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Genetic variation in toll-like receptor 4 gene with primary antiphospholipid syndrome susceptibility: a cohort of Egyptian patients

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Abstract

Background: As toll-like receptor 4 (*TLR4*) plays important roles in cellular immunity and *TLR4* polymorphisms have been shown to be associated with susceptibility to a range of diseases, the present study aimed to investigate the association between *TLR4* gene polymorphisms and the incidence of primary antiphospholipid syndrome (PAPS).

Methods: Two *TLR4* single nucleotide polymorphisms (rs4986790 and rs4986791) were assessed in 110 subjects of Egyptian ethnicity, including 65 female patients with PAPS and 45 matched healthy controls, using polymerase chain reaction-restriction fragment length polymorphism. Results were verified using automated sequencing.

Results: The homozygous wild-type (AA, aspartic acid) rs4986790 variant and (CC, threonine) rs4986791 variant were the predominant genotypes in the control and PAPS groups.

Conclusion: The results of this preliminary study of *TLR4* gene variants among patients with PAPS in an Egyptian population found no association between the rs4986790 and rs4986791 variants and susceptibility to PAPS.

Keywords: Primary antiphospholipid antibody syndrome, Toll-like receptor 4, Polymerase chain reaction-restriction fragment length polymorphism, rs4986790, rs4986791, Polymorphism

Background

Antiphospholipid syndrome (APS) is an acquired autoimmune disease of coagulopathy characterized by thrombosis in the microvascular, arterial, or venous systems and pregnancy complications in the presence of persistent antiphospholipid antibodies (aPLs), such as anti-beta2glycoprotein I (anti-β2GPI), anticardiolipin (aCL), and lupus anticoagulant (LA) antibodies. Our understanding of the management, phenotypes, and pathogenesis of APS has increased markedly in recent years. Moreover, international trans-disciplinary research collaborations have contributed to improved care and health outcomes among patients with APS [1–3].

In 1998, the Sapporo criteria of classifying cases of APS were proposed [4]. These criteria were updated in 2006 following a workshop held in Sydney (known as the Sydney criteria) to state APS patients must have both a clinical manifestation (arterial or venous thrombosis and/or pregnancy morbidity) and one or more repeatedly positive circulating antiphospholipid antibodies (aPLs) [5].

APS may arise in isolation without underlying disease in more than 50% of patients, a condition known as PAPS [6]. However, there is a lack of population-based studies assessing the prevalence and incidence of APS, with the estimated incidence in previous studies ranging between 40 and 50 cases per 100,000 in the general population [4]. PAPS is reportedly more common in females than in males [7].

The precise mechanisms underlying the pathogenesis of APS remain unclear; however, a multifactorial process similar to other autoimmune diseases has been posited

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involving a complex interplay between genetic predisposition, antigenic stimuli, and the presence of specific autoantibodies. APS is thought to involve the activation of several cell types, such as endothelial cells, monocytes, platelets, and trophoblast cells, the activation of the coagulation and complement systems, and the inhibition of fibrinolytic processes by antiphospholipid antibodies (aPLs) via cell surface receptors, including toll-like receptors (TLRs) [5, 8].

Toll-like receptors (TLRs) are members of the pattern recognition receptor (PRR) family and play crucial functions in the innate immune system. TLRs are expressed by a range of important cell types in the immune system. Human TLRs were first recognized as homologs of the toll receptor in *Drosophila* and were first identified as a regulator of dorsal–ventral polarity in *Drosophila* embryogenesis. However, TLRs were later shown to influence antifungal immunity [9]. There are 10 TLR family members (TLR1–10) in human beings which consist of type I transmembrane proteins containing 20–27 external leucine-rich repeats (LRRs). TLRs are able to recognize conserved structures in microorganisms including pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs), transmembrane domains, and intracellular toll–interleukin 1 (IL-1) receptor (TIR) domains and activate signal transduction pathways [10]. Several studies have demonstrated that dysregulation of TLR signaling contributes to a range of diseases including cancer and chronic inflammatory, autoimmune, and infectious diseases [11].

Toll-like receptor 4 (*TLR4)*, the first human TLR to be discovered [12, 13], is a type I transmembrane glycoprotein containing an extracellular domain composed of 22 LRRs which mediates lipopolysaccharides (LPS) recognition and receptor dimerization, a transmembrane domain, and a TIR domain responsible for downstream signal transduction. Activation of *TLR4* stimulates a number of inflammatory cascades predominantly via the adapter molecule myeloid differentiation protein 88 (MyD88) to activate the nuclear factor—kappa beta (NF-kB) pathway and promote transcription of pro-inflammatory cytokines such as interleukin-1 (IL-1), IL-6, and IL-8 [14, 15].

SNPs in TLR genes, particularly *TLR4*, have been shown to be associated with a number of inflammatory and autoimmune diseases including rheumatoid arthritis, asthma, ulcerative colitis, systemic lupus erythematosus, Crohn's disease, and rheumatoid arthritis. Accordingly, SNPs within the TIR or LRRs may disrupt intracellular signaling in mononuclear cells, leading to immune responses [16, 17].

According to the national center for biotechnology (ncbi.nlm.nih.gov), the human *TLR4* gene is found on chromosome 9 (9q33.1), has three exons, and encodes 839 amino acid residues. There are two missense SNPs in the coding area (exon number 3) of the *TLR4* gene in the extracellular domain of *TLR4*: an A–G substitution at position 896 of *TLR4* cDNA causing an aspartic-to-glycine substitution at position 299 (c.896A > G, p. Asp299Gly; rs4986790); and a C–T substitution at position 1196 of *TLR4* cDNA resulting in a threonine-to-isoleucine change at 399 (c.1196 C > T, p. Thr399Ile; rs4986791). *TLR4* c.896A > G and c.1196 C > T SNPs are the two most studied co-segregating functional extracellular domain variants of *TLR4* and are reported to have a positive correlation with susceptibility to several infectious diseases [18]. Nevertheless, the association between *TLR4* gene polymorphisms and PAPS is yet to be evaluated in the Egyptian population. Due to the clinical importance of predicting the development of PAPS, the present study assessed the association of *TLR4* c.896A > G and c.1196C > T polymorphisms with the risk of developing PAPS in an Egyptian population.

Materials and methods

Study participants

The present study included 65 unrelated Egyptian adult females diagnosed with PAPS according to the Sydney criteria which state that "the patient must have pregnancy morbidity, or arterial or venous thrombosis confirmed by one positive laboratory result at least repeated on two or more occasions (12 weeks apart), including (LA), IgG or IgM (aCL) antibodies and\or IgG or IgM (anti-β2GPI) antibodies" in the absence of any underlying autoimmune diseases. PAPS was confirmed by the absence of antinuclear antibodies (ANAs). Patients with PAPS were under the care of obstetrics, gynecology, and vascular clinics at Ain Shams University hospitals, Cairo, Egypt. The overall health of control participants was assessed at the time of recruitment taking into consideration clinical history over the preceding 5 years. The control group comprised 45 healthy subjects recruited from colleges and committees in the same region and were matched to the study group for gender, age, and ethnicity. Control participants were enrolled in the present study after confirming the absence of underlying bleeding disorders, thrombosis, pregnancy loss, and autoimmune diseases.

Biochemical analyses

After clinical examination in obstetrics, gynecology, and vascular clinics, 10 ml of venous blood was collected from each participant into different vacutainer tubes (Kemico, Egypt) by venipuncture. A portion of venous blood was drawn into gel vacutainer tubes, allowed to clot, and then centrifuged for 15 min at 2500 rpm to obtain sera. Serum aCL (IgG and IgM) and anti-β2GPI

(IgG and IgM) antibodies were qualitatively measured using enzyme-linked immunosorbent assay (ELISA) kits (Demeditec Diagnostics GmbH, Kiel, Germany). Serum ANA levels were measured by indirect immunofluorescence using Aesku slides-ANA-HEp2 kits (Aesku diagnostics, Wendelsheim, Germany). A portion of the venous blood sample was collected into trisodium citrate (3.2%) anticoagulant-coated vacutainers and centrifuged at 2500 rpm for 15 min to obtain plasma. Plasma LA was detected using coagulation assays, the dilute Russell viper venom time (dRVVT) assay, and an activated partial thromboplastin time lupus anticoagulant sensitive (aPTT/LA sensitive) assay using kits provided by Diagnostica Stago Inc. (NJ, USA).

Genotyping of *TLR4* c.896A > G and c.1196 C > T polymorphisms

Whole venous blood samples were drawn into vacutainers coated with dipotassium ethylene diamine tetraacetic acid (K_2EDTA) and were utilized to extract DNA from peripheral blood leukocytes using Gentra Puregene blood kits (Qiagen, Hilden, Germany). The purity and concentration of extracted DNA were determined using a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, USA).

TLR4 c.896A > G and c.1196 C > T polymorphisms were detected by PCR–RFLP. SNPs of interest were selected using the database of SNP (dbSNP) (https://www.ncbi.nlm.nih.gov/snp). *The TLR4* c.896A > G (rs4986790) variant is located at nucleotide 896 of TLR4 cDNA, while the TLR4 c.1196 C > T (rs4986791) variant is located at nucleotide 1196 of TLR4 cDNA. Primer sequences used to amplify DNA fragments containing TLR4 polymorphic sites, PCR product sizes, and restriction enzymes (REs) used in the present study were adapted from previously described method [19] with minor modifications, as given in Table 1.

PCR was performed in a total reaction volume of 25 µl containing 100 ng of template DNA, 1 µl (20 Pmol/µl) of each of the forward and reverse primers (Thermo Fisher Scientific, USA), and 12.5 µl of (2x) dream Taq Green PCR Master Mix (Thermo Fisher Scientific, USA) with the final volume made up with nuclease-free water. PCR was performed using a Thermal Cycler (Applied Biosystems, Thermo Fisher Scientific, USA) at 95 °C for 5 min for initial denaturation, then 45 cycles of denaturation for 30 s at 95 °C, annealing at 52 °C for both SNPs for 30 s, and 30 s of extension at 72 °C, and 10 min of final elongation at 72 °C.

PCR products were electrophoresed on 1% agarose gel. Amplified PCR products were 249 bp and 406 bp for *TLR4* c.896A > G and c.1196C > T polymorphisms, respectively. Amplified PCR products were eluted from agarose gels using Gene JETTM extraction kits (Thermo Fisher Scientific, USA). A 10 µl aliquot of purified PCR product was then digested for 60 min at 37 °C utilizing 1 µl of NcoI (10U/µl) for *TLR4* c.896A > G and 1 µl of HinfI (10U/µl) for *TLR4* c.1196 C > T. Restriction enzymes were obtained from Thermo Fisher Scientific (USA). Digested and purified PCR products were electrophoresed on 2% agarose gels.

Table 1 Oligonucleotide primer sequences, PCR product sizes, and restriction enzymes

SNP	Primer sequence	PCR product size (bp)	RE
TLR4 c.896A > G, Asp299Gly(rs4986790)	F:5′- GATTAGCAT ACTTAGACTACTACC TC**C**ATG-3′ R:5′-GATCAACTTCTG AAAAAGCATTCC CAC-3′	249	NcoI
TLR4 c.1196 C > T, Thr399Ile (rs4986791)	F:5′-GGTTGCTGTTCT CAAAGTGATTTTGGG A**G**AA -3′ R:5′-CCTGAAGAC TGGAGAGTGAGTTAA ATGCT -3′	406	HinfI

SNP single nucleotide polymorphism, *RE* restriction enzyme, *TLR4* toll-like receptor 4,

F, forward, *R* reverse, Underlined bases in both forward primers indicate restriction sites

Genotyping by sequencing the two successive *TLR4* gene SNPs (c.896A > G and c.1196 C > T) using a common PCR product

PCR primers used for RFLP targeting the *TLR4* gene variants were deemed unsuitable for sequencing for the following two reasons: the sequence of the forward primer for each SNP was modified to create a restriction recognition site for only one SNP allele; and the SNP was located at the first nucleotide following the primer (thereby preventing accurate sequencing). Accordingly, a new set of primers was designed for the sequencing experiment.

The two successive SNPs (rs4986790) and (rs4986791) were amplified using a newly designed common PCR primer set comprising a forward primer; 5′-GAAATT AGGCTTCATAAGCTGACTTTAAG-3′, and a reverse primer; 5′-CCTGAAGACTGGAGAGTGAGTTAA ATGCT-3′, to produce a PCR product with an estimated length of 898 bp. This PCR product was referred to as the "common PCR product."

PCR was performed in a total reaction volume of 25 µl containing 100 ng of template DNA, 1 µl (20 pmol/µl) each of the forward and reverse primers, and 12.5 µl of (2x) dream Taq Green PCR Master Mix made up to a

final volume with nuclease-free water. PCR was performed on a Thermal Cycler at 95 °C for 5 min for initial denaturation, then 45 cycles of denaturation for 30 s at 95 °C, annealing at 53 °C for 30 s, and 30 s of extension at 72 °C, and 10 min of final elongation at 72 °C. Amplified PCR products were electrophoresed on 1% agarose gels and eluted using Gene JETTM extraction kits.

More than 20% of eluted common PCR product for each SNP was randomly selected from both the control and the patient groups and sequenced to validate the RFLP results of both SNPs (rs4986790) and (rs4986791) using a 3500 Genetic Analyzer (Applied Biosystems, Thermo Fisher Scientific, USA) at Colors Medical Labs, Cairo, Egypt.

Consistency between sequenced products and the reference DNA sequences of *TLR4* c.896A > G and c.1196 C > T polymorphisms was evaluated by performing multiple sequence alignment (MSA) between the *TLR4* reference DNA sequence (AC; NC-000009) and both the forward and reverse DNA sequences (with the reverse complementary sequence was used for the latter) using ClustalW in molecular evolutionary genetics analysis (Mega X) software.

Statistical analyses

Statistical analyses were performed using SPSS version 23.0 (IBM Corp, NY, USA). Data distribution was assessed using the Shapiro–Wilk test. Categorical variables were expressed as frequencies (%), and Gaussian data were expressed as the mean ± standard deviation (SD). Continuous variables were compared between several groups using independent t tests. Two-sided *P* values less than 0.05 were considered statistically significant.

Results

Study participant demographics

Study participants in the control and PAPS groups had a mean age of 32.66 ± 1.42 and 35.7 ± 1.42 years, respectively, with no significant difference in age between groups ($P = 0.243$).

The majority of patients with PAPS had obstetric morbidity (52.3%). On the other hand, 47.7% of patients with PAPS had previously suffered thrombotic events. Obstetric events included one or more fetal death at 10 weeks of gestation or greater in 21 cases (32.3%), three or more consecutive pregnancy losses at less than 10 weeks of gestation (18.5%), and one or more premature births occurring at less than 34 weeks due to severe preeclampsia or placental insufficiency (1.5%). Among patients with thrombotic complications, 22 subjects had venous thrombosis while nine participants had arterial thrombosis. Of these, 21 participants had vein thrombosis (DVT) in a lower extremity (32.3%) and one participant had DVT in an upper extremity (1.5%). Regarding aPL positivity, the majority of cases (76.9%) had single positivity for aPL, 10 cases (15.4%) had double positivity for aPL, and five cases (7.7%) had triple positivity for aPL (Fig. 1). The distributions of positivity for antiphospholipid antibodies (aPLs) among PAPS and control participants are shown in Table 2.

TLR4 c.896A > G and c.1196 C > T RFLP and sequencing analyses

Digestion of the *TLR4* c.896A > G PCR product by NcoI resulted in a 249 bp fragment (AA genotype, homozygous wild; Fig. 2a), while digestion of the *TLR4* c.1196C > T PCR product by HinfI resulted in a 406 bp fragment (CC genotype, homozygous wild; Fig. 2b). The wild alleles for both *TLR4* SNPs were identified in all samples by RFLP. Confirmation of the RFLP results was conducted by sequencing randomly selected samples, with results corroborating the results of the RFLP analysis for all samples.

MSA demonstrated consistency between all tested sequences with the reference DNA sequences of *TLR4* c.896A > G and c.1196C > T polymorphisms and the chromatogram of *TLR4* c.896A > G and c.1196C > T polymorphisms for one of the studied samples, as illustrated in Fig. 3a and b, respectively. SNP locations are highlighted with black arrows. The exact location of SNPs was determined according to the nucleotides neighboring SNPs using MSA.

Genotypic distribution and allelic frequencies of *TLR4* c.896A > G and c.1196C > T polymorphisms

The distributions of genotypes and alleles of the *TLR4* c.896A > G and c.1196C > T polymorphisms in the control and PAPS groups are shown in Table 3. The AA genotype of *TLR4* + 896A/G was detected in all subjects, while the AG and GG genotypes were not detected

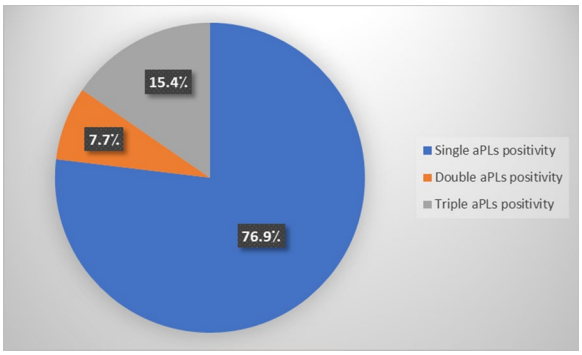

Fig. 1 Serological positivity of different screened autoantibodies among PAPS sufferers

Table 2 Distribution of aPL positivity among healthy controls and patients with PAPS

Group	ANA, *n* (%)	aCL		anti-β2GPI		LA	
		IgG, *n* (%)	IgM, *n* (%)	IgG, *n* (%)	IgM, *n* (%)	aPTT-LA sensitive, *n* (%)	dRVVT, *n* (%)
PAPS (65 cases)	0 (0%)	9 (13.8%)	12 (18.5%)	2 (3.1%)	2 (3.1%)	61 (93.8%)	39 (60%)
Control (45 cases)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)

Categorical variables are expressed as frequencies (percentage). *aPLs* antiphospholipid antibodies, *PAPS* primary antiphospholipid syndrome, *aCL* anticardiolipin, *ANA* antinuclear antibody, *anti-β2GPI* anti-β2 glycoprotein I, *dRVVT* dilute Russell viper venom time test, *LA* lupus anticoagulant, *aPTT-LA sensitive* activated partial thromboplastin time lupus anticoagulant sensitive

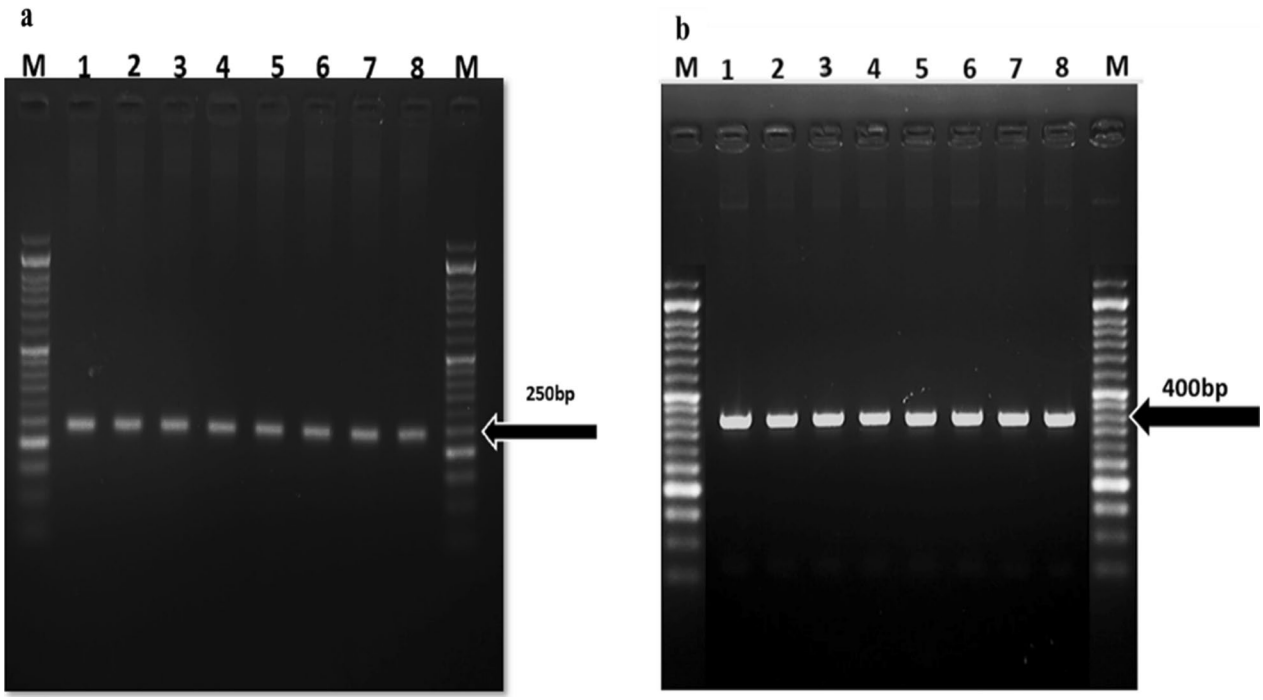

Fig. 2 Detection of the polymorphisms' genotypes for representative digested samples using 2% agarose gel electrophoresis. **a** All lanes represent AA genotypes of *TLR4* c.896A > G (249 bp bands), **b** all lanes represent CC genotypes of *TLR4* c.1196C > T (406 bp bands) and M is a 50 bp molecular weight marker

in any subject. The CC genotype of *TLR4* + 1196C/T was detected in all subjects, while the CT and TT genotypes were not detected in any subject. No significant changes in allele frequency or genotype distribution were observed between groups.

Discussion

Antiphospholipid syndrome (APS) is characterized by the presence of autoantibodies directed against phospholipids (PLs) and/or phospholipid-binding proteins. APS can be diagnosed by detecting aCL and anti-β2GPI antibodies or using LA coagulation test [20–22]. APS is commonly associated with obstetric events, such as complications of pregnancy, and vascular or thrombotic events including venous and arterial thrombosis [6, 23].

Antiphospholipid syndrome (APS) is an autoimmune disease characterized by T cell hyperactivity and B cell overstimulation leading to the overproduction of autoantibodies [24]. Antiphospholipid antibodies (aPLs) activate endothelial cells, monocytes, platelets, trophoblasts, and the complement system. aPLs have also been shown to inhibit fibrinolytic processes and alter coagulation pathways, thereby increasing the risks of thrombotic events and obstetric complications [3, 5].

Previous studies have implicated TLRs in the pathogenesis of autoimmune diseases [25, 26]. TLRs represent the first line of the innate immune system and perform

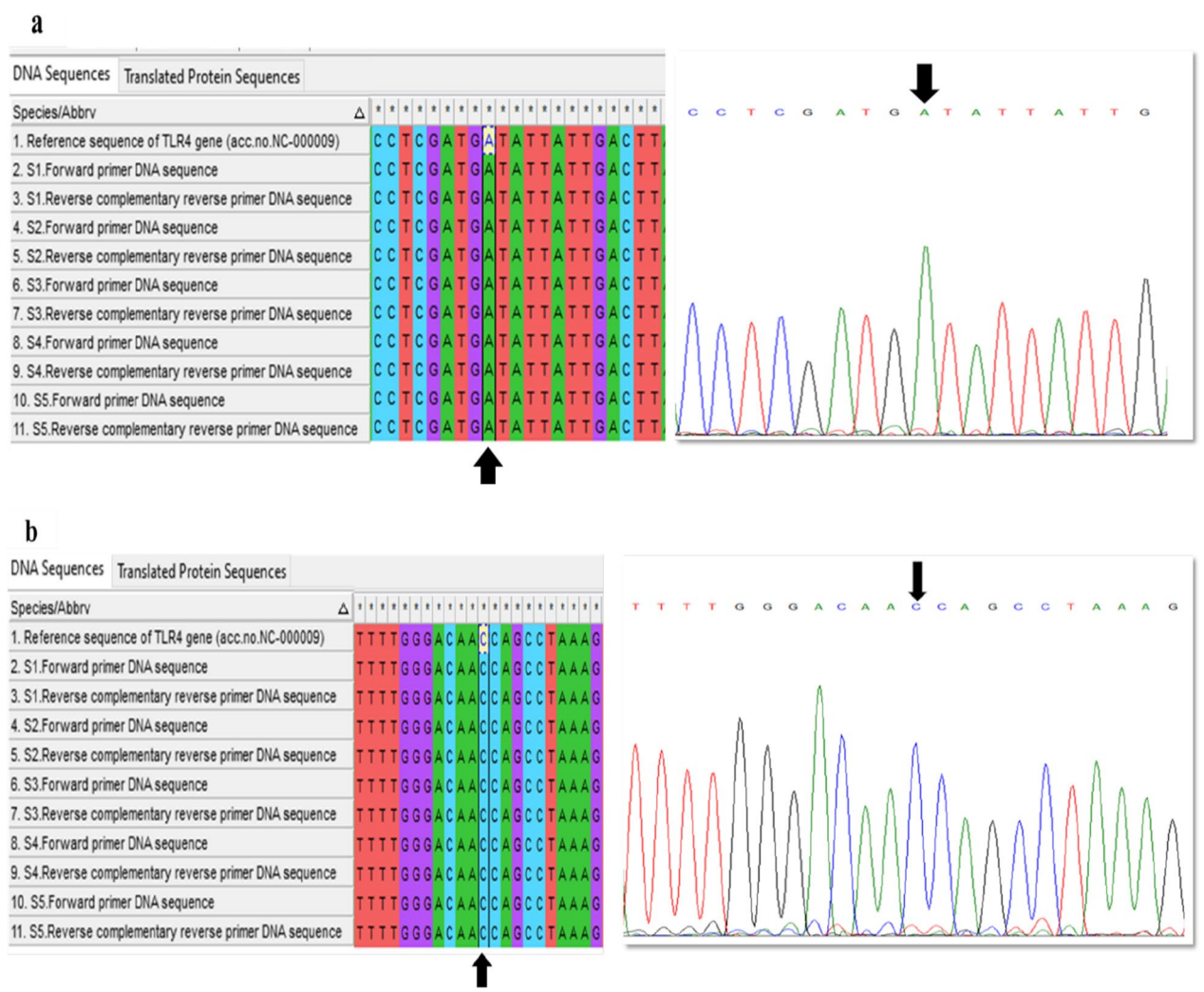

Fig. 3 Multiple sequence alignment of *TLR4* gene sequences and a representative sequence chromatogram for each SNP visualized by Mega X software. **a** *TLR4* c.896AA genotype, **b** *TLR4* c.1196CC genotype. Each of the included five samples was represented as forward and reverse DNA sequences (the latter in the reverse complementary form) for each genotype, and the location of the nucleotide in each SNP was determined based on the database sequence of the *TLR4* gene (AC; NC-000009), and it was highlighted by the black arrow in each SNP

Table 3 Genotypes and allele frequencies of SNPs among patients with PAPS and control subjects

SNP	Group	Genotype frequency *n* (%)			Allele frequency (%)	
		AA	AG	GG	A	G
TLR4 c.896A > G (rs4986790)	Control	45 (100%)	0 (0%)	0 (0%)	100%	0%
	PAPS	65 (100%)	0 (0%)	0 (0%)	100%	0%
		CC	CT	TT	C	T
TLR4 c.1196 C > T (rs4986791)	Control	45 (100%)	0 (0%)	0 (0%)	100%	0%
	PAPS	65 (100%)	0 (0%)	0 (0%)	100%	0%

Data are expressed as frequencies (percentage). *TLR4* toll-like receptor 4, *SNP* single nucleotide polymorphism, *PAPS* primary antiphospholipid syndrome

crucial roles in protecting against invading pathogens [27]. TLRs detect conserved molecules that are specific to individual microbial species, termed PAMPs, as well as endogenous molecules known as DAMPs. Activation of TLRs may trigger immune responses and inflammatory processes [28, 29].

TLR4 is one the most important and well-characterized TLRs. Structural analysis has demonstrated *TLR4* consists of an extracellular LRR domain, a transmembrane domain, and an intracellular TIR domain. The *TLR4* signaling pathway is considered the most robust of the TLR pathways, possibly due to its ability to recruit all four of the known TIR-containing adaptor molecules [30]. *TLR4* signaling can be initiated by the binding of PAMPs or putative endogenous ligands produced during tissue injury and inflammation.

Ligand binding to the extracellular domain of *TLR4* initiates a signal transduction cascade, with activation of the *TLR4* signaling pathway ultimately leading to the activation of innate immune cells via the production of cytokines. Aberrant activation of the *TLR4* signaling pathway has been shown to be involved in the pathogenesis of a number of disease types, particularly autoimmune diseases [13, 31].

Previous studies have demonstrated genetic variations in genes related to the immune system may increase individual susceptibility to particular diseases. Such mutations may alter immune regulation and therefore affect disease outcomes. SNPs are the most frequent type of genetic variation observed within the general population [32].

The *TLR4* gene was sequenced from 348 human samples and 35 mouse strains [33] and is considered to be highly polymorphic. The frequency of genetic variations is highest in the extracellular LRR domain which is responsible for the recognition of PAMPs. Accordingly, evolutionary pressure in pathogens may induce more frequent polymorphisms in this domain [32, 34].

The *TLR4* extracellular domain variants (4,986,790 and rs4986791) have been widely studied in a number of autoimmune and inflammatory disorders including rheumatoid arthritis, asthma, ulcerative colitis, Crohn's disease, gastric cancer, and gastric lymphoma in different cohorts [16, 35]. These SNPs occur at substantial frequencies (>5%) in populations across the globe [36]. However, no previous studies have investigated the relationship between *TLR4* gene polymorphisms and PAPS in Middle Eastern populations, particularly Egyptian populations. As a result, the present study aimed to determine the association of *TLR4* c.896A>G (rs4986790) and c.1196C>T (rs4986791) polymorphisms with the risk of developing PAPS in an Egyptian population.

The present study cohort was comprised of females only, which corroborates the results of previous studies. This female predominance is likely multifactorial; however, a number of X-linked genes, such as FOX-P3 and CD40L, play important roles in immune responses [37, 38]. Further, sex hormones and pregnancy, likely due to increased progesterone levels, are known to influence the immune system. In addition, estrogens bind to specific receptors on lymphocytes and can modulate both the innate and adaptive immune systems [39].

The median age of PAPS participants in the present study was 32.66 ± 1.42 years, indicating that the frequency of aPLs increases with age. This observation is in keeping with the known effects of aging on the immune systems which include a complex series of restructuring events leading to immune dysregulation which may include immune response reduction, increased inflammatory and oxidation processes, and autoantibody production. The generation of autoantibodies is an important cause of morbidity and mortality. However, younger populations have fewer risk factors for chronic diseases such as dyslipidemia, atherosclerosis, the use of estrogen-containing contraceptives, and smoking [40].

The detection of persistently elevated levels of aPLs is a requisite laboratory feature for the diagnosis of APS. Positivity for at least one aPL test must be detected on two or more occasions at least 12 weeks apart. The 2006 updated APS criteria advised patients with APS should be classified into one of two categories: category I comprising patients with more than one positive aPL test; and category II comprising patients with single aPL positivity [20].

Triple positivity (LA/aCL/anti-β2GPI) has been posited to confer a higher risk of thromboembolic events and pregnancy loss according to the results of several retrospective and prospective studies in patients with and without systemic lupus erythematosus (SLE).

Our serological analyses demonstrate that 85% of PAPS participants in the present study had single positivity for aPL. Double positivity and triple positivity for aPL were observed in 10% and 5% of patients with PAPS, respectively. These results indicate triple positivity for aPL may not be associated with severe disease; however, the present study had a limited sample size.

Several previous studies have posited polymorphisms in TLR genes encoding factors related to the innate immune response are involved in the pathogenesis of a range of autoimmune disease types. The findings of the present study conducted a statistical analysis of *TLR4* SNPs (rs4986790 and rs4986791) and found a predominance of homozygous wild-type (AA, aspartic acid) rs4986790 and wild-type (CC, threonine) rs4986791 variants in the control and PAPS groups. The allele frequencies of these *TLR4* variants corroborate the global minor allele frequency (GMAF) reported by the "1000 Genomes Project Phase 3" in the Ensembl database (www.ensembl.org). Furthermore, we observed no significant differences in allelic and genotypic frequencies between controls and patients with PAPS, indicating no significant association

of either rs4986790 or rs4986791 SNPs with the risk of developing PAPS.

Our data also corroborate previous studies that evaluated other autoimmune diseases in different populations. A study performed in the Tunisian population reported the prevalence of the A allele of the rs4986790 variant and C allele of the rs4986791 variant in patients with SLE and controls [41]. A separate study conducted in the Hungarian population assessed the association of *TLR4* Asp299Gly &Thr399Ile polymorphisms with ankylosing spondylitis (AS) and reactive arthritis (ReA) and found no significant difference in allele or genotype frequencies between controls and patients with AS or ReA. Moreover, no significant association was observed between these two common variants of *TLR4* and the risk of developing AS or ReA [42].

A further study [43] reported similar frequencies of the wild-type Asp299Gly and Thr399Ile *TLR4* polymorphisms between patients with RA, patients with SLE, and a control group. Accordingly, there appears to no association between the common *TLR4* Asp299Gly and Thr399Ile variants and the risk of developing SLE and RA in the Egyptian population.

Contrary to the results of the present study, a significant correlation has been reported between the *TLR4* Thr399Ile polymorphism and the incidence of gastric cancer in Italian patients ($P=0.023$) [44]. Consistent with our results, no significant correlation was observed between the presence of the *TLR4* Asp299Gly polymorphism and the risk of developing gastric cancer ($P=0.832$).

On the other hand, a separate study of the prevalence of these two SNPs in the *TLR4* gene in 110 Caucasian APS patients reported the frequency of these two SNPs was significantly reduced in APS patients with thrombotic manifestations compared to healthy controls after matching for ethnic origin ($P=0.038$) [19]. This finding may be attributable to these SNPs changing the extracellular domain of the *TLR4* receptor and modulating ligand binding, thereby reducing pro-inflammatory cytokine production and preventing aPL-mediated activation of the endothelium. Accordingly, these SNPs are considered to be protective against thrombosis with the prevalence of non-protective wild-type alleles in these polymorphisms overrepresented in patients with thrombosis increasing susceptibility to clotting disorders [7, 19].

The distribution of human *TLR4* polymorphisms varies between populations due to differences in social life patterns, environmental exposures, genetic backgrounds, habits, and disease etiologies.

Ethnicity has persistently been posited as a factor underlying the association between *TLR4* variations and a range of diseases. The multiethnic composition of the present Egyptian population [32] may explain some of the parallels and contrasts between the findings of the present study and published data from other populations [45].

Studies with larger sample sizes including a range of diverse ethnicities are required to validate the findings of the present study and elucidate the relationships between other *TLR4* SNPs and the risk of developing PAPS. Further, an intensive evaluation of *TLR4* gene variations may identify noninvasive biomarkers for the early detection and prognosis of PAPS and improve decision-making protocols used in the treatment of PAPS.

Conclusion

Despite previous studies positing a role for *TLR4* in the pathogenesis of PAPS, the present study revealed observed no significant association between variants in the extracellular domain of *TLR4* (Asp299Gly and Thr399Ile) and the risk of developing PAPS in an Egyptian population.

Abbreviations

TLR4: Toll-like receptor 4; PAPS: Primary antiphospholipid syndrome; SNPs: Single nucleotide polymorphisms; PCR–RFLP: Polymerase chain reaction-restriction fragment length polymorphism; aPLs: Antiphospholipid antibodies; anti-β2GPI: Anti-beta2glycoprotein I; aCL: Anticardiolipin antibodies; LA: Lupus anticoagulant antibodies; PRR: Pattern recognition receptor; LRRs: Leucine-rich repeats; PAMPs: Pathogen-associated molecular patterns; DAMP: Damage-associated molecular patterns; TIR: Intracellular toll–interleukin 1 (IL-1) receptor; LPS: Lipopolysaccharides; TIR: Toll/IL-1 receptor domain; MyD88: Myeloid differentiation protein 88; NF-kB: Nuclear factor kappa beta; ELISA: Enzyme-linked immunosorbent assay; ANAs: Antinuclear antibodies; dRVVT: Dilute Russell viper venom time; aPTT/LA sensitive: Activated partial thromboplastin time/lupus anticoagulant sensitive; REs: Restriction enzymes; MSA: Multiple sequence alignment; Mega X: Molecular evolutionary genetics analysis; SD: Standard deviation; DVT: Deep vein thrombosis; PLs: Phospholipids; SLE: Systemic lupus erythematosus; GMAF: Global minor allele frequency.

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Author contributions

All authors have contributed to designing, performing, analyzing the data, and writing the manuscript. All authors read and approved the final manuscript.

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Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

The study was directed by the guidelines of the 1964 Declaration of Helsinki and approved by the Research Ethics Committee of the Faculty of Medicine, Ain Shams University, Cairo, Egypt (Approval#MS642/2021).

Consent for publication
Not applicable.

Competing interest
The author(s) declared no potential conflicts of interest concerning the research, authorship, and/or publication of this article.

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