**ORIGINAL ARTICLE**

# Exploring the Role of PADI4 rs874881 and rs1635564 in Susceptibility to Systemic Lupus Erythematosus and Lupus Nephritis in Egyptian Patients

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***Key words:***
***Systemic lupus erythematosus; lupus nephritis; PADI4 polymorphisms; rs874881; rs1635564; NETosis***

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## ABSTRACT

***Background:*** *Systemic lupus erythematosus (SLE) is an autoimmune disease with pathogenesis involving immune complex deposition in various organs resulting in inflammation and tissue damage. Lupus nephritis (LN) is a crucial clinical manifestation of SLE. Immune complex deposition leads to neutrophil activation and death with neutrophil extracellular traps (NETs) generation. NETs are formed of histones, chromatin fibrils and antimicrobial peptides. Chronic NETs formation (NETosis) aggravates inflammatory tissue and is believed to be dependent on histone citrullination. Peptidylarginine deiminase-4 (PADI4) enzyme is essential for citrullination and highly expressed by neutrophils. PADI4 functionality and expression might be influenced by single-nucleotide polymorphisms (SNPs) in PADI4 gene.* ***Objective:*** *The aim of this work is to study the possible association of PADI4 rs874881 and rs1635564 SNPs with SLE and LN susceptibility in the Egyptian population.* ***Methodology****: this case control study encompasses 102 SLE patients (including 52 LN patients) and 50 healthy controls, all were subjected to genotyping analysis of PADI4 rs874881 and rs1635564 SNPs by Real-Time Polymerase Chain Reaction (PCR) technique.* ***Results:*** *Concerning PADI4 rs874881 polymorphism, genotype and allele frequencies did not show any significant differences between SLE patients and healthy controls (p=0.1 and 0.2 respectively), or between SLE patients with or without LN (p= 0.1 and 0.9 respectively). Regarding PADI4 rs1635564 there was no statistical significance between SLE patients and healthy controls in distribution of genotypes or alleles (p=0.07 and 0.8 respectively), and the same for SLE patients with or without LN with p=0.2 for both of them.* ***Conclusion:*** *PADI4 gene polymorphisms rs874881 and rs1635564 did not show any association with SLE or LN in our sample of Egyptian patients from Cairo.*

## INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune disease with complex pathogenesis involving autoantibodies generation and immune complex deposition in various organs resulting in inflammation and organ tissue damage. This damage can range from mild skin affection to damaged joints or major organs affection reaching cardiovascular, neuropsychiatric and respiratory systems[1]. Lupus nephritis (LN) is one of the most prevalent and crucial clinical manifestations of SLE[2]. Tissue damage mechanism of SLE and specially LN involve immune complex deposition leading to both complement system and neutrophils activation[3]. Immune complexes' antigens extend to include histones, chromatin fibrils and antimicrobial peptides released as part of the neutrophil extracellular traps (NETs) generation during activated neutrophils apoptosis process[4].

Chronic NETs formation (NETosis) promotes inflammatory responses and autoimmunity resulting in tissue damage[5]. NETosis is believed to be crucially dependent on the citrullination of histone bodies, this is the process of post-translational conversion of proteins' positive arginine residues into neutral citrulline residues[6]. Excessive citrullination was related to various chronic inflammatory diseases including autoimmune diseases such as multiple sclerosis[7], rheumatoid arthritis (RA)[8] and SLE[9]. Citrullination is catalysed by any of the five isoforms of peptidylarginine deiminases (PADIs), however PADI4 is the one considered to be in

charge of NETosis, as it is the most abundant isoform in neutrophils and the only one with nuclear access[10].

PADI4 gene is located on chromosome 1p36.13 and encodes PADI4 enzyme[11]. Single-nucleotide polymorphisms (SNPs) in this gene were nominated to influence the pathogenesis of autoimmune diseases and inflammatory diseases for example osteoarthritis[12], autoimmune thyroiditis[13] and RA[14]. Rs874881 where C allele is replaced by G allele resulting in amino acid substitution and rs1635564 which is an intronic SNP with G allele replaced by the T allele, both are suspected to be associated with SLE and LN susceptibility[15].

Our aim in this work is to study the possible association of PADI4 rs874881 and rs1635564 SNPs with SLE and LN susceptibility in the Egyptian population.

## METHODOLOGY

**Patients:**

This case control study included 2 groups: *SLE group:* One hundred and two patients having SLE, diagnosis and classification were done according to European League Against Rheumatism/American College of Rheumatology (EULAR/ACR) Classification Criteria for SLE[16].*Healthy control group*: Fifty healthy volunteers with matching age and sex.

Patients with any other autoimmune disease or any other chronic disease were excluded.

Healthy controls with family history of autoimmune diseases were excluded.

Participants were selected from the Outpatients' Clinic of the Centre of Medical Excellency, National Research Centre (NRC). Patients were recruited during periodic follow up at the Rheumatology Clinic and healthy controls were volunteers coming for regular checkup.

**Ethical consideration:**

Approval of National Research Centre Ethical Committee, Cairo, Egypt was obtained (number 16288). The institute follows Declaration of Helsinki in 2015 approved World Medical Association code of ethics.

All participants signed a written consent after explanation of the procedures and prior to participation in the study.

**Clinical history and physical examination**

All participants were subjected to full medical history taking and thorough clinical examination

We used Systemic Lupus Erythematosus Disease Activity Index 2000 (SLEDAI-2k) to assess the SLE disease activity[17] and LN was diagnosed according to EULAR/ACR Classification Criteria for LN[16].

**Laboratory investigations:**

***Sample collection:***

Venous blood samples were withdrawn into plain vacutainer for routine laboratory investigations and on EDTA anticoagulant for genotyping of PADI4 gene. Urinary samples were collected to measure albumin/creatinine ratio (ACR).

***Routine laboratory assays:***

Creatinine in serum and urine and urinary albumin were assayed by AU400 Beckman Chemistry analyzer (Beckman Coulter, Inc., USA). Complement C3 and C4 assays were done using MININEPH ZK023.R and ZK025.R kits, respectively (Binding Site Group Ltd., Birmingham, UK) on MininephPlus Analyzer endpoint nephelometry. Qualitative determination of Anti-double stranded DNA (Anti-dsDNA) antibodies and antinuclear antibodies (ANA) in serum were assayed using Indirect immunofluorescence tests with ORG-871 and ORG-870 kits, respectively (ORGENTEC Diagnostika GmbH, Germany).

***Genotyping analysis for PADI4 rs874881 and rs1635564 gene polymorphisms by real time PCR:***

We extracted the DNA from whole-blood samples using QIAamp DNA Blood Kit (Qiagen, Hilden, Germany). PADI4 rs874881 and rs1635564 were genotyped using TaqMan allelic discrimination method with primers and probes designed and manufactured by Applied Biosystems (TaqMan SNP Genotyping Assay, Cat no: 4351379, Foster City, California, USA) (context sequence is shown in Table 1). The reaction mix was of 20 µL volume and composed of 20ng of the tested DNA, 10 µL Master Mix, 0.5 µL of the assay primer and nuclease free water to complete the volume. The thermal profile was as follows; initial activation step for 10 minutes at 95°C then 40 repeated cycles of denaturation at 95°C for 15 seconds and annealing/extension for 1 minute at 60°C. Florescent data acquisition was done during the extension step. PCR was performed by LightCycler 480 Real-Time PCR System (Roche Diagnostics, Basel, Switzerland) and final product analysis was done by LightCycler 480 Real-Time PCR System software program. The procedure was done as manufacturer's instructions. Positive and negative controls were assayed with each run in addition to duplicates of 10% of the samples to ensure quality control[18].

**Table 1: Sequences of PADI4 rs874881 and rs1635564 gene polymorphisms detected by real time PCR**

| SNP ID | Context Sequence |
|---|---|
| Rs874881 | GTCAAAGCTCTACTCTACCTCACCG[C/G]GGTGGGTAAGTGACAACCAGGATCC |
| Rs1635564 | ATGTTGTGTGGCATCAGGAGAGGGC[G/T]GACCTGTTTCTTCCTCTCTCGATGG |

SNP=single-nucleotide polymorphism, ID=identification number

**Statistical methods:**

Data analysis was done using SPSS version 22 software (IBM Corp, Armonk, NY, USA). Mean±SD were used to describe quantitative data while frequencies were used to describe qualitative data. $\chi 2$ test was used for comparison of qualitative parameters and Student's t test was used for quantitative data comparisons. the $\chi 2$ test was used to detect The Hardy–Weinberg equilibrium of the studied SNPs among healthy controls. Comparing means of more than 2 groups was done using Analysis of Variance (ANOVA) test.

## RESULTS

This comparative case-control study consisted of 152 participants including 102 SLE patients and 50 healthy controls. Participants ages (mean± SD) were as follows for SLE patients (26.8 ±8.4) and healthy controls (26.70 ±7.11) (p=0.23), the female to male ratio were 89:13 and 37:13 respectively (p=0.07) with no significant difference between the two groups. Descriptive data of the studied SLE patients are shown in Table 2.

**Table 2: Descriptive data of the SLE patients**

| Variables | SLE (n=102) |
|---|---|
| Age of onset (years) [a] | 18.1±9.0 |
| Disease duration(years)[a] | 8.8±8.1 |
| Family history of SLE [b] (Positive/negative) | 19/83 |
| Associated clinical presentation [b] (Positive/negative) | |
| Arthritis | 85/17 |
| Malar Rash | 71/31 |
| Photosensitivity | 70/32 |
| Alopecia | 47/55 |
| Ulcers | 50/52 |
| Myositis | 7/95 |
| Serositis | 33/69 |
| Carditis | 8/94 |
| Neurological manifestations | 7/94 |
| LN | 52/50 |
| SLEDAI score[a] | 4.4±3.5 |
| ANA[b] (Positive/negative) | 99/3 |
| Anti-dsDNA antibodies [b] (Positive/negative) | 50/52 |
| Serum C3 (mg/dl)[a] | 83.6±43.7 |
| Serum C4 (mg/dl)[a] | 29.3±19.8 |
| Serum creatinine (mg/dl)[a] | 0.9±0.3 |
| ESR[a] (mm/[1st] hr) [a] | 36.5±25.5 |
| ACR (mg/g) [a] (LN patients only) | 314.8±296.9 |

[a] = data presented as mean ± SD, [b] = data presented as frequencies, SLE=systemic lupus erythematosus LN=lupus nephritis, SLEDAI= Systemic lupus erythematosus disease activity index, ANA= antinuclear antibodies, dsDNA= double-stranded deoxyribonucleic acid antibodies, C= complement, ESR=Erythrocyte sedimentation rate. ACR = albumin/creatinine ratio.

The genotype analysis of PADI4 polymorphisms rs874881 and rs1635564 showed no significant deviation from HWE among controls (p= 0.7 and 0.1 respectively). Distribution of the studied genotypes and allele frequencies between SLE patients and healthy controls are shown in Table 3.

Concerning PADI4 rs874881 polymorphism, both genotypes (p=0.1) and alleles (p=0.2) frequencies showed no significant differences between SLE patients and healthy controls, additionally there was no association between rs874881 and SLE susceptibility either under dominant, recessive or additive models (Table 3).

Our study of PADI4 rs1635564 polymorphism showed no statistically significant difference between SLE patients and healthy controls in distribution of genotypes (p= 0.07) or alleles (p=0.8), in addition there was no association between SLE occurrence and rs1635564 under the studied inheritance models shown in Table 3.

**Table 3: Comparison between SLE patients and healthy controls regarding the distribution of PADI4 rs874881 and rs1635564 under different models**

| SNP | Variable | SLE (n=102) | Controls (n=50) | P-value |
|---|---|---|---|---|
| **rs874881** | Genotypes | | | |
| | CC | 15 | 14 | |
| | GC | 76 | 31 | 0.1 |
| | GG | 11 | 5 | |
| | Alleles (2n) | | | |
| | C allele | 106 | 59 | |
| | G allele (minor allele) | 98 | 41 | 0.2 |
| | Dominant model: | | | |
| | GG +GC | 87 | 36 | |
| | CC | 15 | 14 | 0.07 |
| | Recessive model: | | | |
| | GG | 11 | 5 | |
| | GC+CC | 91 | 45 | 0.8 |
| | Additive | | | |
| | CC (Reference) | | | Reference |
| | GC | | | 0.06 |
| | GG | | | 0.2 |
| **rs1635564** | Genotypes | | | |
| | GG | 11 | 12 | |
| | GT | 78 | 30 | 0.07 |
| | TT | 13 | 8 | |
| | Alleles (2n) | | | |
| | G allele | 100 | 46 | |
| | T allele (minor allele) | 104 | 54 | 0.8 |
| | Dominant model: | | | |
| | TT +GT | 91 | 38 | |
| | GG | 11 | 12 | 0.07 |
| | Recessive model: | | | |
| | TT | 13 | 8 | |
| | GT+GG | 89 | 42 | 0.5 |
| | Additive | | | |
| | GG (Reference) | | | Reference |
| | GT | | | 0.3 |
| | TT | | | 0.06 |

Regarding PADI4 rs874881 and rs1635564 polymorphisms, genotypes' distribution did not show any statistical differences concerning age of onset, SLEDAI score, or serum levels of C3, C4 and creatinine within the SLE patients (Table 4).

**Table 4:** Association analysis of PADI4 rs874881 and rs1635564 with different parameters in SLE patients

| | rs874881 | | | | rs1635564 | | | |
|---|---|---|---|---|---|---|---|---|
| | **CC** | **GC** | **GG** | **p value** | **GG** | **GT** | **TT** | **p value** |
| **Age of onset (years)** | 23.7±9.8 | 18.1±8.2 | 10.7±8.1 | 0.01 | 16.8±9.6 | 17.8±9.1 | 21.9±7.7 | 0.34 |
| **SLEDAI score** | 4.9±4.5 | 4.4±2.6 | 3.3±2.0 | 0.67 | 4.9±4.4 | 4.0±0.3 | 3.8±3.5 | 0.37 |
| **Serum C3 (mg/dl)** | 81.2±42.3 | 86.6±45.3 | 65.8±31.2 | 0.33 | 90.1±47.2 | 81.9±43.4 | 87.9±44.9 | 0.78 |
| **Serum C4 (mg/dl)** | 29.5±18.4 | 27.2±20.1 | 24.3±16.1 | 0.78 | 23.8±13.9 | 30.6±24.2 | 32.5±17.1 | 0.26 |
| **Serum creatinine (mg/dl)** | 0.8±0.2 | 0.9±0.3 | 0.9±0.1 | 0.46 | 0.8±0.1 | 0.9±0.3 | 0.7±0.2 | 0.24 |
| **ESR (mm/$^{1st}$ hr)** | 28.2±16.7 | 38.3±27.5 | 35.7±19.1 | 0.38 | 33.4±20.9 | 37.4±27.3 | 34.5±17.2 | 0.84 |

All data are presented as mean ± SD, SLE=systemic lupus erythematosus, SLEDAI= Systemic lupus erythematosus disease activity index, C= complement, ESR=Erythrocyte sedimentation rate.

Out of the 102 SLE patients 52 patients were diagnosed with LN and we studied PADI4 rs874881 and rs1635564 polymorphisms comparing SLE patients with no LN to SLE patients with LN. As regards to rs874881 our results showed no significant difference concerning genotypes' or alleles' frequencies (p= 0.1 and 0.9 respectively) and there was no detected significant association on studying other inheritance models (Table 5).

In addition, the current study showed no significant differences between LN patients and other SLE patients regarding PADI4 rs1635564 neither with genotypes' (p=0.2) nor with alleles' (p=0.2) distribution and we did not detect any significant association on studying other inheritance models (Table 5).

**Table 5:** Comparison between SLE patients with and without LN regarding the distribution of PADI4 rs874881 and rs1635564 under different models

| SNP | Variable | SLE with no LN (n=50) | SLE with LN (n=52) | P-value |
|---|---|---|---|---|
| **rs874881** | Genotypes | | | |
| | CC | 10 | 5 | |
| | GC | 33 | 43 | 0.1 |
| | GG | 7 | 4 | |
| | Alleles (2n) | | | |
| | C allele | 53 | 53 | |
| | G allele (minor allele) | 47 | 51 | 0.9 |
| | Dominant model: | | | |
| | GG +GC | 30 | 47 | |
| | CC | 10 | 5 | 0.1 |
| | Recessive model: | | | |
| | GG | 7 | 4 | |
| | GC+CC | 43 | 48695 | 0.3 |
| | Additive | | | |
| | CC (Reference) | | | Reference |
| | GC | | | 0.1 |
| | GG | | | 0.8 |
| **rs1635564** | Genotypes | | | |
| | GG | 3 | 8 | |
| | GT | 39 | 39 | 0.2 |
| | TT | 8 | 5 | |
| | Alleles (2n) | | | |
| | G allele | 45 | 55 | |
| | T allele (minor allele) | 55 | 49 | 0.2 |
| | Dominant model: | | | |
| | TT +GT | 47 | 44 | |
| | GG | 3 | 8 | 0.1 |
| | Recessive model: | | | |
| | TT | 8 | 5 | |
| | GT+GG | 42 | 47 | 0.3 |
| | Additive | | | |
| | GG (Reference) | | | Reference |
| | GT | | | 0.2 |
| | TT | | | 0.3 |

## DISCUSSION

SLE is an autoimmune multisystemic inflammatory disease characterized by production of autoantibodies targeting nuclear antigens and deposition of immune complexes at classic target organs including kidneys[19]. NETosis, part of the host immune defense, is an extracellular formation of scaffolds of decondensed chromatin encompassing bactericidal granular and cytosolic proteins resulting from neutrophil cell death[20], this process is accomplished by PADI4-dependent citrullination of histones[21]. Dysregulation of NETosis is believed to participate in the loss of tolerance to self-antigens which is one of the earliest manifestations of

SLE[20]. PADI4 enzyme is encoded PADI4 gene, polymorphisms in this gene have been associated with SLE in a previous study[15].

This work is to study, for the first time, the PADI4 gene polymorphisms at rs874881 and rs1635564 loci and their association with susceptibility to SLE and LN in the Egyptian population.

The *PADI4* rs874881 gene polymorphism (also known as PADI4 -92) results into non-synonymous or missense variation at 112th residue of PADI4 protein (Gly112Ala)[22]. This SNP was studied as a part of haplotype comprising three SNPs (rs11203366G, rs11203367T and rs874881G), these susceptibility alleles were thought to be associated with more stable mRNA and increased enzymatic affinity and activity of PADI4 and was linked to diseases as RA[23], LN[15] and COVID-19 mortality[24]. Other studies focused on rs874881 SNP and it was linked to acute chest syndrome in sickle cell anemia patients[25] and RA[22].

Our results for rs874881 did not show any significant differences either in genotypes' or alleles' distribution between SLE and healthy control or between SLE patients with and without LN under any inheritance model.

Our results were in agreement with Massarenti's[15] research group who found that rs874881 was, on its own, not associated with susceptibility to either SLE or LN in Danish patients in spite of its association as a haplotype. Our findings might be explained by the *in-silico* pathogenicity prediction analysis that PADI4 structure and enzymatic activity are not majorly affected by Gly112Ala substitution[22]. Supporting our results, Bakr[26] study on an Egyptian population from Cairo with RA, another autoimmune inflammatory disease, did not show any association between rs874881 and disease occurrence. On the other hand, Abd-Allah's[27] and Bashir's[22] studies showed significant risk with G allele compared to C allele with the same polymorphism in RA Egyptians from Zagazig and Pakistani patients respectively. These discrepancies between results were shown in other studies in different populations confirming different inheritance patterns between different populations[22].

Our results for rs1635564 did not show any significant differences either in genotypes' or alleles' distribution between SLE and healthy control or between SLE patients with and without LN under any inheritance model.

Our results concerning rs1635564 was not in agreement with Massarenti's[15] research group who found SLE increased risk was associated with the presence of minor T allele weather homozygous or heterozygous, they also found LN to be associated with homozygosity of the minor allele (TT), in addition to observed variant allele gene dose effect was observed for both SLE and LN. They were not able to find explanation to those relations since rs1635564 is intronic with no known biological function. On the other hand, rs1635564 was also studied in Chinese with esophageal squamous cell carcinoma and did not show association with the disease on its own though they postulated similar mechanism of diseases pathogeneses[28].

SLE and LN pathogeneses are multifactorial and polygenic nature with known racial and individual variations even in the clinical presentation of the diseases[29]. In addition to the mentioned factors, the PADI4 enzyme activity dysregulation is not only dependent on the gene polymorphism but factors like high calcium levels can even affects its target specificity, tumor necrosis factor alpha in RA can induce its translocation and aberrant levels of citrullinated proteins, autocitrullination of PADI4 can also lead to increase in citrullinated proteins[30]. Which explains the inconsistency of the polymorphism results without undermining the role of PADI4 in the disease pathogenesis.

Future studies on the effect of PADI4 SNPs on PADI4 enzyme functions and its association with SLE and LN in different populations and with larger sample size are recommended.

## CONCLUSION

Our case-control study did not detect any association of PADI4 gene polymorphisms rs874881 and rs1635564 with SLE or LN in a sample of Egyptian patients from Cairo.

**Conflict of interests:**
Authors declare no conflicts of interests

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