ACADEMIA JOURNAL OF BIOLOGY 2021, 43(2): 11–16
DOI: 10.15625/2615-9023/15727

ASSOCIATION BETWEEN GENOTYPES OF *TLR4* GENE RS2149356 POLYMORPHISM AND SERUM LEVEL OF IL-6 IN VIETNAMESE PATIENTS WITH GOUT

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Received 3 December 2020, accepted 28 May 2021

ABSTRACT

Gout is an inflammatory type of arthritis caused by the deposition of crystals of monosodium urate monohydrate (MSU) in the joints. The arthritis is mediated by the release of pro-inflammatory cytokines including IL-1β, TNF-α and IL-6 by leukocytes including macrophages. Toll-like receptor $(TLR)_4$ is expressed in the immune cells to initiate immune response by inducing activation of signaling molecules involved in the transcription of nuclear genes. In this study, mutational analysis of *TLR4* gene was examined to determine the prevalence of gene polymorphism in Vietnamese patients with gout. Molecular analysis of this gene was investigated by PCR amplification and direct DNA sequencing. Level of serum cytokines was measured by ELISA. As a result, no significant difference in frequency of TLR4 gene rs2149356 polymorphism was observed between the patient and control groups. Importantly, level of IL-6 was significantly higher in patients carrying CG genotype as compared with patients carrying TG and TT genotypes of TLR4 gene rs2149356 polymorphism and the patients carrying GG genotype exhibited higher IL-6 level than healthy individuals. The GG genotype of TLR4 gene rs2149356 polymorphism in gout patients were sensitive to IL6-induced inflammatory response. The effect is expected to affect the immune response to treatment for gout patients.

Keywords: Cytokine, ELISA, gout, DNA sequencing and TLR4.

Citation: Nguyen Thi Xuan, Le Thuy Ha, Dang Thanh Chung, 2021. Association between genotypes of *TLR4* gene rs2149356 polymorphism and serum level of IL-6 in Vietnamese patients with gout. *Academia Journal of Biology*, 43(2): 11–16. https://doi.org/10.15625/15727

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INTRODUCTION

Gout is a common form of inflammatory arthritis resulting from the accumulation of monosodium urate monohydrate (MSU) crystals in the joints causing hyperuricemia (VanItallie, 2010). Attacks of gout are triggered by the deposition of MSU crystals in the joints and MSU crystals exert toll-like receptor (TLR)-mediated inflammatory response (Liu-Bryan, Scott, Sydlaske, Rose & Terkeltaub, 2005). The ingestion of MSU crystals by macrophages is mediated through activation of the TLR2/TLR4 signaling, leading to nuclear factor- κB (NF-κB)-mediated gene transcriptions including interleukin-6 (IL-6), IL-1β and TNF-α, which drive acute gouty inflammation (Liu-Bryan et al., 2005; Martinon et al., 2006; Cavalcanti et al., 2016; Crisan et al., 2016).

The host immune response to pathogens is monitored by the appropriate recognition of pathogen-associated molecular patterns (PAMPs), including TLR4 in the innate immune system (Aderem & Ulevitch, 2000). *TLR4* gene is located on chromosome 9 and has a size of 13,3 kb, includes 3 exons and 2 introns with the approximate molecular weight of 95 kDa (Aderem et al., 2000). TLR4 is expressed in immune cells to sense microbial components and initiate the immune response by binding of this receptor with its specific ligand to respond to pathogens (Manicassamy & Pulendran, 2009). TLR4 signals lead to recruitment of intracellular adaptors including the myeloid differentiation factor 88 (MyD88) and activation of downstream signaling and transcription factors to induce transcriptions of target genes. Deficiency in the regulation of TLR4-mediated signaling leads to an excessive and persistent inflammatory response. Thus, TLR4-deficient mice show defects in cytokine productions secreted by immune cells in the response with lipopolysaccharides (Hollingsworth et al., 2006).

Molecular genetic investigations in humans have indicated that polymorphisms in multiple genes such as *TLR4*, *SLC2A9/SLC22A12* and *ABCG2* are associated with risk of gout (Li et al., 2012; Flynn et al., 2013; Qing et al., 2013; Hurba et al., 2014; Rasheed et al., 2016). These genes, exception of *TLR4*, play a crucial role in the regulation of plasma urate levels (Kawamura et al., 2011; Itahana et al., 2015). Recently, a variant in *TLR4* gene TT-genotype rs2149356 is related to a higher risk of gout in Han Chinese and European populations (Qing et al., 2013; Rasheed et al., 2016). In contrast, a protective effect is shown for this genotype in Polynesian patients with gout (Rasheed et al., 2016). This genotype was associated with increased *TLR4* mRNA and interleukin (IL)-1β expressions (Qing et al., 2013). Little is known about the association between variants in *TLR4* gene and cytokine profile in Vietnamese patients with gout (Qing et al., 2013). In this study, we investigated *TLR4* gene variants and their association with the cytokine profile of 53 gout patients and 49 healthy individuals.

MATERIALS AND METHODS

Patients and control subjects

Fresh peripheral blood samples were collected from 53 male patients aged from 30 to 55 years who were diagnosed with gout at the 103 Hospital, Military Medical University, Hanoi, Vietnam. The control group comprised 49 healthy subjects aged from 30 to 55 years. No individuals in the control population took any medication or suffered from other acute or chronic diseases. All patients and volunteers gave a written consent to participate in the study. Person care and experimental procedures were performed according to the Vietnamese law for the welfare of humans and were approved by the Ethical Committee of Institute of Genome Research, Vietnam Academy of Science and Technology.

DNA sequencing

Genomic DNA was isolated from peripheral blood samples using a DNeasy blood and tissue kit (Qiagen, Hilden, Germany). To determine variants of *TLR4* genes, polymerase chain reaction (PCR) and DNA sequencing (3500 Genetic Analyzers, Thermo Scientific) were performed as

previously described (Nguyen et al., 2012). DNA sequence variations were identified by comparing the subject DNA sequence to *TLR4* reference sequence: Genbank Accession Number NC-000009.12. The *TLR4* was amplified by using primers: *TLR4*-F: 5'-TTGGTCCACAACGGTTCTCTG-3' and *TLR4*-R: 5'-CTGGATGGGGTTTC CTGTCA-3'. The amplification product length was 737-bp. All obtained PCR fragments were purified with a GeneJET PCR purification kit (Thermo Scientific, USA). The PCR products were sequenced on both strands with the same primers used for the PCR.

Cytokine quantification in serum

Sera was collected and stored at -20 ºC until used for ELISA. For analysis of IL-12p70, IL-6 and TNF-α concentrations, commercially available ELISA kits (eBioscience) were used according to the manufacturer's instructions.

Statistical analysis

Associations between genotypes carried by patients with gout and healthy controls were estimated by computing the odds ratios (OR) and their 95% confidence intervals (CI) from multivariate logistic regression analysis. The statistical power was calculated using the VassarStats website (http://vassarstats.net/odds2x2.html, Fisher's exact test).

Differences between genotypes and serum levels of cytokines were tested for significance using Student's unpaired two-tailed t-test or ANOVA. Data are provided as means ± SEM, n represents the number of independent experiments. $P < 0.05$ was considered statistically significant.

RESULTS

Analysis of polymorphisms in TLR4 gene

Sequencing of *TLR4* gene identified a nucleotide change in this gene at rs2149356 location in both patients and control groups (Table 1 and Fig. 1). The TG genotype of these single nucleotide polymorphisms (SNP) has been considered as a normal genotype (Qing et al., 2013), which was observed in both patient and control groups in this study with identical carrier frequencies of 26,4% and 34,7%, respectively (adjusted OR=0.68, 95% CI=0.29–1.58). Although, the TT genotype of this variant is indicated to be a high risk of gout in Han Chinese and European populations (Qing et al., 2013; Rasheed et al., 2016), this genotype was identified similarly in both patient and healthy control groups with the carrier frequencies of 0,76% and 0,82%, respectively (adjusted OR=0.92, 95% CI=0.22–3.9). In addition, the appearance of the GG genotype of this variant is reported to occur at similar frequencies between patient and control groups (Qing et al., 2013; Rasheed et al., 2016). Consistently, it was present with the carrier frequencies of 66% and 57,1% in patients and control groups, respectively (adjusted OR=1.46, 95% CI=0.65–3.25). The evidence indicated that no significant difference in frequencies of TLR4 gene rs2149356 polymorphism was observed between the patient and control groups.

Table 1. Frequencies of *TLR4* gene rs2149356 polymorphism in patients with gout and healthy individuals

Genotype	Number (%) of SNPs		P value
	Patient	*Control*	
TG	14/53 (26.4%)	17/49 (34.7%)	P > 0.05
TT	4/53 (0.76%)	4/49 (0.82%)	P > 0.05
GG	35/53 (66%)	28/49 (57.1%)	P > 0.05

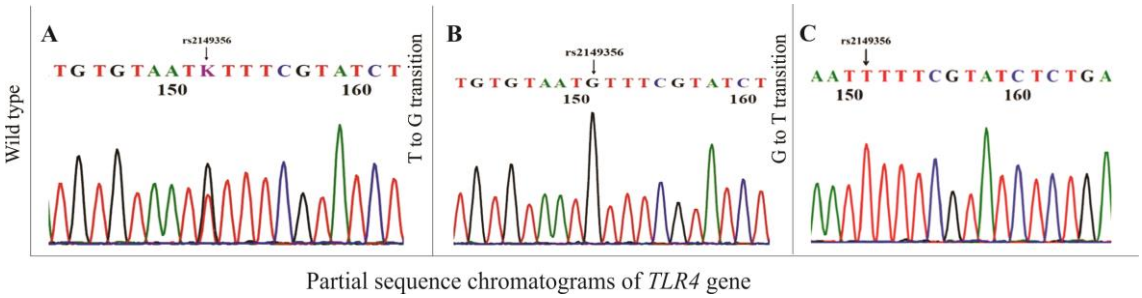

Figure 1. Partial sequence chromatograms of *TLR4* gene rs2149356 polymorphism from the patients with gout and healthy individuals. Arrows indicate the location of the base changes. **A**: Wild type. **B**: T to G transition at position rs2149356. **C**: G to T transition at position rs2149356

Association between GG genotype in TLR4 gene rs2149356 polymorphism and serum cytokine profile

Since acute gout patients exhibit an inflammatory condition via elevated concentration of serum cytokines. Therefore, among 53 participants we examined only 27 acute gout patients, in which 14 patients carrying GG, 10 patients carrying TG and 3 patients carrying TT genotypes (Table 2). As shown in Figure 2, the level of IL-6 was significantly higher in patients carrying CG genotype as compared with patients carrying TG and TT genotypes (Fig. 2A). However, no significant difference in levels of IL-12p70 and TNF-α was found among GG, TG and TT genotypes carried by patients with acute gout (data now shown). Importantly, a significant difference was also observed between patients and control groups who carry GG genotype of TLR4 gene rs2149356 polymorphism (Fig. 2B), pointing out that gout patients carrying GG genotype of *TLR4* gene rs2149356 polymorphism were sensitive to IL6-induced inflammatory response.

Table 2. Genotype frequencies of *TLR4* gene rs2149356 polymorphism carried by patients with acute gout

Genotype	Number (%) of SNPs
TG	10/27 (37%)
TT	3/27 (11.1%)
GG	14/27 (51.9%)

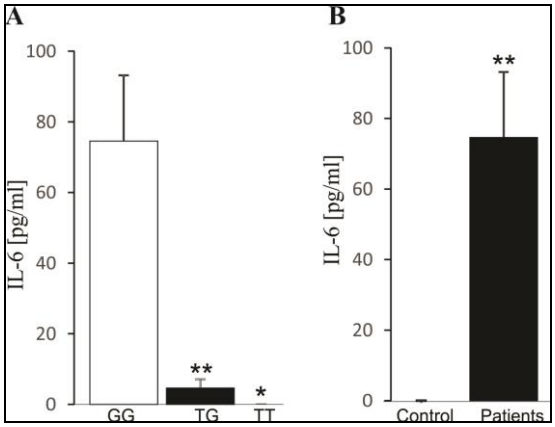

Figure 2. **A**: Arithmetic means ± SEM (n = 3–14) of serum IL-6 level is shown to GG genotype (1st bar), TG genotype (2nd bar) and TT genotype (3rd bar) of *TLR4* gene rs2149356 polymorphism in patients with acute gout. * ($p < 0.05$) and ** ($p < 0.01$) represent significant differences from GG genotype (ANOVA). **B**: Arithmetic means ± SEM (n = 10–14) of serum IL-6 level is shown to GG genotype of *TLR4* gene rs2149356 polymorphism in healthy controls (1st bar) and patients with acute gout (2nd bar). * ($p < 0.05$) represents significant difference from healthy controls (Student's unpaired two-tailed *t*-test)

DISCUSSION

To our knowledge, it is the first study to demonstrate the association among genotype alteration of *TLR4* gene rs2149356 polymorphism, serum cytokine profile and

susceptibility to gout in the Vietnamese population. Our investigation showed that genotype frequencies of the rs2149356 SNP tended not to be different between male patients and control groups. Consistently, a recent study described by Kilding et al. (2003) that genetic alteration in *TLR4* gene is not a risk factor for rheumatoid arthritis. In contrast, other studies report that nucleotide changes in *TLR4* gene are linked to gout, in which the TT-, but not GG- genotypes is associated with an increased risk of gout in Han Chinese and European populations (Qing et al., 2013; Rasheed et al., 2016), whereas the TT genotype of rs2149356 SNP is considered as a reduced risk for gout in Polynesian population (Rasheed et al., 2016). Therefore, this observation suggested that the effect of the genotypes of *TLR4* gene rs2149356 SNP in gout susceptibility is different from one country to another.

Next, our study focused on the association between genetic alteration in *TLR4* gene and serum cytokine profile in male patients with gout, since TLR4 plays a crucial role in the regulation of immune response. Investigation in a mouse model showed that serum cytokine productions secreted by immune cells is defected in *TLR4*-deficient mice when treated with LPS (Hollingsworth et al., 2006). In this study, the GG genotype of rs2149356 SNP carried by patients with acute gout was associated with enhanced serum level of IL-6, but not TNF-α and IL-12p70 as compared to the TG- and TT genotypes of this SNP carried by those (Fig 2A). This study is reported for the first time, whereas the TT genotype of this SNP is previously published that it is related to the expression of IL-1β in gout patients (Qing et al., 2013). The release of IL-6 by immune cells in gout patients is also linked to the presence of tophi and articular deformities (Cavalcanti et al., 2016).

CONCLUSION

This finding indicates that the GG genotype of TLR4 gene rs2149356 SNP might involve in releasing of IL-6 by immune cells in gout patients. The effect is expected to affect the immune response to treatment for male patients with gout.

Acknowledgements: This research was funded by Institute of Genome Research, Vietnam Academy of Science and Technology.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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