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Perica, Marija; Vidović, Mandica; Lamot, Lovro; Tambić Bukovac, Lana; Kapitanović, Sanja; Perić, Magdalena; Barbić, Jerko; Harjaček, Miroslav

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ORIGINAL ARTICLE

Single nucleotide polymorphism of toll-like receptor 4 (TLR4) is associated with juvenile spondyloarthritis in Croatian population

Marija Perica [1] · **Mandica Vidović** [1] · **Lovro Lamot** [1,5] · **Lana Tambić Bukovac** [1] · **Sanja Kapitanović** [2] · **Magdalena Perić** [3] · **Jerko Barbić** [4,6] · **Miroslav Harjaček** [1,5]

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Abstract Single nucleotide polymorphisms (SNP) of toll-like and NOD-like receptors have been associated with altered receptor activity and modified production of proinflammatory cytokines leading to a number of diseases. Our aim was to determine whether SNP of TLR2 (Arg753Gln), TLR4 (Asp299Gly, Thr399Ile), and NLRP3 (Q705K) influence susceptibility to juvenile spondyloarthrtis (jSpA) and juvenile idiopathic arthritis (JIA). After the DNA extraction, 26 patients with jSpA, 11 with oligoarticular, polyarticular, or systemic JIA, and 40 healthy controls were genotyped for Arg753Gln, Asp299Gly, Thr399Ile, and Q705K SNP using real-time PCR–SNP analysis. Statistically significant difference in genotype frequency for Thr399Ile SNP of TLR4 was observed in the jSpA ($\chi2=6.705$, $p=0.035$) and not in the JIA group ($\chi2=3005$, $p=0.223$). Regarding Asp299Gly SNP, no significant difference in genotype frequency was found; however, allele frequency was significant in both jSpA and JIA patients. No significant difference in genotype or allele frequency was observed for Arg735Gln and Q705K SNP. The399Ile polymorphism of TLR4 may be responsible for altered immune response to microbial infection in variant carriers and represent a mechanism of triggering overproduction of proinflammatory cytokines and long-term inflammation in jSpA. SNP of TLR2, NLRP3, and TLR4 (Asp299Gly) were not associated with jSpA or JIA.

Keywords Juvenile idiopathic arthritis · Juvenile spondyloarthritis · Single nucleotide polymorphism · Toll-like receptor

Introduction

Juvenile spondyloarthritis (jSpA) is a goup of inflammatory disorders affecting children under 16 years of age, clinically characterized by enthesitis and arthritis, with a prevalence of 0.7–1.2 % [1]. This family of disorders comprises of the following: ankylosing spondylitis, reactive arthritis, psoriatic arthritis, arthritis associated with inflammatory bowel disease, undifferentiated SpA, and juvenile form of SpA. According to the International League of Associations for Rheumatology (ILAR) criteria, spondyloarthritis is classified as enthesitis-

✉ Marija Perica
marija_perica@hotmail.com

Mandica Vidović
vidovicmand@yahoo.com

Lovro Lamot
lovro.lamot@gmail.com

Lana Tambić Bukovac
lanabukovac@gmail.com

Sanja Kapitanović
kapitan@irb.hr

Magdalena Perić
magdalena0706@gmail.com

Jerko Barbić
jerko.barbic@mefos.hr

Miroslav Harjaček
miroslav.harjacek@zg.t-com.hr

1 Department of Pediatric Rheumatology, Children's Hospital Srebrnjak, Srebrnjak 100, 10000 Zagreb, Croatia

2 Ruđer Bošković Institute, Zagreb, Croatia

3 Institute for Public Health, Osijek, Croatia

4 Clinical Hospital Centre Osijek, Osijek, Croatia

5 School of Medicine, University of Zagreb, Zagreb, Croatia

6 School of Medicine, University of Osijek, Zagreb, Croatia

related arthritis or psoriatic arthritis [2]. Key factors in the pathogenesis of these disorders are genetic background, immune system disturbances, and environmental factors; however, their exact role has not been completely clarified [3]. Genetic background mostly refers to HLA B27 positivity, which varies in different types of jSpA (the highest proportion of HLA B27 positivity is found in ankylosing spondylitis and the lowest in undifferentiated jSpA) [4, 5]. The study by Lamot et al. indicates that jSpA could be a polygenic disease with a possible malfunction in antigen recognition and activation of immunological response, migration of inflammatory cells, and regulation of the immune system [3]. Bacterial infection is an environmental factor associated with jSpA, especially with reactive arthritis. Arthrogenic bacteria such as *Salmonella*, *Yersinia*, and *Shigella* trigger disease in about 80 % of patients with reactive arthritis [6–8]. Additional bacteria have been investigated in patients with jSpA; *Chlamydia trachomatis*, *Mycobacterium tuberculosis*, *Borrelia burgdorferi*, and *Mycoplasma pneumoniae* [9–11]. Both parts of the immune system, adaptive and innate immunity, are involved in the pathogenesis of the disease. Toll-like receptors (TLR) and NOD-like receptors (NLR), as a part of the innate immune system, are initiators of the immune response after invasion of microorganisms or following the release of endogenous molecules as a result of necrosis, injury, or lysosomal damage. Although they connect key factors of the pathogenesis of jSpA, their role has not been thoroughly investigated.

TLRs comprise a family of transmembrane proteins which play a key role in microbial recognition and regulation of innate immune response. So far, ten members of the TLR family have been described in humans. Even though toll proteins share a similar structure, each TLR interacts with a different combination of adapter proteins and activates various transcription factors such as NF-κB, activating protein-1, and interferon regulatory factors, driving a specific immune response [12, 13]. Over the past several years, studies have identified a number of common TLR single nucleotide polymorphisms (SNP) which are the result of single nucleotide alteration in the genome sequence. Many SNPs of TLRs have no effect on cell function; however, some of them modify cellular immune response and can be associated with susceptibility to a spectrum of diseases [14].

TLR2, among the TLRs, recognizes the most diverse set of pathogens. Currently, 24 polymorphisms within the human TLR2 are described, among which Arg753Gln is one of the most common. [15] Arg753Gln has been associated with an increased risk of restenosis after percutaneous transcoronary angioplasty [16], an increased risk of acute rheumatic fever [17], and risk of CMV infection after liver transplantation [18], as well as susceptibility to a number of other infections.

TLR4 is involved in the recognition of lipopolysaccharide (LPS) of Gramm negative bacteria, and it interacts with heat-shock proteins [19], fragments of hyaluronic acid [20], and fibronectin [21]. The role of Asp299Gly and Thr399Ile SNP has been studied both in infectious and noninfectious diseases such as inflammatory bowel disease, asthma, autoimmune diseases, and cancer [22, 13, 23].

NLRs are intracellular receptors, capable of recognizing microbial invasion and intracellular molecules associated with cell injury [24]. After the signal is sensed by the LRR domain, unfolding of NLRP3 enables the effector domain to recruit ASC (apoptosis-associated speck-like protein containing caspase recruitment domain family member 8 (CARD)) and the enzyme caspase, forming a cytoplasmatic complex known as the inflammasome. Active caspase 1 is responsible for processing precursors of proinflammatory cytokines IL-1 and IL-18 into their active forms. Adequate downstream signaling results in efficient host defense; however, excess activity and overproduction of proinflammatory cytokines contribute to a number of diseases. Mutations in the NAHCT domain of NLRP3 are responsible for cryopyrin-associated periodic syndromes (CAPS); familial cold autoinflammatory syndrome (FCAS), Muckle–Wells syndrome (MWS), and neonatal onset multi-systemic inflammatory disease (NOMID) [25, 26]. Several studies have associated SNP polymorphism of NLRP3 (Q705K) with various diseases including Crohn's [27] and celiac disease [28], abdominal aortic aneurysms [29], diabetes type I [30], and rheumatoid arthritis [31].

So far, the role of the most common SNP of TLR2, TLR4, and NLRP3 in patients with juvenile spondyloarthropaties has not been revealed. To our knowledge, this is the first study to investigate the relationship between Arg753Gln, Asp299Gly, Thr399Ile, Q705K polymorphisms, and susceptibility to jSpA and JIA.

Materials and methods

Subjects The study population comprised of patients diagnosed with juvenile spondyloarthritis or juvenile idopathic arthritis at the Children's Hospital Srebrnjak, Department of Rheumatology, Zagreb, Croatia and control group of healthy blood donors. Samples were collected from 26 patients diagnosed with jSpA, 11 patients diagnosed with oligoarticular, polyarticular, or systemic form of JIA and 40 healthy individuals as the control group. All patients included met the ILAR criteria for jSpA or JIA and had not been treated with disease modifying antirheumatic drugs (DMARD) or biologic agents prior to the study enrollment. Patients diagnosed with reactive arthritis were not included in the study.

Demographics, clinical data, and blood samples were collected from the patients fulfilling criteria for study enrollment. Informed consent was obtained from each participant/participants' parents. The study was approved by the Children's Hospital Srebrnjak Ethics committee and conducted according to the guiding principles of the World Medical Association

Declaration of Helsinki of 1975, as revised in 2000 and Good Clinical Practice.

Methods Three common polymorphisms of TLR2 (Arg753Gln) and TLR4 (Asp299Gly, Thr399Ile) were investigated in collected blood samples of 26 jSpA, 11 JIA patients, and 40 healthy controls. JSpA and JIA patients were additionally genotyped for NLRP3 (Q705K) polymorphism.

DNA isolation

Genomic DNAs were isolated from peripheral EDTA blood of patients using proteinase K (Macherey-Nagel, Germany) digestion followed by phenol chloroform extraction.

Real-time PCR genotyping

Real-time PCR–SNP analysis of TLR4 Asp299Gly (rs4986790), TLR4 Thr399Ile (rs4986791), and NLRP3 Q705K (rs35829419) was performed using an ABI PRISM 7300 SDS (Applied Biosystems) and predeveloped TaqMan SNP genotyping assays (Applied Biosystems) according to the manufacturer's instructions. TLR2 (rs5743708) SNPs were analyzed using LightMix (Tib MolBiol, Germany), LightCycler DNA Master Hybridization Probes (Roche Diagnostics GmbH, Germany), and LightCycler Instrument v 1.5 (Roche, Germany). Polymorphisms of the TLR2 were determined using specific melting point (Tm) of A for the mutant and G for the wild type in the channel F2 of the LightCycler Instrument according to the manufacturer's instructions.

For quality control, 15 % of randomly selected samples of both cases and control were re-analyzed the second time, without finding any discrepancies. Control samples covering four possible SNP genotypes and no template control were run in parallel with tested samples in each experiment.

Statistical analysis Significant differences in genotype and allele frequencies of TLR4, TLR2, and NLRP3 SNPs between jSpA, JIA, and control group were determined using χ^2 test. All SNPs were tested for Hardy–Weinberg equilibrium. The magnitude of association was expressed as odds ratio with 95 % confidence interval. A p value of <0.05 was considered statistically significant. Statistical analysis was performed using STATISTICA for Windows, version 7.1 (StatSoft, Inc., Tulsa, OK, USA).

Results

The study population consisted of 77 participants, 26 diagnosed with jSpA, 11 diagnosed with JIA (either oligoarthritis, polyarthritis, or systemic arthritis), and 40 healthy controls. The mean age of participants in groups were as follows: 15.37±4.2 for jSpA, 11.68±4.8 for JIA and 41±12 in the control group (Table 1.) The genotype and allele distribution in both groups of patients and controls is given in Table 2.

Asp299Gly TLR4 polymorphism

In the JIA patient group, all participants were homozygous for the wild type allele of TLR4 Asp299Gly polymorphism. There were three heterozygous and no homozygous variant allele carriers in the jSpA group. In the control group, both heterozygous (two participants) and homozygous variant allele carriers (six participants) were observed. There was no significant difference in genotype frequency between controls and both groups of patients. A statistically significant difference was observed in variant allele frequency when comparing both groups of patients with controls (jSpA $\chi2=3.865$, $p=0.049$, JIA $\chi2=4.463$, $p=0.035$) (Table 2.).

Thr399Ile TLR4 polymorphism

All JIA patients were homozygous for the wild type allele of TLR4 Thr399Ile polymorphism. In the jSpA group, six heterozygous carriers and no homozygotes of variant allele were observed; while in the control group, both homozygous variant allele carriers (six participants) and heterozygotes carriers (three participants) were observed. Statistical analysis revealed significant difference in genotype frequency for Thr399Ile polymorphism in the jSpA group in comparison with controls ($\chi2=6.705$, $p=0.035$). No significant difference was observed for genotype frequency in the JIA group; however, allele frequency was significant.

Table 1 Characterisitics of patients with jSpA, JIA, and controls

	jSpA	JIA	Controls
Patients, *n*	26	11	40
Mean age	15.37±4.2	11.68±4.8	41±12
Male gender (%)	38.46 %	18.18 %	60 %
HLA B27 positivity (N/N of genotyped patients)	20/25	1/3	
HLA B7 posititiy (N/N of genotyped patients)	8/25	1/3	
HLA B27/B7 positivity (N/N of genotyped patients)	6/25	0/3	

Table 2 Allele and genotype frequencies of the Asp299Gly and Thr399Ile TLR4, Arg735Gly TLR2, and Q705K NLRP3 polymorphism in jSpA, JIA, and the control group

	Control group		jSpA		Statistics	JIA		Statistics
	n	%	*n*	%		*n*	%	
TLR4 (Asp299Gly) genotype								
AA	32	80.0	23	88.46	$\chi2=4.925$	11	100	$\chi2=2.609$
AG	2	5.0	3	11.54	$p=0.085$	0	0	$p=0.271$
GG	6	15.0	0			0	0	
Allele					$\chi2=3.865$			$\chi2=4.463$
A	66	82.5	49	94.23	$p=0.049$	22	100	$p=0.035$
G	14	17.5	3	5.77	OR=0.289 (95 % CI, 0.079–1.06)	0	0	OR=0.021 (95 % CI, 0.000–10.97
TLR4 (Thr399Ile) genotype								
CC	31	77.5	20	76.92	$\chi2=6.705$	11	100	$\chi2=3.005$
CT	3	7.5	6	23.08	$p=0.035$	0	0	$p=0.223$
TT	6	15.0	0	0		0	0	
Allel e					$\chi2=1.225$			$\chi2=4.6$
C	65	81.25	46	88.46	$p=0.269$	22	100	$p=0.032$
T	15	18.75	6	11.54	OR=0.565 (95 % CI, 0.204–1.566)	0	0	OR=0.020 (95 % CI, 0.000–10.07)
TLR2 (Arg735Gly) genotype								
GG	38	95.0	17	94.4	$\chi2=0.01$	10	100	$\chi2=0.52$
GA	2	5.0	1	5.6	$p=0.995$	0	0	$p=0.771$
AA	0		0	0		0	0	
Allele					$\chi2=0.008$			$\chi2=0.314$
G	78	97.5	35	97.2	$p=0.931$	20	100	$p=0.576$
A	2	2.5	1	2.8	OR=1.114 (95 % CI, 0.098–12.7)	0	0	OR=0.195 (95 % CI, 0.000–113.8)
NLRP3 (Q705K) genotype								
CC			24	92.3		9	81.8	$\chi2=0.882$
CA			2	7.7		2	18.2	$p=0.348$
AA			0	0		0	0	
Allele								$\chi2=0.832$
C			50	96.2		20	90.9	$p=0.362$
A			2	3.8		2	9.1	OR=0.4 (95 % CI, 0.053–3.037)

Arg735Gln TLR2 polymorphism

No homozygotes for the variant allele were found in all three groups. One heterozygote carrier was found in the jSpA group and two in the control group. No significant difference in allele or genotype frequency was observed among groups.

Q705K NLRP3 polymorphism

Additional analysis of Q705K polymorphism of NLRP3 was done in samples of JIA and jSpA patients; however, due to a small sample size of controls, the same analysis could not be performed in the control group. There were no homozygotes for the variant allele in any of the groups, and only two participants from each group had heterozygous genotype. The difference in allele and genotype frequency was not statistically significant.

Discussion

The aim of our study was to investigate whether polymorphisms of TLR2, TLR4, and NLRP3 influence susceptibility to JIA and jSpA. We have found a statistically significant correlation between polymorphism of TLR4 Thr399Ile and susceptibility to jSpA. Similar correlation was not found for JIA. Our results differ from Myles et al. [32] who have found a lack of association for both polymorphisms of TLR4 in patients with enthesitis-related arthritis in the Indian population. Lack of correlation of TLR4 polymorphisms in JIA patients has been previously reported by Lamb et al. [33] on the UK population. Previously mentioned two studies are among a few rare studies in the pediatric population. This is, to our knowledge, the first study on TLR polymorphisms in children of European origin with jSpA.

Studies investigating the role of TLR polymorphisms in an adult population of different ethnic background diagnosed with ankylosing spondilitis (AS), rheumatoid arthritis (RA), and reactive arthritis (ReA) have found opposite results. Van der Paard et al. [34] reported a lack of association of TLR4 polymorphism (A896G) with AS in the Dutch population, which was confirmed by Gergely et al. [35] for Asp299Gly and Thr399Ile polymorphism in the Hungarian population, Adam et al. [36] in the UK population, and by Na et al. [37] in the Korean population. On the contrary, Snelgrove et al. [38] found minor allele frequency of TLR4 variant to be statistically significant in the AS population and have emphasized necessity for larger studies.

A similar discrepancy regarding the association of TLR4 polymorphisms and disease susceptibility has been described for RA. Radstake et al. [15] found a protective role of Asp299Gly in a RA population from the Netherlands while other studies from the UK (Kilding et al.) [39], Spain (Sanchez et al.) [40], (Alvarez-Rodriguez et al.) [41], and France (Jean et al.) [42] did not reveal any correlation of TLR4 or TLR2 polymorphisms with susceptibility to RA.

Conflicting results could be explained by variation of polymorphism presence in different ethnic groups. Absence of TLR polymorphism in the Asian population has been confirmed by several studies [37, 43]. Differences in TLR SNP distribution among ethnic groups has also been noticed in inflammatory bowel disease studies (IBD) [44–47]. By analyzing the TLR polymorphism presence in 2491 individuals from Africa, Asia, and Europe, Ferweda et al. [48] showed that Asp299Gly and Thr399Ile polymorphism have unique distribution in each continent. African population showed a high prevalence of Asp299Gly allele (10–18 %), while a population from Asia was practically missing TLR4 polymorphisms. Allele frequency among Indo-European individuals was 3–7 %. The clustering pattern is related to out-of-Africa migration and local environmental conditions, infectious pressure [48]. However, a larger analysis of the different ethnic groups should reveal whether described discrepancies regarding susceptibility to RA and AS are the result of different ethnic background, or whether the role of TLR receptors is different for each rheumatologic disease and additionally is different for children in comparison with adults.

Although the genotype frequency for TLR4 (Asp299Gly) was not statistically significant for the jSpA group in our research ($\chi 2=4.925$, $p=0.085$), a large sample size should be estimated to elucidate whether the Asp299Gly polymorphism, as well as Thr399Ile, influence susceptibility to JIA. We did not find correlation between genotype frequencies of both TLR4 polymorphisms with JIA.

As it is postulated that pathogen exposure plays an important role not only in the pathogenesis of spondyloarthropaties (SpA), especially reactive arthritis and ankylosing spondilitis, but also jSpA, polymorphisms of TLR4 could be the underlying reason for altered immune response to pathogen invasion resulting in overproduction of inflammatory cytokines and consequently resulting in predisposition to spondyloarthropathy development in susceptible individuals. Increased expression of TLR4 on mononuclear cells of peripheral blood and synovial tissue has been previously reported in patients with SpA [49]. Furthermore, linkage of TLR polymorphisms with the disease susceptibility has been reported for inflammatory bowel disease which is present in about two thirds of patients with SpA as a subclinical form of gut inflammation [50]. Inflammatory changes in the gut have been found in 65 % patients with undifferentiated SpA, 90 % of ReA patients, and 60 % of AS patients [51].

Currently, there are two theories explaining the link between the connection of intestinal and joint inflammation. The first refers to intestinal activated lymphocyte or macrophage movement disorder [32], while activated lymphocyte expressing $\alpha4\beta7$ i $\alpha E\beta7$ [42, 40] adhesive molecules and macrophages with CD163 receptor, suggesting their origin from intestinal mucosa, have been discovered in synovial tissue of patients with spondyloarthritis [52]. The second theory suggests the overproduction and constant exposure to high levels of TNFα affecting synovial fibroblast and intestinal myofibroblast, leading to inflammation development [53].

Our results suggest that polymorphism of TLR2 (Arg735Gln) is not associated with jSpA or JIA. Lack of correlation between TLR2 polymorphisms and ERA was also reported by Myles et al. [53] Previous studies found no correlation of Asp753Gln SNP with RA [54], gout [55], Behçet's disease [56], rheumatic heart disease [57], or development of secondary amyloidosis in patients with Familial Mediterranean Fever (FMF) [58]. However, it may affect the severity of FMF [59].

As the cascade of microbial recognition involves both transmembrane and intracellular receptors, we additionally screened our patient population (JIA and jSpA patients) for Q705K polymorphism of NLRP3 and found no significant correlation in either group. A literature search revealed only one report on NLRP3 SNP by Yang et al. [56] in the pediatric population (of Taiwanese origin) which indicated that OR2B11 SNP might contribute to the pathophysiology of JIA. We found no other research in the pediatric population. Studies in the adult population revealed no susceptibility to AS development in carriers of Q705K variant, but C10X SNP seem to influence susceptibility in the same group of Swedish AS patients. Healthy individuals with Q705K and C10X SNP had increased levels of IL1β and IL33, while carriers with only one of the mentioned SNP had normal cytokine levels [58]. A functional in vitro study in a human monocyte cell line showed increased production of IL-1β and IL-18 by cells expressing Q705K variant in comparison with wild type cells, thereby demonstrating the possible effect of SNP in elevating the basal state of the innate immune response [59].

The limitations of our study lay in the small sample size and inability to perform NLRP3 SNP analysis in the control patient group. Our results suggesting the lack of susceptibility of variant carriers of NLRP3 to jSpA and JIA development should be confirmed in a larger study.

In conclusion, genotype frequency The399Ile was statistically significant in patients with jSpA in comparison with the control population. Similar correlation was not found for JIA patients. We have not found any correlation of Asp299Gly, Arg735Gln, or Q705K SNP with jSpA or JIA. The399Ile polymorphism may be responsible for altered immune response to microbial infection in variant carriers and represent a mechanism of triggering overproduction of proinflammatory cytokines and long term inflammation in jSpA. Our presumption is that the locus of minor resistentiae is the gut and lack of adequate cleavage of bacteria in the gut leads to subclinical gut inflammation typical for jSpA patients.

Disclosures None.

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