Genotypic analysis of Asp299Gly and Thr399Ile polymorphisms of TLR4 in Egyptian patients with rheumatoid arthritis and systemic lupus erythematosus
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Introduction
Rheumatoid arthritis (RA), the most common inflammatory joint disease, exacts a huge toll of disability, deformities, quality-of-life alterations, premature deaths, and economic costs [1]. RA is an autoimmune disease characterized by chronic inflammation of the synovial membrane, which is infiltrated by activated immune cells including CD4+ T cells, B cells, and antigen-presenting cells such as dendritic cells and macrophages. The factors responsible for RA induction and progression are poorly understood but may involve interactions between innate and adaptive immunity [2]. It has been suggested that viruses and bacteria may contribute to initiate or exacerbate RA by binding to toll-like receptors (TLRs) [3].

Systemic lupus erythematosus (SLE) is a chronic multisystem and autoimmune disease characterized by the production of autoantibodies against a relatively limited range of nuclear antigens. These autoantibodies result in the formation of immune complexes that deposit in the tissues and induce inflammation, thereby contributing to disease pathology [4]. Growing evidence suggests that recognition of nucleic acid motifs by TLRs may play a role in both the activation of antinuclear B cells and in the subsequent disease progression after immune complex formation. The endosomal localization of the nucleic acid-sensing TLRs is believed to contribute to the distinction between endogenous nucleic acids and those of foreign origin [4].

TLRs constitute a family of transmembrane proteins expressed by various cell types, including immune cells, to identify pathogens and initiate inflammatory signaling pathways [5]. Stimulation of the TLR pathway culminates in nuclear factor κB activation and the transcription of immune response genes [6].

Keywords:
Egypt, polymorphism, rheumatoid arthritis, systemic lupus erythematosus, toll-like receptor

Background
Toll-like receptors (TLRs) are essential molecules of the innate immune system that stimulate numerous inflammatory pathways and harmonize systemic defense against a wide array of pathogens. TLRs may also recognize a number of self-proteins and endogenous nucleic acids. Inappropriate stimulation of the TLR pathway by endogenous or exogenous ligands or as a consequence of mutation in the TLR genes may lead to induction and/or prolongation of autoimmune response and tissue injury. This study was designed to detect the TLR4 (Asp299Gly and Thr399Ile) gene polymorphism in Egyptian patients with rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) as well as its correlation with disease activity.

Participants and methods
This study enrolled 50 patients who were attending the internal medicine and immunology out-patient clinic of Beni-Suef University Hospital. They were classified into three groups: 20 patients with RA, 20 patients with SLE, and 10 healthy controls. All studied persons were subjected to complete clinical evaluation, laboratory investigations, and detection of mutation in the TLR4 (Asp299Gly and Thr399Ile) gene by PCR technique.

Results
No individual in the RA patients group, the SLE patients group, or control population was identified carrying the Asp299Gly and Thr399Ile polymorphisms; in other words, all RA patients, SLE patients, and controls were with the same wild genotype.

Conclusion
The similarity in genotype between the patients group and control population concluded that these two missense polymorphisms do not contribute to RA and SLE in a group of Egyptian population.

Keywords:
Egypt, polymorphism, rheumatoid arthritis, systemic lupus erythematosus, toll-like receptor

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lipoproteins, flagellin, and viral RNA or DNA [7]. Furthermore, the administration of endogenous RNA or DNA has been shown to activate TLRs and induce autoimmune reactions [8]. On the basis of the immune-regulating and immune response-initiating effects of TLRs, they are considered candidate genes for RA [9].

Associations between different polymorphisms within the TLR2 and TLR4 genes or within the promoter region of the TLR9 gene and susceptibility for RA and SLE are subjects of great controversy [10]. Few studies exist to date on the importance of innate immunity and, in particular, TLRs in the pathogenesis of juvenile idiopathic arthritis (JIA) and SLE. Recently, two common cosegregating missense mutations, Asp299Gly and Thr399Ile, affecting the extracellular domain of the TLR4 protein have been characterized [11]. Both mutations lead to an attenuated efficacy of lipopolysaccharide signaling and a reduced capacity to elicit inflammation [12].

This study was designed to detect the TLR4 (Asp299Gly and Thr399Ile) gene polymorphism in Egyptian patients with RA and SLE and its correlation with disease activity.

Participants and methods
The study protocol was agreed by ethical committee of Beni-Suef University. Participants who were interviewed received information about our study idea and signed written consent for the study. This study enrolled 50 participants who were attending the internal medicine and immunology out-patient clinic of Beni-Suef University Hospital during the period between October 2010 and December 2011. They comprised two groups: patients and the control group.

Group I included 20 patients with RA. Group II included 20 patients with SLE. Group III included 10 healthy volunteers of matched age and sex, without history of chronic or acute arthritis or any chronic illnesses such as hypertension, diabetes mellitus, and liver or renal diseases. Patients below 16 years or above 60 years, who were pregnant, taking oral contraceptive pills, and having infections were excluded from this study.

All patients and control group were subjected to the following:

(1) Clinical evaluation including identification data: age, sex, body weight and height, BMI (kg/m²), complete history taking, and proper physical examination. Diagnosis of RA according to the American College of Rheumatology [13] and of SLE according to the American College of Rheumatology [14]. Disease activity in our patients with RA was assessed by the DAS28 scoring system [15], and in patients with SLE was assessed by the SLEDAI scoring system [16].

(2) Laboratory evaluation: ESR, C-reactive protein, complete blood profile, alanine transaminase (ALT), aspartate transaminase (AST), rheumatoid factor, anti-CCP, antinuclear antibody (ANA), anti-ds DNA, C3 levels, and C4 levels.

(3) Radiological investigation: radiograph on the affected joints needed for diagnosis and follow-up.

(4) Laboratory specific test for the study: detection of mutation in TLR4 (Asp299Gly and Thr399Ile) by PCR.

Methods
Blood sampling: The skin was cleaned with an alcoholic swab and the vein was palpated and punctured with the needle; 5 ml of venous blood was drawn into a syringe, then the sample was placed in a laboratory sampling tube containing 15% EDTA anticoagulant. DNA isolation: Genomic DNA was extracted from peripheral blood leukocytes [17] using the QUIAmp blood kit (Qiagen, Hilden, Germany). Allele-specific PCRs were used to detect TLR4 Asp299Gly and Thr399Ile polymorphisms in DNA samples extracted from 5 ml of total blood samples, as reported by Lorenz et al. [18]. The TLR4 polymorphism was amplified by PCR. Briefly, a fragment containing the repeats was amplified using the following primers (Table 1).

After DNA extraction, genomic DNA was detected by 1% agarose gel electrophoresis. Two microliters of loading dye was mixed with gDNA, and then loaded in each well and the electrophoresis was performed with 100 V for 1 h. The gDNA in the gel was stained with ethidium bromide and visualized by UV transilluminator (Fig. 1).

Statistical analysis
The following tests were used: Descriptive analysis of the results was in the form of percentage distribution for qualitative data. Minimum, maximum, mean,
and SD were calculated for quantitative data. Cross-tabulation test was performed for comparison between percentage values. The Student t-test was performed for comparison between means of two groups. The F-test (one-way analysis of variance), a test statistics, was calculated for comparison between means of three groups. The probability/significance value: P value greater than 0.05 was considered nonsignificant, P value less than 0.05 was considered significant at 0.05 level, and P value less than 0.01 was considered significant at 0.01 level. The χ²-test was used to determine whether there is a significant difference between the expected frequencies and the observed frequencies in one or more categories. For statistical analysis, statistical package for social science software, version 15 (SPSS v15, Chicago, IL, USA) was used.

**Results**

Fifty participants enrolled in this study were classified into three groups: group I included 20 patients with RA (19 women and one man) with mean age of 39 ± 14 years; group II included 20 patients with SLE (18 women and two men) with mean age of 26.1 ± 6.6 years; and group III included 10 healthy volunteers of matched age and sex (six women and four men) with mean age of 26.1±5.8 years (Figs 2–4 and Tables 2–4).

No individual in the RA patients group, SLE patients group, or control population was identified carrying the Asp299Gly and Thr399Ile polymorphisms; in other words, all RA patients, SLE patients, and controls were with the same wild genotype (Table 5 and Fig. 5).

**Discussion**

The roles of TLRs in development of autoimmune diseases have been studied recently. Autoreactive B cells are present in the lymphoid tissues of healthy individuals, but as they are subject to self-tolerance mechanisms, they remain silent. However, when tolerance to self-antigens fails, a complex of self-reactive antibodies against self-reactive or cross-reactive DNA coengage the antigen receptor and the TLRs, leading to a continuous activation of these autoreactive B cells and the development of autoimmune diseases. The maturation of antigen-presenting cells (APCs), in response to signals received by the innate immune system, may lead to the breakdown of tolerance. This process is mainly activated by TLRs that have been triggered by self-antigens [19].

TLRs are a key link between infection, injury, and inflammation [20]. They recognize pathogen-associated and danger-associated molecular patterns (PAMPs and DAMPs), and subsequently trigger a proinflammatory

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**Table 2 Demographic data of all studied groups**

| Variables               | RA group (n = 20) | SLE group (n = 20) | Control group (n = 10) | P value          |
|-------------------------|------------------|-------------------|------------------------|------------------|
| Age (years)             | 39 ± 14          | 26.1 ± 6.6        | 26.1 ± 5.8             |                  |
| Sex [n (%)]             |                  |                   |                        |                  |
| Males                   | 1 (5)            | 2 (10)            | 4 (40)                 | P<1: 0.01* P<2: 1.0 (NS) |
| Females                 | 19 (95)          | 18 (90)           | 6 (60)                 | P<2: 0.002*      |
| Disease duration (years)| 6.8 ± 6.6        | 1.6 ± 1.3         | –                      | P<2: 0.223 (NS)  |

RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; P<1, Comparison between the RA and control groups; P<2, Comparison between the SLE and control groups; *P < 0.05, significant; P > 0.05, NS.

**Figure 1**

TLR4 gDNA expression visualized by UV – transilluminator.

**Figure 2**

DAS28 score of the rheumatoid arthritis (RA) group.
cascade, which induces the transcription of multiple proinflammatory cytokine genes as well as dendritic cell maturation [21]. Cell wall components of various Gram-positive and Gram-negative pathogens, stress proteins, and cell decomposition products are predominantly identified by the TLRs 2 and 4 [22]. Although TLR-mediated inflammation is an important aspect of host defense, it is also linked to pathogenesis of several autoimmune diseases, such as RA and SLE [23].

Given the known genetic predisposition displayed in RA patients, TLR polymorphism studies have been conducted to determine whether TLR is involved in the pathogenesis of RA [9].

The TLR4 Asp299Gly (rs4986790) polymorphism is the most frequently studied polymorphism in RA, and it displays ethnic differences. Africans have the highest frequency (16%) [24] followed by Europeans (4–10%) [25], whereas Asians do not have this polymorphism [26].

Most studies either deal with the Asp299Gly or the Thr399Ile polymorphism but neglect the fact that these polymorphisms also exist in a cosegregated (Asp299Gly/Thr399Ile) way [27].

In our study, 50 participants were included: 20 with RA, 20 with SLE, and 10 healthy control individuals.

### Table 3 DAS28 score of the rheumatoid arthritis group

| DAS28 score                      | RA group (n = 20) [n (%)] |
|----------------------------------|---------------------------|
| ≤3.2 remission                   | 0 (0)                     |
| >3.3 and ≤5.1 low disease activity | 4 (20)                    |
| >5.1 high disease activity       | 16 (80)                   |

RA, rheumatoid arthritis.

### Table 4 Systemic lupus erythematosus disease activity index SLEDAI

| SLEDAI                          | SLE group (n = 20) [n (%)] |
|---------------------------------|---------------------------|
| Inactive ≤2                    | 5 (25)                    |
| Persistent active ≥8           | 14 (70)                   |
| Flare ≥3 change in SLEDAI       | 1 (5)                     |

SLE, systemic lupus erythematosus.

### Table 5 Toll-like receptor 4 genotype frequencies in the RA, SLE, and control groups

| Parameters          | RA group (n = 20) | SLE group (n = 20) | Control group (n = 10) |
|---------------------|-------------------|--------------------|------------------------|
| TLR4 [n (%)]        |                   |                    |                        |
| Thr399Ile Normal    | 20 (100)          | 20 (100)           | 10 (100)               |
| Abnormal            | 0                 | 0                  | 0                      |
| Asp299Gly Normal    | 20 (100)          | 20 (100)           | 10 (100)               |
| Abnormal            | 0                 | 0                  | 0                      |

RA, rheumatoid arthritis; SLE, systemic lupus erythematosus.

The aim of our study was to detect the polymorphism of TLR4 gene in Egyptian patients with RA and SLE and its correlation with disease activity. Disease activity in our patients with RA was assessed by the DAS28 scoring system and in patients with SLE was assessed by the SLEDAI scoring system.

In the present study, no individual in the RA patients group, SLE patients group, or control population was identified carrying the Asp299Gly and Thr399Ile.
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polymorphisms; in other words, all RA patients, SLE patients, and controls were with the same wild genotype. The similarity in the genotype between the patients group and control population concluded that these two missense polymorphisms do not contribute to RA and SLE in Egyptian population.

**TLR** gene polymorphisms have been tested in several cohorts. A case–control study was conducted in Spain on 122 patients with SLE, 224 patients with RA, and a control group of 199 healthy individuals to evaluate the possible association between the polymorphisms of TLR4 (Asp299Gly and Thr399Ile) genes with the susceptibility or severity of RA and SLE. They found no statistically significant differences in the TLR4 Asp299Gly and the TLR4 Thr399Ile genotype or allele distribution between SLE patients, RA patients, and control individuals. However, they observed a difference in the distribution of TLR4 Asp299Gly heterozygous individuals between RA patients (9%) and controls (13%), which did not reach statistical significance [28].

Another case–control study to determine whether TLR4 Asp299Gly polymorphism influences susceptibility to RA was conducted on 212 RA patients and 879 healthy controls in Northern England, which also found no association, even in the subgroup of patients negative for the shared epitope [29].

Interestingly, heterozygous Asp299Gly status was protective in early untreated RA in a case–control study performed in the Netherlands. They presented data showing a lower frequency of the TLR4 heterozygous condition for the polymorphism in 282 patients with RA (10.6%) than in 314 control individuals (17.2%). They found that the TLR4 Asp299Gly functional variant is associated with decreased RA disease susceptibility but does not influences disease severity and/or outcome [30].

The aim of the case-only study, which was performed on 169 RA patients by Kuuliala et al. [31], was to determine whether the TLR4 Asp299Gly (rs4986790) polymorphism influences treatment response. The authors found that DAS28 was significantly different in RA patients with and without this polymorphism who were treated with a disease-modifying antirheumatic drug ($P = 0.019$) but not in RA patients with and without this polymorphism who were treated with three disease-modifying antirheumatic drugs and prednisolone ($P = 0.31$).

Another French Caucasians study ruled out that the common Asp299Gly and Thr399Ile variant and SLE and RA in Egyptian population. Despite the fact that our results were negative, the role for TLR4 in the pathogenesis of RA and SLE remains uncertain. Altogether, published data and our data lead to the prediction that, to improve these data, analyses in larger cohorts with more than

no association between RA and **TLR** polymorphisms in more severe subgroups with RF, or anti-CCP antibodies, or joint erosions. In addition, they ruled out a protective role for TLR4 Asp299Gly in their French Caucasian cohort [3].

Enevold et al. [32] supposed that an important way to investigate the role of TLRs themselves in RA is to study genetic variants [e.g. single nucleotide polymorphism (SNP)] in the TLR genes that might lead to an altered ligand binding capacity and/or expression leading to an altered TLR-mediated response that might subsequently translate into variations in disease activity and/or severity. Nevertheless, a well-documented prospective cohort was unable to show any significant effect of studied TLR SNP on RA disease variability and/or severity [32].

Both TLR4 gene Asp299Gly (rs4986790) and Thr399Ile (rs4986791) polymorphisms with RA were investigated in two Asian studies, one Korean [33], and one Chinese study [26], but in agreement with our results these polymorphisms were not detected in all RA patients and controls.

Meta-analysis, performed by Lee et al. [9], of the TLR4 Asp299Gly polymorphism and RA in three European studies [29,30,34] failed to reveal any association with RA in terms of disease susceptibility and severity in Europeans.

Recently, in a case–control study conducted in Germany 2013, there was no correlation between the TLR4 genotypes and the observed significant reduction in the level of TLR4 expression ($P \leq 0.001$) on monocytes of patients with JIA and SLE compared with that of healthy control individuals. This finding indicates that the observed reduced TLR4 protein expression on monocytes of JIA and SLE patients is not due to a known functional SNP in the TLR4 gene, but instead, it is supportive of a kind of tolerance to PAMPs and cellular danger signals [12].

**Conclusion**

The present study is, to our knowledge, the first to demonstrate no association between the common Asp299Gly and Thr399Ile variant and SLE and RA in Egyptian population. Despite the fact that our results were negative, the role for **TLR** in the pathogenesis of RA and SLE remains uncertain. Altogether, published data and our data lead to the prediction that, to improve these data, analyses in larger cohorts with more than
500 patients would be required. As this sample size is not easy to reach in single-center or even multicenter studies, meta-analytic analysis could probably be the only feasible approach. With that method, our results might help to spread light on the overall contribution of TLR genes in RA and SLE.

**Recommendation**

We recommend that further studies with extended SNPs within TLR4 should be performed to disclose its possible role in the common autoimmune diseases in Egyptian patients.

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*Conflicts of interest*

There are no conflicts of interest.

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