X-linked TLR7 gene polymorphisms are associated with diverse immunological conditions but not with discoid lupus erythematosus in Polish patients

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Abstract

Introduction: Toll-like receptor 7 (TLR7) is an important molecule involved in the development of autoimmunity and the response to different pathogens. Several polymorphisms within the TLR7 gene were previously found to be associated with systemic lupus erythematosus (SLE). However, none of those studies investigated the TLR7 promoter flanking variants rs1634318 and rs1616583. TLR7 gene diversity has not been analyzed with respect to discoid lupus erythematosus (DLE) development, while its role in the human immunological response to fungal infection is not fully known.

Aim: To clarify the potential involvement of two novel single-nucleotide polymorphisms (SNPs) located in the TLR7 gene (rs1634318 and rs1616583) in a variety of immune-related conditions, we studied the variability of these loci in patients from a Polish population with SLE and DLE, as well as in immunocompromised patients who were affected by invasive aspergillosis (IA) and those who were not affected.

Material and methods: Real-time polymerase chain reaction was used to genotype SNPs. Statistically significant differences between case and control groups for both allele and genotype frequencies were assessed using the \( \chi^2 \) test with Yates’ correction or two-tailed Fisher’s exact test. The results were Bonferroni-corrected for multiple comparisons and odds ratios were calculated.

Results: Two polymorphisms located in TLR7 might be associated with the development of SLE but not DLE within the Polish population. Moreover, variation of the two investigated SNPs was found to be associated with IA in immunocompromised Polish patients.

Conclusions: In Polish patients, TLR7 promoter flanking gene polymorphisms might be associated with IA and SLE but not DLE.

Key words: discoid lupus erythematosus, systemic lupus erythematosus, aspergillosis, single-nucleotide polymorphism, allele, polymorphism.

Introduction

Toll-like receptor 7 (TLR7) is a crucial component of the innate immune system. It plays an essential role in pathogen recognition and the development of autoimmunity. Studies in mice have shown that TLR7 is important for host immunity against various fungal species [1–6]. However, very little is known about its involvement in human responses to fungal infection. The human TLR7 gene, which is about 23 kbp in size, is located on the X chromosome and consists of three exons. Our massively parallel targeted resequencing (MPS) of fifteen innate immunity genes in leukemia patients who were affected by invasive aspergillosis (IA) and control patients

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Notably, both sequence variability and the expression of TLR7 have been previously implicated in the development of systemic lupus erythematosus (SLE), a heterogeneous autoimmune disease that primarily affects women (female-to-male ratio of approximately 9 : 1) and manifests with a variety of clinical symptoms [9, 10]. Interestingly, males with an additional X chromosome have a risk of developing SLE that is approximately 14 times higher than XY males. Conversely, females with only one X chromosome and SLE are very rare [11–14]. Rs3853839 (located within the 3′ UTR of TLR7) has been found to be strongly associated with SLE in Asian populations [15]. Moreover, the rs3853839 “G” allele (associated with SLE) has been reported to increase the expressions of both TLR7 and type I interferon-regulated genes [15], the latter being the molecular signature of SLE pathogenesis. TLR7 sequence variability in discoid lupus erythematosus (DLE) has not yet been studied, although we recently reported that DLE and SLE might have different molecular signatures in the Polish population [16]. Overall, previous findings suggest that TLR7 might be involved in a variety of immune-related conditions. This indicates the need for further research into TLR7 gene variability in ethnically homogeneous groups of patients affected by different immune disorders.

Aim

In this study we investigated whether two TLR7 promoter flanking variants (rs1634318 and rs1616583), suggested to be potentially functional by our previous research [7], are associated with the development of either SLE or DLE within the Polish population. We also included enlarged samples of immunocompromised Polish individuals to verify whether these two SNPs are associated with IA.

Material and methods

The study was approved by the Bioethics Committee of the Ludwik Rydygier Collegium Medicum, Nicolaus Copernicus University in Bydgoszcz, Poland (statements no. KB 605/2011, KB 223/2013, and KB 562/2013). All adult patients and healthy subjects gave written informed consent to participate in the study. Parents of all adolescent patients gave written informed consent for their child to participate in the research.

Patients and clinical data

Sixty-five unrelated pediatric patients with leukemia (31 individuals diagnosed with IA and 34 persons without IA) were recruited to the study from the Polish population. None of the pediatric patients underwent hematopoietic stem cell transplantation. Buccal swabs from patients were collected at the Department of Pediatrics, Hematology and Oncology in Bydgoszcz (Poland). The clinical characteristics of the study subjects are given in Table 1.

Discoid lupus erythematosus was diagnosed according to commonly accepted clinical, histological, and immunofluorescence findings [9]. Systemic lupus erythematosus was diagnosed according to revised American College of Rheumatology criteria [10]. Altogether, 35 patients with DLE and 84 patients with SLE from the Polish population were recruited to the study. Clinical data were obtained from medical records (Table 2). The control group consisted of 100 unrelated, healthy adults from Poland (50% female). Buccal swabs or blood samples were collected from subjects with DLE and SLE, and from healthy subjects at the Department of Dermatology, Sexually Transmitted Diseases and Immunodermatology in Bydgoszcz (Poland) and the Department of Dermatology and Venereology in Lodz (Poland).

Genotyping

DNA was extracted from blood samples or buccal swabs using the GeneMatrix Bio-Trace DNA Purification Kit (Eurx, Gdansk, Poland) according to the manufacturer’s instructions. The TLR7 gene polymorphisms rs1634318 and rs1616583 were genotyped using real-time PCR using TaqMan assays (Life Technologies, Carlsbad, CA, USA) on a ViiA 7 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Genotyping was performed according to the manufacturer’s protocols. Allele discrimination was achieved by fluorescence detection.

Table 1. Clinical and demographic characteristics of Polish patients with leukemia used as subjects in this study

| Parameter | Cases, n (%) | Controls, n (%) |
|-----------|--------------|----------------|
| Total     | 31           | 34             |
| Females   | 11 (35.5)    | 9 (26.5)       |
| Mean age [years] | 14.1 ±4.32 | 10.2 ±4.1 |
| AML       | 9 (29.0)     | 2 (5.9)        |
| ALL       | 22 (71.0)    | 32 (94.1)      |
| Pulmonary aspergillosis | 27 (87.1) | 0 (0)         |
| Pulmonary + extrapulmonary aspergillosis | 4 (12.9) | 0 (0) |

Cases – leukemia patients with invasive aspergillosis, Controls – leukemia patients without invasive aspergillosis, AML – acute myeloid leukemia, ALL – acute lymphoblastic leukemia.
Table 2. Clinical and demographic characteristics of patients from a Polish population with DLE and SLE

| Parameter                  | DLE, n (%) | SLE, n (%) |
|----------------------------|------------|------------|
| Total                      | 35         | 84         |
| Females                    | 20 (57.1)  | 74 (88.1)  |
| Mean age [years]           | 56.6 ±13.4 | 52.4 ±12.7 |
| Malar rash                 | 13 (37.1)  | 66 (78.6)  |
| Discoid rash               | 29 (82.9)  | 11 (13.1)  |
| Photosensitivity           | 30 (85.7)  | 80 (95.2)  |
| Oral ulcers                | 5 (14.3)   | 22 (26.2)  |
| Arthritis                  | 0 (0.0)    | 36 (42.9)  |
| Serositis                  | 0 (0.0)    | 4 (4.8)    |
| Renal disorder             | 0 (0.0)    | 21 (25.0)  |
| Neurological disorder      | 0 (0.0)    | 19 (22.6)  |
| Hematological disorder     | 0 (0.0)    | 51 (60.7)  |
| ANA positive               | 24 (68.6)  | 80 (95.2)  |

**Figure 1.** Linkage disequilibrium plot of the analyzed SNPs displayed with $R^2$ indicated by a black and white color scheme. Black color indicates very high linkage disequilibrium ($R^2$ = 1). By contrast, white color ($R^2$ = 0) illustrates the absence of a correlation between SNPs.

**Data analysis**

Genotype distributions of each SNP were tested for deviation from Hardy-Weinberg equilibrium using Haploview software, v. 4.2. [17]. The same software was used to determine linkage disequilibrium (LD) patterns of neighboring SNPs. Statistically significant differences between case and control groups in both allele (in males and females) and genotype frequencies (females only) were assessed using the two-tailed Fisher’s exact test or the $\chi^2$ test with Yates’ correction. Associations between clinical manifestations and allele or genotype distributions in patients with SLE vs control subjects were determined by the two-tailed Fisher’s exact test. The odds ratios (OR) and 95% confidence intervals (95% CI) were also calculated. A $p$-value $< 0.05$ was considered to be statistically significant. Correction for multiple testing was performed using the Bonferroni adjustment.

**Statistical analysis**

Haplotypes were determined using Haploview software v. 4.2. [17] and statistically significant differences between case and control groups in haplotype frequencies were calculated. Unless otherwise indicated, all analyses were performed using Statistica, v. 13.1 software (StatSoft Inc, Round Rock, TX, USA).

**Results**

Two SNPs (rs1634318 and rs1616583) located in the TLR7 gene were successfully genotyped in all patients with leukemia and lupus erythematosus, as well as in healthy subjects. The allelic frequencies of rs1634318 and rs1616583 loci were in Hardy-Weinberg equilibrium in females with leukemia who had IA and in those without IA, and in patients who had DLE and SLE and healthy subjects. The allele and genotype frequencies in all groups are given in Tables 3 and 4. The investigated SNPs showed strong linkage disequilibrium (Figure 1).

**Associations of TLR7 polymorphisms with aspergillosis**

First, we examined the potential associations between genetic variability in rs1634318 and rs1616583 loci and acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), and IA. The differences in allele frequencies at the two analyzed TLR7 loci were not significantly different between healthy controls and either group of leukemia patients. The only statistically significant differences in allele frequencies that were found were between leukemia patients diagnosed with aspergillosis and those without aspergillosis (Table 3). Indeed, the C allele in rs1634318 and the G allele in rs1616583 were found in leukemia patients diagnosed with aspergillosis at frequencies that were approximately four times higher than those of leukemia patients without aspergillosis ($p = 0.0261$, OR = 5.5909 with 95% CI: 1.1296–27.6725). However, these differences did not reach statistical significance after Bonferroni correction ($p_B = 0.0522$; Table 3). Furthermore, when males and females were considered separately there was no statistically significant difference in allele frequencies between the case and control groups.
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Next, we checked for potential associations between genotype or haplotype distributions and AML, ALL, and IA in females. The differences in genotype or haplotype frequencies between the female case and control groups were not statistically significant.

Associations of TLR7 gene polymorphisms with SLE

We also investigated whether rs1634318 and rs1616583 loci were associated with SLE development. We found that the frequencies of the C allele in rs1634318 and the G allele in rs1616583 were approximately twice as high in all patients with SLE as those of healthy subjects ($p = 0.0306, OR = 2.1833$ with 95% CI: 1.498–112.8141) in females with SLE, but the results were not significant after Bonferroni correction ($p_B = 0.0848$). Allele frequencies were not significantly different between male subjects with SLE and healthy individuals (Table 4).

Differences between females with SLE and healthy women were not statistically significantly different for any genotype (Table 4).

We subsequently analyzed haplotypes formed by these two SNPs in females in order to assess potential associations with susceptibility to SLE. Comparison of haplotype distributions in females with SLE and healthy women showed that the “C-G” haplotype (formed by two SNPs, rs1634318 and rs1616583, respectively) was associated with SLE ($p = 0.028, p$-value after 10 000 permutations = 0.0497, OR = 2.1511 with 95% CI: 1.0754–4.3029). However, none of the reconstructed haplotypes were associated with clinical outcomes in female patients with SLE.

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Table 3. Associations between SNPs and IA in Polish patients with leukemia

| SNP      | Allele/genotype* | Frequency | Fisher's p-value | Bonferroni corrected p-value ($p_B$) |
|----------|------------------|-----------|-----------------|-------------------------------------|
|          | IA positive | IA negative |                  |                                     |
| rs1634318 | T (all)     | 0.786 | 0.953            | 0.0261                              | 0.0522                              |
|          | C (all)     | 0.214 | 0.047            |                                    |                                     |
|          | T (female) | 0.727 | 0.944            | 0.1048                              | 0.2096                              |
| rs1616583 | C (female) | 0.273 | 0.056            |                                    |                                     |
|          | T (male)   | 0.850 | 0.960            | 0.3087                              | 0.6174                              |
|          | C (male)   | 0.150 | 0.040            |                                    |                                     |
|          | TT (female)| 0.545 | 0.889            | 0.1571                              | 0.3142                              |
|          | CT (female)| 0.364 | 0.111            | 0.3189                              | 0.6378                              |
|          | CC (female)| 0.091 | 0.000            | 1.000                               | 1.000                               |

*Genotyping results for 20 IA-positive and 20 IA-negative individuals were previously published in an MPS-based (massively parallel sequencing) study dedicated to the discovery of novel variants in fifteen complete human immunity genes [7].
Associations of TLR7 gene polymorphisms with DLE

The analysis of allele distributions in patients with DLE and healthy controls performed for all populations, as well as for each sex separately, showed no statistically significant differences in the allele frequencies of rs1634318 and rs1616583 (Table 4). Moreover, the genotype frequencies were not significantly different between healthy individuals and female subjects with DLE (Table 4). Furthermore, we did not identify any haplotype that was associated with or protective against the development of DLE in females.

Discussion

Toll-like receptors are considered to be key players in regulating inflammatory reactions and immune responses to pathogens due to their ability to recognize nucleic acids derived from both the host and infectious agents. In autoimmune diseases such as SLE, TLRs 7, 8, and 9 are believed to play a pivotal role in the activation and regulation of dendritic cells and B cells responsible for antibody production [18]. Because SLE predominantly affects women, there is particular interest in research on the X-linked TLR7 gene and its functionally related TLR8 gene. Indeed, a study of TLR7 rs3853839 conducted on approximately 9000 patients with SLE and 10,000 control subjects of Asian ancestry showed a strong association of the G allele with the development of SLE [15]. Moreover, this genetic variant in Asians was associated with an increased TLR7 mRNA level, which further led to increased expression of type I interferon-regulated genes [15]. Interestingly, TLR7 is expressed in plasmacytoid dendritic cells and recognizes a single-stranded RNA motif. Notably, the immune complexes containing RNA motifs are often observed in the sera of patients with SLE. These complexes can further activate TLR7 signaling, which in turn stimulates plasmacytoid dendritic cells to produce type I interferon, the latter being frequently observed in patients with SLE. The results of other studies have also confirmed the involvement of TLR7 rs3853839 in the development of SLE in Japanese, Taiwanese, and Danish populations.

Table 4. Associations between SNPs and DLE or SLE in a Polish population

| SNP     | Allele/genotype | Frequency Controls | Frequency SLE | Fisher's p-value | Bonferroni corrected p-value (pF) | Frequency DLE | Fisher's p-value | Bonferroni corrected p-value (pF) |
|---------|-----------------|-------------------|---------------|-----------------|-----------------------------------|---------------|-----------------|-----------------------------------|
| rs1634318 | T (all)         | 0.087            | 0.759         | 0.0153*         | 0.0306                             | 0.893         | 0.8875*         | 1.000                             |
|         | C (all)         | 0.127            | 0.241         |                 |                                    |               |                 |                                    |
|         | T (female)      | 0.087            | 0.757         | 0.0419*         | 0.0838                             | 0.875         | 1.0000          | 1.0000                            |
|         | C (female)      | 0.130            | 0.243         |                 |                                    | 0.125         |                 |                                    |
|         | T (male)        | 0.088            | 0.800         | 0.6096         | 1.0000                             | 0.933         | 0.4372         | 0.8744                            |
|         | C (male)        | 0.120            | 0.200         |                 |                                    | 0.067         |                 |                                    |
|         | TT (female)     | 0.076            | 0.608         | 0.1168*         | 0.2336                             | 0.762         | 0.7773         | 1.0000                            |
|         | CT (female)     | 0.127            | 0.297         | 0.4543*         | 0.9086                             | 0.238         | 1.0000         | 1.0000                            |
|         | CC (female)     | 0.020            | 0.095         | 0.1984*         | 0.3968                             | 0.000         | 0.5149         | 1.0000                            |
|         | C (all)         | 0.087            | 0.759         | 0.0153*         | 0.0306                             | 0.893         | 0.8875*         | 1.0000                            |
|         | G (all)         | 0.127            | 0.241         |                 |                                    | 0.107         |                 |                                    |
|         | C (female)      | 0.087            | 0.757         | 0.0419*         | 0.0838                             | 0.875         | 1.0000         | 1.0000                            |
|         | G (female)      | 0.130            | 0.243         |                 |                                    | 0.125         |                 |                                    |
|         | C (male)        | 0.088            | 0.800         | 0.6096         | 1.0000                             | 0.933         | 0.4372         | 0.8744                            |
|         | G (male)        | 0.120            | 0.200         |                 |                                    | 0.067         |                 |                                    |
|         | CC (female)     | 0.076            | 0.608         | 0.1168*         | 0.2336                             | 0.762         | 0.7773         | 1.0000                            |
|         | CG (female)     | 0.127            | 0.297         | 0.4543*         | 0.9086                             | 0.238         | 1.0000         | 1.0000                            |
|         | GG (female)     | 0.020            | 0.095         | 0.1984*         | 0.3968                             | 0.000         | 0.5149         | 1.0000                            |

*p-value for χ² test with Yates correction.
TLR7 gene polymorphisms are associated with diverse immunological conditions but not with discoid lupus erythematosus in Polish patients

Further studies revealed two novel TLR7 SNPs, rs179010 and rs179019, that were associated with SLE in Japanese patients. However, the analysis of rs179010 in a Taiwanese population did not reveal an association between this SNP and SLE. Moreover, rs1634318 was also found to be associated with SLE in a Chinese population [22]. A recent meta-analysis also indicated an association between TLR7 rs179008 and SLE in Africanans, but not in a population of European ancestry [18]. Taken together, these data implicate TLR7 variability in SLE pathogenesis and simultaneously suggest the importance of population structure and the biogeographical ancestry of the study subjects.

In this paper we present, for the first time, an association of two novel TLR7 variants, rs1634318 and rs1616583, with SLE in a population of Polish patients. We found that the frequencies of the C allele in rs1634318 and the G allele in rs1616583 were approximately twice as high in all SLE patients compared with healthy subjects. However, the statistical power of these results was only ~44%; further studies performed on at least 258 cases and 258 controls are needed to fully elucidate the associations between rs1634318 and rs1616583 and SLE in the Polish population. Moreover, associations between rs1634318 and rs1616583 and SLE were found when allele frequencies were compared only in females. Additionally, we revealed in this study that both SNPs are in strong linkage disequilibrium and the “C-G” haplotype (formed by rs1634318 and subsequent rs1616583, respectively) was associated with SLE in females. As these two SNPs are located in a regulatory element of the TLR7 gene [7], genetic variation at these positions may potentially influence binding of the transcription factors in the TLR7 gene. In consequence, these two TLR7 gene polymorphisms could potentially affect TLR7 transcription, which could further change the expression of type I interferon-regulated genes and cause an SLE phenotype. However, additional association-based and functional studies are required to test this hypothesis.

By contrast, the allele frequencies of rs1634318 and rs1616583 in patients with DLE were similar to those observed in healthy individuals. This observation suggests that the studied variation in TLR7 is not related to the development of DLE in the Polish population. Remarkably, our previous research into SNPs located in ITGAM, TNXB, and STAT4 genes showed that only STAT4 might be associated with DLE in the Polish population. Therefore, the SNPs in ITGAM and TNXB [16], as well as the two TLR7 SNPs reported here, appear to differentiate patients with SLE from those with DLE. Thus, the results of our study reveal further differences in the molecular backgrounds of DLE and SLE. Nevertheless, further studies performed on larger groups of patients representing different ethnicities are still needed to clarify the significance of TLR7 polymorphisms in the susceptibility of patients to DLE.

Because TLR7 variants rs1634318 and rs1616583 were initially discovered in our previous screening research focused on IA in immunocompromised individuals [7], in this study we used enlarged samples of leukemia patients to verify the putative implication of these SNPs in the development of IA. In this respect, it is worth noting that most of the TLR7 gene polymorphisms (five out of seven) suggested by our previous study as being associated with IA were located within intron regions but their functional in silico analysis showed no consequences on the splicing process or gene expression [7]. However, TLR7 variants rs1634318 and rs1616583 constituted notable exceptions due to their localization in promoter flanking regions that may contain transcription factor binding sites. Our present results confirm associations between IA and the C allele in rs1634318 and the G allele in rs1616583. However, these associations should be further evaluated based on a larger cohort of subjects (at least 96 individuals in each group) because the statistical power of the current data is only ~38% and differences between cases and controls in allele frequencies did not reach statistical significance after Bonferroni correction. We also note that data indicating a lack of association between genotype or haplotype frequencies and IA should be considered preliminary, because the case and control groups consisted of only small numbers of females (11 vs. 9 individuals, respectively). However, the implicated role of the investigated TLR7 SNPs in an autoimmune condition (SLE) shown in this study, as well as their putative involvement in pathogen-host interactions in IA, suggests that a common mechanism underlies the action of these potentially functional variants, presumably based on regulation of TLR7 expression. Considering these and previous findings, further studies based on large sample sizes of patients affected by a variety of immune-related conditions are needed to reconstruct with more precision the involvement of TLR7 variability in autoimmunity and pathogen recognition.

Conclusions

Overall, the findings of this study indicate the involvement of two novel TLR7 variants, rs1634318 and rs1616583, in the development of SLE (but not DLE) and their putative role in pathogen recognition in IA by a common mechanism based, presumably, on regulation of TLR7 gene expression.

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Conflict of interest

The authors declare no conflict of interest.
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