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Özgün Araştırma / Original Article

Investigation of the Asp299Gly and Thr399Ile polymorphisms of TLR4 gene in Rheumatoid Arthritis

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Abstract

Objective: Rheumatoid arthritis (RA) is a chronic and inflammatory disease characterized by synovial inflammation that causes cartilage and bone destruction as well as systemic defects, including cardiovascular, pulmonary, psychological, and skeletal disorders. The etiology of RA is unclear. Evidence suggests that RA is influenced by both genetic and environmental factors and the inflammatory and autoimmune activities take important roles in the development of this disease. In the onset of RA, an interaction between the resident cells of synovium and cells of the innate and adaptive immune system reported. Fibroblast-like synoviocytes (FLS) are one of the resident cells and they play a central role, with a tumor-like behavior, in joint destruction and development of chronic inflammation. Toll-like receptors (TLRs) are transmembrane glycoproteins that are related to inflammation via synthesis of proinflammatory cytokines like TNF-α, IL-6, and IL-1β. Some studies report an association between the activation of FLS and the cytokine environment, cell-to-cell contacts, or the activation of TLR2, TLR4, and TLR3. TLRs and especially TLR4 is involved in the recognition of endogenous molecules released by injured tissues and necrotic cells. TLR4 gene involved in a wide variety of both infectious and non-infectious diseases and two polymorphisms of TLR4, Asp299Gly and Thr399Ile, changed the binding capacity and electrical charge of the protein. There are conflicting or even contradictory results about these polymorphisms and we aimed to determine the distribution of the allele frequencies of these polymorphisms and compare the result of RA patients with healthy subjects.

Methods: DNA extraction was realized by salting out method from peripheral blood lymphocytes of RA patients and healthy controls. PCR amplification carried out with appropriate primer pairs against the related DNA sequences. Genotyping performed by the restriction fragment length polymorphism method.

Results and Conclusion: According to the results obtained from RA patients and healthy controls, we have not found any statistical difference between both groups. Including the other polymorphisms of the TLR family into this type of studies, will give more information about the role of TLR family in rheumatoid arthritis.

Keywords: Rheumatoid Arthritis, Toll-like receptor 4, polymorphism, inflammation.

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Romatoid artrit hastlarında TLR4 geni Asp299Gly ve Thr399Ile polimorfizmlerinin araştırılması

Öz

Amaç: Romatoid artrit (RA), kardiyovasküler, pulmoner, psikolojik ve iskelet bozuklukları dahil olmak üzere sistemik kusurların yanı sıra kıkırdak ve kemik tahribatına neden olan sinovyal enflamasyonla karakterize kronik ve enflamatuar bir hastalıktır. RA'nın etiyolojisi tam olarak bilinmemektedir. Kanıtlar RA'nın hem genetik hem de çevresel faktörlerden etkilendiğini ve enflamatuar ve otoimmün aktivitelerin bu hastalığın gelişiminde önemli rol aldığını göstermektedir. RA'nın başlangıcında, yerleşik sinovyum hücreleri ile doğal ve adaptif bağışıklık sisteminin hücreleri arasında bir etkileşim olduğu bildirilmektedir. Fibroblast benzeri sinoviyositler (FBS), yerleşik hücrelerdir ve eklem yıkımı ve kronik enflamasyonun gelişmesinde- tümör benzeri bir davranışla- merkezi rol oynarlar. Toll-like reseptörler (TLR'ler), TNF-a, IL-6 ve IL-1 gibi proenflamatuar sitokinlerin senteziyle ilişkili transmembran glikoproteinlerdir. Bazı çalışmalarda FLS aktivasyonu ile sitokin, hücre teması veya TLR2, TLR4 ve TLR3 aktivasyonu arasında bir ilişki olduğunu bildirmektedir. TLR'ler ve özellikle TLR4, yaralı dokular ve nekrotik hücreler tarafından salınan endojen moleküllerin tanınmasında rol oynar. TLR4 geni, hem enfeksiyöz hem de enfeksiyöz olmayan hastalıklarda rol almakta olup, bu gendeki iki polimorfizm, Asp299Gly ve Thr399Ile, TLR4 proteinin bağlanma kapasitesini ve elektrik yükünü değiştirir. Bu polimorfizmlerle ilgili çelişkili ve uyumsuz sonuçlar bulunmaktadır, biz bu çalışmamızda bu polimorfizmlerin allel sıklıklarını belirlemeyi ve RA hastalarının sonuçlarını sağlıklı deneklerle karşılaştırmayı amaçladık.

Yöntemler: DNA ekstraksiyonu, RA hastaları ve sağlıklı kontrollerin periferik kanlarından 'salting out' metoduyla gerçekleştirildi. PCR amplifikasyonu, çalışılan DNA dizilerine uygun primer çifleri kullanılarak yapıldı. Genotip belirlenmesinde 'restriksiyon enzimi parça uzunluk polimorfizmi metodu' kullanıldı.

Bulgular ve Sonuç: RA hastalarından ve sağlıklı kontrollerden elde edilen sonuçlara göre, her iki grup arasında istatistiksel bir fark bulamadık. TLR ailesinin diğer polimorfizmlerinin bu tür çalışmalara dahil edilmesi, TLR ailesinin romatoid artritteki rolü hakkında daha fazla bilgi verecektir.

Anahtar kelimler: Romatoid artrit, Toll-like reseptör 4, polimorfizm, enflamasyon.

NTRODUCTION

Rheumatoid arthritis (RA) is an age-related disorder, with a prevalence range differ from 1% to 5%[1]. It is affecting women two to three times more than men[1]. RA is a chronic and inflammatory disease characterized by synovial inflammation that causes cartilage and bone destruction as well as systemic defects, including cardiovascular, pulmonary, psychological, and skeletal disorders[2,3]. Although the pathophysiology of RA is not clear, evidence suggests that RA is influenced by both genetic and environmental factors. Twin studies have shown heritability of RA is ~60%[4]. The general consensus about the RA is it has a spectrum of disease stages that can begin many years before the onset of clinical symptoms and genetic markers associated to disease, influence the transition from one disease stage to another with the stochastic environmental factors[4].

In the onset of RA, an interaction between the resident cells of synovium and cells of the innate and adaptive immune system reported[5]. Fibroblast-like synoviocytes (FLS) are one of the resident cells and they play a central role, with a tumor-like behavior, in joint destruction and development of chronic inflammation. During the chronic inflammation, FLS migrate to healthy tissue and invade to the extracellular matrix and lead to joint damage by contributing to the destruction of the cartilage and bone tissues[5]. There are some studies that report an association between the activation of FLS and the cytokine environment, cell-to-cell contacts, or the activation of Toll-like receptors (TLRs)[5,6]. It has been shown that the expression levels of TLRs were higher in 'rheumatoid

arthritis synovial fibroblasts' than in 'osteoarthritis synovial fibroblasts' and ligation of TLRs, such as TLR2, TLR3, and TLR4 resulted to the production of inflammatory cytokines, MMPs and VEGF[6].

TLRs are transmembrane glycoproteins that are expressed on the surface of leukocytes and non-immune epithelial cells[7,8]. TLRs are related to inflammation via synthesis of proinflammatory cytokines like TNF-α, IL-6, and IL-1β and inflammatory enzymes including inducible nitric acid synthase and cyclooxygenase-2 by NF-κB pathway[7,9]. TLRs and especially TLR4 is involved in the recognition of endogenous molecules released by injured tissues and necrotic cells[7,8]. Activated TLR4 may cause cytokine production and this may lead to inflammation or increase the existing inflammation.

Synovial inflammation is the main character of the RA and TLR4 is an important key effector of the immune system and inflammation. Genetic variations of TLR4 like as Asp299Gly and Thr399Ile are linked to susceptibility to various disease associated with age and these variations may alter the ligand recognition, binding character, interaction and transport of TLR4 protein to the cell membrane[10]. Therefore, in this study, we aimed to evaluate the Asp299Gly and Thr399Ile frequencies of TLR4 gene in RA patients to determine whether is there any relation between these genetic variations and RA.

METHODS

This study was performed on RA patients and healthy volunteers. The protocol of the study and the consent procedure were approved by the Institutional Medical Ethics Committee of Dicle University Medical Faculty (No:2019-44). The blood samples were collected from RA patients and age-/sex-matched healthy controls. Patients with another inflammatory, autoimmune, acute, or chronic infectious diseases and diabetes mellitus were excluded from the study. DNA extraction was realized from peripheral blood lymphocytes by salting out method. Concentrations of isolated DNAs were determined in the spectrophotometer. PCR amplification carried out with the primer pairs of a previous study[11]. Genotyping realized by Restriction Fragment Length Polymorphism (PCR-RFLP) method. Primer pairs, restriction endonucleases, and expected fragment sizes after cleavage were given in Table 1. DNA fragments after cleavage were monitored by using agarose gel electrophoresis. The number of patients and control groups varies for the two studied polymorphic structures, the number of patients and volunteers were given in Table 2.

Statistical Analyzes

Descriptive statistics were expressed as count and percent. Chi-square test was used to test the significance of the distribution of variations between RA patients and control group. Statistical significance levels were considered as 5%, and the Statistical Package for the Social Sciences (SPSS) (ver. 21) statistical program was used for all statistical computations.

RESULTS

According to the results obtained from RA patients and healthy controls, Asp299Gly (rs4986790) and The399Ile (rs4986791) conversions have not shown any statistical difference between both groups. The genotype frequencies of RA patients and healthy control groups were almost the same. These results are also the same for allele frequencies too. Chi-square results and p values were shown in Table 2.

Table 1: The primer pairs and restriction endonucleases used for detection of the Asp299Gly and Thr399Ile polymorphisms.

SNPs	Primer Pairs	Restriction endonuclease	Expected fragment sizes
Asp299Gly rs4986790	F 5′-GATTAGCATACTTAGACTACCTCCATG R 5′-GATCAACTTCTGAAAAAGCATTCCCAC	*Nco1*	AA 249 bp AG 249+223+26 bp GG 223+26 bp
Thr399Ile rs4986791	F 5-GGTTGCTGTTCTCAAAGTGATTTTGGGAGAA R 5′-ACCTGAAGACTGGAGAGTGAGTTAAATGCT	*Hinf1*	CC 407 bp CT 407+378+29 bp TT 378+29 bp

Table 2: Genotype and allele frequencies and statistical summary of the study.

	Genotypes	Case n 112	Controls n 97	Genotype frequencies of case %	Genotype frequencies of control %	χ^2	*p*
TLR4 rs4986790 Asp299Gly	AA	105	89	93,8	91.8	0.322	0.8515
	AG	6	7	5.4	7.2		
	GG	1	1	0.8	1.0		
	Alleles						
	A	218	187	OR (95% CI)		0.298	0.5838
	G	6	7	0.7353 (0.2428-2.2261)			
	Genotypes	Case n 110	Controls n 146	Genotype frequencies of case %	Genotype frequencies of control %	χ^2	*p*
TLR4 rs4986791 **The399Ile**	CC	99	132	90.0	90.4	0.727	0.6953
	CT	9	13	8.2	8.9		
	TT	2	1	1.8	0.7		
	Alleles						
	C	207	277	OR (95% CI)		0.145	0.7037
	T	13	15	1.1597 (0.5401-2.4904)			

DISCUSSION

In this study, we hypothesized that there may be a relation between TLR4, which is associated with inflammation and immunity, and the RA disease. RA is a chronic autoimmune disease characterized by hyper-proliferated synovial fibroblast and dysregulated activation of the inflammatory responses. In RA, hyper-proliferated synovial fibroblasts express a

series of TLRs and these TLRs stimulate synovial fibroblasts to synthase high levels of VEGF, IL-8, COX-2, TNF-α, IL-17, and IL-336,[12].

The mediators synthesized from synovial fibroblasts lead to the infiltration of immune cells, chronic inflammation and bone lesions6. These situations contribute to the onset of RA and also maintain the inflammation.

TLR4 is a member of the TLR family and first recognized as a sensing receptor for gram-negative lipopolysaccharide[7,13]. After a while, it has found that TLR4 binds to the endogenous molecules produced from injured tissues and also necrotic cells[7]. Hence, TLR4 is a key molecule of the proinflammatory response7 and it has been shown that synovial fibroblasts express mediators which cause chronic synovial inflammation that depends on NF-κB activity after the activation of TLR4[13,14]. Therefore, TLR4 leads to inflammation and some products that are the results of inflammation such as hyaluronan induce TLR4 to maintain the proinflammatory response[7].

Interestingly, TLR4 is also the only known member of TLR family that capable of inducing the production of type 1 interferons and type 1 interferons important in inflammation[15].

TLR4 is a transmembrane protein composed of a 608 amino acid in the extracellular domain and a 187 amino acid in the intracellular domain. After an induction, a dimerization of two TLR4 occurs and this dimerization results in a conformational change to induce the recruitment of adaptor proteins below the cascade[7]. The Asp299Gly polymorphism results to change the charge of TLR4 protein and also increase the rotational capability of the peptide bond[10]. This may change the binding capacity of the protein to other related proteins. It has been reported that the Asp299Gly and Thr399Ile polymorphisms containing TLR4 has decreased binding capacity to HMGB1 (high mobility group box 1) protein[10]. It has shown that patients with Asp299Gly or Thr399Ile have increased TNF-alpha, IL-10, monocyte chemotactic protein–1 and macrophage inflammatory protein–1 expression but decreased IL-1 beta, IL-6, IL-8, and GRO-expression[16]. Reports indicate that there may be blunted response to inhaled LPS in heterozygous individuals for Asp299Gly and Thr399Ile conversions[17]. Some other studies revealed that both these polymorphisms reduce the binding and signaling capacity of TLR4 and presence of these polymorphisms increased the risk of tissue infection[18]. Interestingly, according to our results, there was no statistical difference between patient and control groups for both polymorphisms studied.

The two polymorphisms of TLR4, Asp299Gly and Thr399Ile, are located in the fourth exon of TLR4 are special polymorphisms, because most of the polymorphisms in human beings is low (<1%), but the frequency of these two variations differ between ethnic groups. The Asp299Gly allele is most frequent in African populations, the frequency of G allele differ between 10% to 18%, whereas only 2% are carriers for T allele of Thr399Ile and these individuals always have Asp299Gly as well10. The incidence of these polymorphisms are rare in Asian, Papuan and American populations10. According to our results the allele frequency of Asp299Gly for RA patients was 2.68 and for healthy controls was 3.61. The frequency of Thr399Ile was found 5.91 for RA patients and 5.14 for healthy controls.

In conclusion, although the polymorphisms of TLR4 have been shown to alter the electric charge or binding capacity of the protein, we have not found any relation between these polymorphisms and RA disease. Our study contributes to revealing the frequency of these polymorphisms in our country.

Conflicts of interest: The authors have no conflict of interests to declare.

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