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 χ^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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revealed seven *TLR7* single-nucleotide polymorphisms (SNPs) or indels that could potentially be associated with IA [7]. Among them, two SNPs, rs1634318 and rs1616583, are located in the promoter-flanking region, a regulatory part of the *TLR7* gene that may contain binding sites for transcription factors [8]. Considering this, genetic variability in this promoter-flanking region could have a modulatory influence on *TLR7* gene expression, ultimately affecting pathogen recognition.

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)

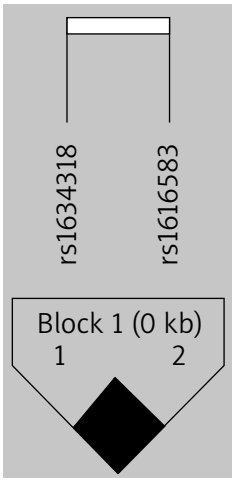

Figure 1. Linkage disequilibrium plot of the analyzed SNPs displayed with R^2 indicated by a black and white color scheme. Black color indicates very high linkage disequilibrium ($R^2 = 1$). By contrast, white color ($R^2 = 0$) illustrates the absence of a correlation between SNPs

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 χ^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. Moreover, the allele distributions in AML patients were similar to those observed in ALL 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.

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
	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
rs1616583	C (all)	0.786	0.953	0.0261	0.0522
	G (all)	0.214	0.047		
	C (female)	0.727	0.944	0.1048	0.2096
	G (female)	0.273	0.056		
	C (male)	0.850	0.960	0.3087	0.6174
	G (male)	0.150	0.040		
	CC (female)	0.545	0.889	0.1571	0.3142
	CG (female)	0.364	0.111	0.3189	0.6378
	GG (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].*

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_B = 0.0306, OR = 2.1833 with 95% CI: 1.1936–3.9936). However, we did not uncover any associations between these alleles and the clinical outcomes of patients with SLE (specified in Table 2). Frequencies of the C allele in rs1634318 and the G allele in rs1616583 were also found to be nearly two times higher when compared only for females (p = 0.0419, OR = 2.1511 with 95% CI: 1.0754–4.3029). However, these differences were not statistically significant after Bonferroni correction (p_B = 0.0838). Moreover, we found associations of these alleles with serositis (p = 0.0424, OR = 13 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.

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

SNP	Allele/ genotype	Frequency		Fisher's *p*-value	Bonferroni corrected *p*-value (p_B)	Frequency DLE	Fisher's *p*-value	Bonferroni corrected *p*-value (p_B)
		Controls	SLE					
rs1634318	T (all)	0.873	0.759	0.0153*	0.0306	0.893	0.8875*	1.0000
	C (all)	0.127	0.241			0.107		
	T (female)	0.870	0.757	0.0419*	0.0838	0.875	1.0000	1.0000
	C (female)	0.130	0.243			0.125		
	T (male)	0.880	0.800	0.6096	1.0000	0.933	0.4372	0.8744
	C (male)	0.120	0.200			0.067		
	TT (female)	0.760	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
rs1616583	C (all)	0.873	0.759	0.0153*	0.0306	0.893	0.8875*	1.0000
	G (all)	0.127	0.241			0.107		
	C (female)	0.870	0.757	0.0419*	0.0838	0.875	1.0000	1.0000
	G (female)	0.130	0.243			0.125		
	C (male)	0.880	0.800	0.6096	1.0000	0.933	0.4372	0.8744
	G (male)	0.120	0.200			0.067		
	CC (female)	0.760	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 χ^2 test with Yates correction.*

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 [19–21]. 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, rs1634323 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 Africans, 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 autoimmunological 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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