In the Brazilian population, the presence of this antigen is around 69.5% [5, 6].

The innate immune response in RD can be stimulated in several cell types through the recognition of molecular patterns from external or internal sources, such as PAMPs or DAMPs (pathogen-associated molecular patterns and damage-associated molecular patterns, respectively) [7–11].

Toll-like receptors (TLRs) are the most well-characterized pattern recognition receptors (PRRs) and are a transmembrane protein coded by the toll genes family [7]. TLRs are expressed in different cell types including immune and nonimmune cells; they play a crucial role not only in the detection of many PAMPs and DAMPs but also in the activation and steering of the adaptive immune system by the upregulation of costimulatory molecules of the antigen-presenting cells [4, 7–10].

The endogenous TLR ligand-mediated signaling has an important role in autoimmune disorders [8]. The activation of some TLRs through the interaction of DAMPs can facilitate the repair of damaged tissues and the elimination of cell debris; on the other hand, this same interaction was associated with the chronic inflammatory process involved in RD [12]. Therefore, activation of TLR by DAMPS seems to play a role in the self-sustained inflammatory cycle and the progression of these chronic diseases [7].

The SpA can be considered as multifactorial diseases that have a pathogenesis involving interactions between the environment and genes [2]. The exact mechanisms of SpA immunopathogenesis have not yet been fully elucidated, and probably that other genes outside the MHC also contribute to this complex process.

There is still insufficient data to prove that polymorphisms in the TLRs genes are involved in autoimmunity processes and the development of RD [11]. However, some polymorphisms have already been associated with the pathogenesis of RD [4].

In light of this, the purpose of this study was to analyze the influence of important polymorphisms in TLR2, TLR6, and TLR9 genes in the immunopathogenesis of the two most common SpA, whereas no study involving these polymorphisms in SpA has been performed in the Brazilian population.

2. Materials and Methods

2.1. Ethics Statement. This study was approved by the Human Research Ethics Committee of the State University of Maringá—CEP-UEM 687.222/2014, and all volunteer participants signed the informed consent term.

2.2. Clinical Characterization. In this case-control study were included 529 subjects living in the south of Brazil. The patient group is composed of 149 unrelated subjects diagnosed with SpA by a single rheumatologist using the ASAS criteria [13], and in patients with PsA we also used the CASPAR criteria [14] to complement the ASAS criteria and thus provide greater security in the data obtained. The patients were attended at the rheumatology outpatient clinic of the University Hospital of Maringá (PR, Brazil), who presented one of the two most frequent clinical forms of SpA in the population (AS or PsA). All patients had magnetic resonance imaging of the sacroiliac joints and were evaluated for the presence of HLA-B27.

The control group consists of 380 healthy subjects, and the inclusion criteria in this group were absences of SpA or autoimmune and/or rheumatic disease, unrelated to subjects from the same group or patient group, and residence in the same geographical area as the patients. Both groups were age and sex matched.

2.3. Genotyping of TLR Genes. Based on previous published data on the association of TLR genes with autoimmune and inflammatory diseases, four SNPs were selected from three TLR genes. For the TLR2 gene (ENSG00000137462), the SNP rs5743708: G>A (2258G>A, Arg753Gln); for TLR6 gene (ENSG00000174130), the SNP rs5743810: C>T (745C>T, Ser249Pro); and for TLR9 gene (ENSG00000239732), the rs5743836: T>C (-1237T>C) and rs187084: T>C (-1486T>C) SNPs, both located in the promoter region of the TLR9. The primer design was according to Folwaczny et al. [15] for the TLR2 and to Selvaraj et al. [16] for TLR6 and TLR9 [15, 16].

The DNA was extracted from the buffy coat by using the salting-out technique [17]. The genotyping of the TLR SNPs was performed by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP). The PCR was carried out with 100 ng/μL DNA, 200 μM of each dNTP, 0.1 μM of each primer, 1.68 mM of MgCl2, 3 μL 10x PCR buffer, and 1.5 U Taq DNA polymerase (GoTaq® DNA Polymerase, Promega, USA), in a final volume of 15 μL. PCR products were digested using restriction enzymes, temperature, and time of digestion specifically for each SNP (see Table 1).

2.4. Genotyping of HLA-B*27. Genotyping of the HLA-B*27 was performed by PCR-SSP according to Oliveira et al. [18], and a genetic association analysis was performed between the presence or absence of HLA-B*27 and the TLR polymorphisms in both clinical forms [18].

2.5. Analysis of Disease Activity. Disease activity was assessed by BASDAI (Bath Ankylosing Spondylitis Disease Activity Index), considering patients with high disease activity when BASDAI ≥ 4 [1, 5]. The association analysis was performed among BASDAI and TLR polymorphisms.

2.6. Ethnic Classification. The ethnic composition of the Brazilian population is influenced by some races [19, 20], which is due to the high ethnic diversity, and considering the Paraná population according to Probst et al. [19], which is of predominantly European origin (80.5%) and has a small but significant contribution of African (12.5%) and Amerindian (7.0%) genes [19, 20].

In this study, the population was considered a mixed ethnic group. Thus, the risk of population stratification bias due to genetic differences presented by different ethnic groups was minimized with a comparison between patients and controls of the same ethnic origin.
| Gene | SNP       | Primer sequences                  | Restriction enzymes | Restriction temperature (°C) (time) | Length of the restriction fragments |
|------|-----------|-----------------------------------|---------------------|------------------------------------|-----------------------------------|
| TLR2 | rs5743708 | F: 5′-CATTCCCCAGCGCTTCTGCAAGCTCC-3′<br>R: 5′-GGAACCTAGGACTTTATCGCAGCTC-3′ | Msp I               | 37°C (overnight)                   | Allele G—104 bp + 25 bp<br>Allele A—210 bp |
|      |           |                                   |                     |                                    |                                   |
| TLR6 | rs5743810 | F: 5′-GCATTTCGAGCTTTCTATGTGC-3′<br>R: 5′-GCAAAAACCCCTCAACCTTTGT-3′ | Ava II              | 37°C (3 hours)                    | Allele C—50 bp + 160 bp<br>Allele T—210 bp |
|      |           |                                   |                     |                                    |                                   |
| TLR9 | rs5743836 | F: 5′-CTGTTTCATGGACTGTGT-3′<br>R: 5′-ATGGGAGCAGACAGACATAATGGA-3′ | Mva I               | 37°C (3 hours)                    | Allele C—27 bp + 48 bp + 60 bp<br>Allele T—34 bp + 111 bp |
|      |           |                                   |                     |                                    |                                   |
|      | rs187084  | F: 5′-TATCGTCTTTATGCTGAGCTGGATGT-3′<br>R: 5′-TGCCAGAGCTGACTGCTGG-3′ | Afl II              | 37°C (1 hour)                     | Allele T—34 bp + 111 bp<br>Allele C—145 bp |

F: forward standard; R: reverse standard; TLR: toll-like receptor; °C: degrees Celsius; bp: base pair.
Polymorphisms (including the two clinical forms) are shown in Table 4. Results and allelic groups. All tests were performed at a significance level of 5%.

3. Results

The mean age for the patient group was 49.5 ± 15 years, composed of 47% men and 53% women (see Table 2). The patients analyzed presented two clinical forms of SpA: AS (95/63.8%) and PsA (54/36.2%) (see Table 3).

The genotypic frequency distribution of all SNPs was in the Hardy–Weinberg equilibrium in both analyzed groups. The codominant model was selected as best inheritance model to analyze the association between SNPs and the risk of developing SpA.

No association was observed among HLA-B*27 and TLR polymorphisms ($p = 0.72$), nor among BASDAI and TLR polymorphisms ($p = 0.85$). The allele and genotype frequencies of the polymorphisms analyzed in patients and controls (including the two clinical forms) are shown in Table 4.

The variables analyzed in the logistic regression test were gender and age between controls and patients; these results are described below.

The presence of the rs5743708*G/A for the TLR2 gene was a susceptibility factor to SpA for males (OR = 12.27, CI = 3.95 – 38.12) and females (OR = 3.56, CI = 1.29 – 9.86), but the risk is higher in men with SpA. However, the polymorphism of the TLR6 gene was not significantly related to the susceptibility to SpA.

The rs5743836*C for the TLR9 gene was associated with susceptibility to SpA (OR = 1.62, CI = 1.20 – 2.21, and the rs5743836*T/C presented risk for males (OR = 1.84, CI = 1.30 – 3.28), but the rs5743836*C/C presented risk for females (OR = 9.75, CI = 1.88 – 15.43). Both genotypes associated with risk of SpA development present the rs5743836*C; however, two copies of this allele seem to only affect women SpA.

The TLR9 rs187084*T/C was associated with susceptibility to develop SpA only in male patients (OR = 2.51, CI = 1.12 – 5.65).

### Table 2: Demographic data in control and SpA patients for the analyzed SNPs.

|                | TLR2 rs5743708 | TLR6 rs5743810 | TLR9 rs5743836 | TLR9 rs187084 |
|----------------|---------------|---------------|----------------|---------------|
|                | Controls $n = 380$ | Patients $n = 149$ | Controls $n = 221$ | Patients $n = 149$ | Controls $n = 380$ | Patients $n = 149$ | Controls $n = 221$ | Patients $n = 149$ |
| Age (years) ± mean | 58.4 ± 15.4 | 49.5 ± 15 | 48.9 ± 13.7 | 49.5 ± 15 | 58.4 ± 15.4 | 49.5 ± 15 | 48.9 ± 13.7 | 49.5 ± 15 |
| Gender (n as %) Female | 200 (53) | 78 (53) | 113 (51.3) | 78 (53) | 200 (53) | 78 (53) | 113 (51.3) | 78 (53) |
| Male | 180 (47) | 71 (47) | 108 (48.7) | 71 (47) | 180 (47) | 71 (47) | 108 (48.7) | 71 (47) |

### Table 3: Clinical characteristics of patient groups.

|                | SpA $n = 149$ | AS $n = 95$ | PsA $n = 54$ |
|----------------|--------------|-------------|-------------|
| Female/male (n as %) | 79/70 (53.0/47.0) | 51/44 (52.7/47.3) | 28/26 (51.8/48.1) |
| Family history SpA (n as %) | 39 (58.1) | 9 (13.4) | 30 (44.7) |
| BASDAI ≥ 4 (n as %) | 104 (69.8) | 69 (73.4) | 35 (65.5) |
| Disease duration-years (mean ± SD) | 11.6 ± 8.6 | 10.8 ± 8 | 13.2 ± 10 |
| Treatment time-years (mean ± SD) | 4 ± 3.7 | 7.8 ± 7.9 | 6.3 ± 5.9 |
| HLA-B*27 (n as %) Present | 83 (55.7) | 53 (55.8) | 30 (55.5) |
| Absent | 66 (44.3) | 42 (44.2) | 24 (44.5) |

SpA: patients with spondyloarthritis; AS: ankylosing spondylitis; PsA: psoriatic arthritis; BASDAI: Bath Ankylosing Spondylitis Disease Activity Index.

2.7. Statistical Analysis. The allele and genotype frequencies were estimated and compared by chi-square distribution tables with Fisher’s corrections, and the Hardy–Weinberg equilibrium was tested using the Arlequin and SNPStats software [21].

The logistic regression analysis was performed, and the choice of the best inheritance model was performed using the Akaike information (AIC) in order to minimize the expected entropy. Analysis of the linkage disequilibrium (Δ′) between the TLR SNPs was calculated by the SNPStats software. The EM algorithm or the Markov chain Monte Carlo method was used for the estimation of haplotypes and allelic groups. All tests were performed at a significance level of 5%.

The three TLR9 haplotypes (rs5743836_ rs187084) were associated with risk to SpA: the CC haplotype (OR = 4.65; CI = 2.44-8.89; $p < 0.0001$); the CT (OR = 3.02; CI = 1.62-5.63; $p = 0.00006$); and the TC (OR = 1.67; CI = 1.20-2.75, $p = 0.043$), and CC haplotype showed linkage disequilibrium...
Δ′ = 0.897, \( p < 0.05 \). However, the analysis of allelic groups \( \text{rs5743836}_\text{rs187084}_\text{rs5743708}_\text{rs5743810} \) did not show linkage disequilibrium (Δ′ = 0.1223, \( p > 0.05 \)).

### 4. Discussion

To the best of our knowledge, this is the first study evaluating TLR2, TLR6, and TLR9 gene polymorphisms in the immunopathogenesis of SpA. The genetic variability of TLRs was featured with an involvement in the susceptibility in inflammatory diseases [7, 22].

Our findings suggest that the polymorphisms analyzed for Toll-like receptor genes may contribute to the development of the immunopathogenesis of SpA, without influence of the presence of antigen HLA-B27 or disease activity. The association of other genes in the development of SpA without presence of HLA-B27 antigen was described in the literature by our group, which may indicate that other genetic markers may be involved in SpA [23].

In this present study, the TLR2 gene rs5743708*A polymorphism increased the chance of developing SpA by 10-fold. Furthermore, the presence of homozygote \( \text{rs5743708}_A/A \) was not observed among the controls, confirming our findings that the presence of the altered allele \( A \) may influence the development of SpA. This polymorphism has a very low frequency in the population (<1%) and this allele has been implicated in the risk
phenotype [24]. In this way, our data confirm that this polymorphism is a susceptibility factor, also among the spondyloarthritides, in both clinical forms analyzed.

The TLR2 rs5743708 is a missense variant which affects the structure of the TLR-2 protein in the intracellular region and generates a nonfunctional protein, due to a replacement of an amino acid arginine for glycine at position 753 of the protein (Arg753Gln) [25–27]. This change in TLR-2 reduces the activation of NF-κB pathway and compromises the intracellular signaling cascade [25, 26]. The recognition of PAMPs by these nonfunctional TLR-2 is impaired, leading to failures in the recognition mechanism of the extracellular pathogens, such as the Gram negative/positive bacteria [25, 26]. However, the consequences of this nonfunctional protein are not restricted only to the recognition of pathogens, since TLR-2 activates inflammation through canonical and noncanonical NF-κB pathway [28].

The NF-κB pathway is an important cellular pathway of innate and adaptive immune response, and influences the expression of many genes involved in the regulation of the major processes of activation of the immune response [28]. And the expression of this factor is a major regulator of inflammation and can be active by TLRs (mainly TLR-2 and TLR-9) [28]. There are increasing evidences that suggest a role of NF-κB signaling in the development of various RD; the main genes associated with RD by genome-wide association studies (GWAS) show a correlation with this transcription factor and the production of proinflammatory cytokines [11].

Niebuhr et al. [29] demonstrated a change in the cytokine profile produced by monocytes in patients with the polymorphism rs5743708 [29]. Levels of IL-12 and IL-6 cytokines were significantly increased, which could explain the increased inflammation in the skin of patients with atopic dermatitis who presented this TLR2 mutation [29, 30]. Thus, this polymorphism may also affect the profile of the IL-12 cytokine profile in patients with SpA, since high levels of IL- 12 are found in psoriatic lesions and synovial involvement, and the Th1 inflammatory cascade is supported by high levels of IL-12 and IL-6 [31].

Another pattern of cytokines that is modified by the presence of rs5743708 is the IL-8 profile; as discussed by Nedoszytko and Renke [25], the Th2 response appears to be enhanced by this polymorphism, impairing IL-8 production and may also affect neutrophil adhesion by an increase in IgE levels [25].

The TLR6 polymorphisms suggest that this variation does not present a direct involvement in the development of SpA. However, the frequencies observed for the rs5743810 in this study for patients and controls (21% and 23%) were higher than the world population frequency of 12% [24].

The two analyzed polymorphisms in the TLR9 gene promoter region have been linked to autoimmunity and gene transcription rate [25, 32–34]. These polymorphisms are regulatory region variants of the TLR9 gene; the C allele of both rs5743836 and rs187084 polymorphisms is located in the regions of CTF-binding sites and sites of several transcription factors (TF) [24].

The rs5743836*C creates new NF-κB sites in the promoter region of TLR9 gene [25], and in silico, it has been observed that this allele generates new multiple binding sites for different transcription factors [34]. Furthermore, there is an increase in the expression rate of TLR9 mRNA by stimulation of IL-6, creating a positive feedback loop that amplifies TLR9 signaling through IL-6 in the presence of the rs5743836*T/C genotype and also interfering in proliferation rate of B cells [34].

Our data suggest that TLR9 rs5743836*C is a susceptibility factor regardless of gender or age, increasing by 1.69-fold the susceptibility to SpA. In addition, rs5743836*T/C and rs5743836*C/C genotypes are an even greater risk factor for patients with PsA. Although there are lacking studies confirming the role of TLR9 rs5743836 in SpA, there are studies that have previously associated the rs5743836*C to the development of rheumatoid arthritis (RA) in women [28]. These data corroborate to ours, once that women with SpA, carrier of rs5743836*C, are at a higher risk than men with rs5743836*C.

The rs187084 is associated with increased production of INF-γ and TNF-α in rs187084*T/T subjects, and the rs187084*C increases the transcriptional rates of TLR9 [28]. This polymorphism has been associated with susceptibility to diseases, and in the present study a susceptibility was observed only for men with rs187084*T/C, according to the predisposition already described for men to the development of SpA [28, 35, 36].

Gebura et al. [28] discussed the association among rs187084*T and increased IFN-γ and TNF-α productions in RA patients compared to healthy subjects; however, in RA patients, this allele presented a less favorable response to therapies with TNF-α inhibitors [28]. Likewise, patients with PsA do not respond to anti-TNF treatment [37]; this polymorphism in the TLR9 promoter could be influencing cellular and molecular mechanisms in this disease.

In this study, the rs187084, rs5743836, and rs5743708 polymorphisms were associated with the risk of SpA development, wherein, according to the literature, these mutations may lead to significant changes in the innate and adaptive immune response profile, as well as in the maintenance of the regulation of immunological mechanisms. In light of these, we may be inferring that these polymorphisms contribute to potentiate the Th1, Th2, and Th17 immune response seen in SpA, which may confer to individuals carrying the polymorphisms a predisposition to the development of SpA.

5. Conclusions

Our findings suggest that the polymorphisms analyzed for Toll-like receptor genes may contribute to the development of the immunopathogenesis of SpA, independently of the presence of antigen HLA-B27.

The polymorphisms analyzed for Toll-like receptors genes may contribute to the development of the immunopathogenesis of ankylosing spondylitis and psoriatic arthritis. The polymorphisms rs5743708 for the TLR2 and the rs187084_r5743836 TLR9 haplotypes appear to be involved in the
development of clinical forms of PsA and can be a possible therapeutic target for the rheumatic diseases. However, further studies are needed to more clearly understand the influence of the immunogenetics of these polymorphisms in the development of SpA, and these observations should be interpreted with caution due to limitations found in this study, such as the relatively small sample size and the fact that there were no analyses of the expression of the TLR genes nor of the cytokines involved in the SpA development process.

Data Availability

The authors declare that all the data that support the results of this study are available in the article.

Disclosure

Preliminary data was presented, in part, at the 2016 and 2017 ID Week and 2016 International Society for Disease Surveillance Annual Conferences.

Conflicts of Interest

All authors state that potential conflicts do not exist.

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