

Relation between Vitamin D Level and Cyclin-Dependent Kinase-1 Gene Expression in Egyptian Patients with Lupus Nephritis and their Impact on Disease Activity

Abstract

Introduction: Lupus nephritis (LN) is a common complication of systemic lupus erythematosus. Vitamin D and cyclin-dependent kinase-1 (CDK1) have been implicated in its pathogenesis. The aim of this study was to determine the relation between vitamin D level and CDK-1 in lupus nephritis patients and their impact on disease activity. **Patients and Methods:** The current study was conducted on 50 LN patients and 20 control subjects from Egyptian population using ELISA to assess vitamin D level in serum and TaqMan assay for CDK1 gene expression. **Results:** Serum vitamin D level was significantly lower in LN patients and *CDK-1* gene was down expressed in the majority of LN patients. A significant inverse correlation was found between vitamin D level and 24 h protein in urine, ANA, anti-dsDNA, CRP, with a significant positive correlation with renal biopsy indices, eGFR. There was a non-significant inverse correlation between vitamin D and *CDK-1* (before RO-3306 addition) and a positive correlation after RO-3306. A significant positive correlation was found between *CDK-1* gene expressions with urinary albumin/creatinine ratio. However, a significant positive correlation was found between *CDK-1* (after RO-3306 addition) and proteinuria. While a significant positive correlation was found between *CDK-1* expression (after RO-3306 addition) and ANA, a significant positive correlation was found between *CDK-1* expression (before RO-3306 addition) and anti-dsDNA but *CDK-1* is not associated with renal biopsy indices nor with activity indices of SLE. There was a positive correlation between *CDK-1* gene expression and CRP before and after RO-3306 addition. **Conclusions:** Vitamin D deficiency acts as a risk factor for developing LN. *CDK-1* may have an association with the diagnosis of LN but its association with the progression of staging of LN is still confusing

Keywords: *CDK-1, lupus nephritis, systemic lupus erythematosus, vitamin D*

Introduction

Systemic lupus erythematosus (SLE) is one of the most heterogeneous autoimmune diseases with a wide range of clinical and serological manifestations.^[1,2] The disease varies from relatively mild manifestations of skin and joints to life-threatening renal involvement, as lupus nephritis (LN).^[2] The term LN encompasses diverse patterns of renal injury through immune-mediated mechanisms such as immune complexes formation, infiltration of leukocyte with intrarenal inflammation, and dual facets of fibrosis/scarring.^[3,4] In addition to clinical heterogeneity, SLE patients also demonstrate immunological heterogeneity; different autoantibodies have been described in SLE such as autoantibodies directed against single-stranded (ss)

and double-stranded (ds) DNA, Ro/La antigens, and ribonuclear protein (RNP).^[5,6] SLE pathogenesis entails a complex interplay of environmental factors such as ultraviolet (UV) light, genetic and epigenetic factors. In the last decade, vitamin D with its broad effects on the immune system has been studied extensively in autoimmune diseases.^[7] About 80% of patients with SLE show a photosensitivity, so vitamin D deficiency was found in these patients.^[8] 1, 25-dihydroxy vitamin D₃ (1, 25(OH)₂ D₃) regulates the immune system by inhibition of interleukin-2, antibodies production, and lymphocyte proliferation and also inhibits IFN- γ secretion.^[9-11] Many of the immunomodulatory effects of vitamin D are opposite to the immunological abnormalities observed with disease activity in SLE patients, so it can be concluded

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that vitamin D deficiency is a risk factor for the onset and development of disease activity in SLE.^[12]

Defective innate and adaptive immune system presented by overproduction of autoantibodies, increased production of cytokines and inflammatory mediators contributes to the activity of the disease.^[13] The type I interferon (IFN) production is classically induced by viruses, bacteria, or microbial nucleic acids when sensed by pattern recognition receptors (PRRs). It is mainly produced by plasmacytoid dendritic cell.^[14] Type I IFN mechanisms including the development of myeloid dendritic cells from monocytes, production of B-lymphocyte stimulator (BLyS) which stimulates B cell differentiation and autoantibody production, suppression of regulatory T cells and T helper 2 cells development, and promoting podocytes loss via suppressed renal progenitor differentiation into mature podocytes have direct pathogenic effect in LN.^[15]

Because of its mechanism, Type I IFN acts as central mediators in the pathogenesis of SLE and its clinical outcome such as LN. Wu *et al.*^[16] have found that type I IFN signaling pathway is regulated by cyclin-dependent kinase 1 (CDK-1) via induced phosphorylation of signal transducer and activator of transcription-1 (STAT-1) and the expression of interferon-stimulated genes (ISGs). *CDK-1* is member of the CDK subfamily that drives cell cycle progression.^[17]

Therefore, the aim of this study was to determine the relation between vitamin D level and *CDK-1* gene expression with disease activity scores, serological and biopsy parameters in patients with lupus nephritis.

Patients and Methods

The present study was conducted on seventy subjects who were grouped as:-

- 50 patients with LN fulfilling the Systemic Lupus International Collaborating Clinics (SLICC) classification criteria 2012.^[18]
- 20 normal healthy subjects' sex and age matched used as control group.

Patients were recruited from the outpatient clinic of Internal Medicine Department, Medical Research Institute hospital, Alexandria University. Patients with LN were diagnosed by the presence of proteinuria greater than 0.5 g/day or abnormal urinary casts and classified according to the scheme of the International Society of Nephrology and the Renal Pathology Society (ISN/RPS) 2003 classification of LN.^[19,20] Patients with infectious or other autoimmune diseases were excluded from the study. All patients were subjected to full history taking including age, sex, duration of the disease, and renal complications. Disease activity was assessed using SLE disease activity index (SLEDAI).^[21] Assessment of organ damage was done by applying SLICC/American College of Rheumatology (SLICC/ACR) criteria.^[22]

Laboratory investigations were done for all subjects in the study including complete blood count (CBC) and erythrocytes sedimentation rate (ESR), complete urine analysis, urinary albumin/creatinine ratio in spot urine sample, 24 h proteins in urine, serum creatinine, and blood urea. Calculation of estimated glomerular filtration rate (eGFR) using Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation was done.^[23] Immunological investigations including measurement of C-reactive protein (CRP) and complement components C3 and C4 were done by nephelometry. Autoantibodies analysis (ANA and anti-ds DNA) in addition to vitamin D serum level was done by ELISA.

Cell culture: Peripheral blood mononuclear cells (PBMCs) from all subjects were isolated using Ficoll-Hypaque and cultured in RPMI (PBMCs); they were subjected to a short-term culture (24 h) in 5% CO₂ incubator at 37 °C both in the presence and absence of the *CDK-1* gene inhibitor (RO-3306). After 24 h, cells suspended in culture media were collected for RNA extraction.

Detection of *CDK-1* gene expression: Total RNA was purified from PBMCs using Qiagen RNeasy Mini Kit Spin Technology according to manufacturer's instructions. (Qiagen, Valencia, CA, USA). The concentration of extracted RNA was determined using Nano Drop Thermo Spectrophotometry (Thermo Scientific) by measuring the absorbance at 260 nm A260. The purity of RNA was assessed using ratio A260/A280 ratio. Ratio between 1.7–1.9 was considered acceptable. RNA was reverse transcribed into cDNA and amplification of *CDK-1* gene was done by TaqMan® probes using One-Step real time PCR on Applied biosystems StepOne™ Real-Time PCR System (with Real MODTM Probe (W2 2x qPCR mix iNtRON Biotechnology, South Korea) according to the manufacturer's protocol using the following thermal profile, reverse transcription of RNA extract to cDNA was initially done at 50°C for 30 mins followed by incubation at 95°C for 10 min to activate the DNA Polymerase. This was followed by 40 cycles of 3 PCR-step amplification, denaturation for 95° C for 20 sec, followed by annealing at 55°C for 40 sec and elongation at 72°C for 30 sec, with real time fluorescence detection.

Relative expression of *CDK-1* gene was calculated using the comparative CT method (2^{-ΔΔCT} method).^[24] CT values of the target genes were normalized against the CT value of housekeeping gene (GAPDH). The fold changes of LN patients with and without the addition of CDK-1 inhibitor was compared to the control healthy group which was set to 1.^[25]

Statistical analysis was done using the statistical package for social sciences (SPSS software version 20, Chicago, IL). The Student's t-test (t-test) and one-way analysis of variance were used to assess the statistical significance of quantitative data of two groups and more than two groups,

respectively. Mann–Whitney and Kruskal–Wallis tests were used for statistical comparison between the various groups. The positivity rates were compared by the X² test. Pearson correlation coefficient (r) was used to investigate the relationship between variables. Statistical significance was set at $P < 0.05$.

Results

There were no significant differences between patients and controls regarding sex and age. The mean disease duration of the patients was 5.0 ± 3.46 years. LN patients were grouped using SLEDAI into those with moderate disease activity from 6–10 and high disease activity from 11–19. The mean value of SLEDAI in LN patients was 10.2 ± 3.8 . Group with moderate disease activity included 16 (32%) patients; group with high disease activity included 34 (68%) patients. The mean value of SLICC/ACR in LN patients was 1.34 ± 0.69 while the mean value of renal SLEDAI was 9.44 ± 1.49

Regarding the histological classification of renal biopsy in patients with LN, 34 (68%) patients had a renal biopsy and classified as class II in 2 patients (5.9%), class III in 4 (11.8%), class IV in 22 (64.7%), and class V in 6 (17.6%) patients. The mean value of chronicity index was 2.8 ± 2.3 while the mean value of activity index was 10.2 ± 3.8 [Table 1].

About 18% of LN patients were suffering from severe deficiency, 24% of them were with insufficient vitamin D level, and the rest of patients (8%) were of optimal vitamin D level. There was a decrease in *CDK-1* gene expression after RO-3306 addition in LN patients but with nonstatistical significant difference [Table 2 and Figure 1].

There was a significant inverse correlation between vitamin D and 24 h protein in urine, ANA, anti-dsDNA, and CRP. While there was a significant positive correlation between vitamin D and renal biopsy indices, eGFR, *CDK-1* (after RO-3306 addition) [Table 3], there was a significant positive correlation between *CDK-1* gene expression (before and after RO-3306 addition) and urinary albumin/creatinine ratio and a significant positive correlation between *CDK-1* gene expression (before RO-3306 addition) and 24 h protein in urine. In addition, there was a significant positive correlation between *CDK-1* expression (after RO-3306 addition) and ANA, a significant positive correlation between *CDK-1* expression (before RO-3306 addition) and anti-dsDNA. In addition, there was a significant positive correlation between *CDK-1* gene expression before and after (RO-3306 addition) and CRP [Table 4].

Discussion

SLE is an autoimmune disease with multisystemic manifestations. LN is one of the serious complications of SLE that effect on the patient's outcomes. Vitamin D is a steroid hormone which has multiple effects on host immune

Table 1: Distribution of patients according to renal biopsy

	Patients (n=34)	
Histological classification		
II	2	5.9
III	4	11.8
IV	22	64.7
V	6	17.6
Chronicity index		
Mean±SD		2.8±2.3
Median (min.-max.)		2.0 (0.0-7.0)
Activity index		
Mean±SD		10.2±3.8
Median (min.-max.)		11.0 (1.0-15.0)

Table 2: *CDK-1* gene expression after and before RO-3306

	Patients (n=50)	P
Before Ro-3306 Addition		0.493
Mean±SD.	0.48±0.51	
Median (min.-max.)	0.37 (0.07-2.17)	
After Ro-3306 addition		
Mean±SD.	0.44±0.33	
Median (min.-max.)	0.37 (0.05-1.22)	

CDK-1=Cyclin-Dependent Kinase 1

Table 3: Correlation analysis between vitamin D and clinical, laboratory data

	Vitamin D level	
	r _s	P
SLEDAI	-0.119	0.409
SLIC/ACR	-0.038	0.793
Renal SLEDAI	-0.153	0.288
Urinary albumin/creatinine ratio	-0.108	0.454
24 h protein in urine	-0.364*	0.009*
Serum creatinine	0.131	0.363
Blood urea	0.050	0.731
eGFR	0.462*	0.001*
Chronicity index	0.496*	0.003*
Activity index	0.411*	0.016*
ANA	-0.564*	<0.001*
Anti-dsDNA	-0.420*	0.002*
C3	-0.218	0.128
C4	-0.099	0.492
CRP	-0.490*	<0.001*
CDK-1 gene expression		
Before Ro-3306 addition	-0.137	0.344
After Ro-3306 addition	0.396*	0.004*

response. This study illustrated the levels of vitamin D in LN patients; there were three levels of vitamin D, about 36% of LN patients were suffering from severe deficiency, 48% of them were with insufficient level, and the rest of patients were of optimal level. Our results agree with

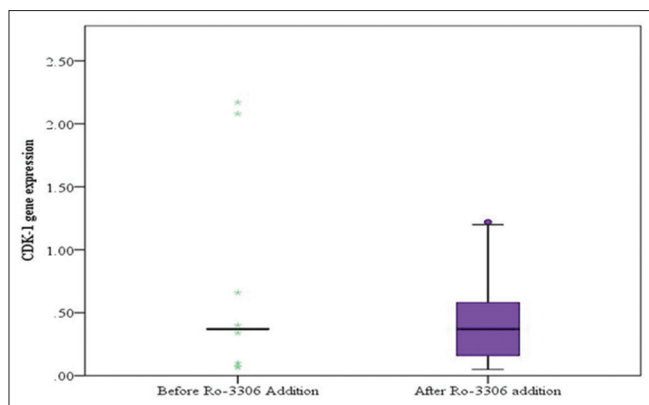


Figure 1: *CDK-1* gene expression before and after addition of Ro-3306

Mahto *et al.* (2018)^[26] and Gado *et al.*, (2017)^[27] who found that vitamin D level was significantly lower in LN patients.

Correlation analysis of the clinical data revealed a highly significant inverse correlation between vitamin D level and CRP. This can be explained that inflammatory markers were aggravated by vitamin D deficiency. CRP is a plasma protein that is considered an acute phase reactant which serves clinically as a marker of inflammation and tissue destruction. Our findings were in accordance with Buleu *et al.* (2015)^[28] and De Souza *et al.* (2014).^[29]

Another correlation analysis showed a highly significant inverse correlation between vitamin D level and both ANA, anti-dsDNA. This is clarified by the fact that autoantibodies are amplified by low vitamin D level and comparable results were found by Abaza *et al.* (2016),^[30] while Miskovic *et al.* (2015)^[31] and Reynolds *et al.* (2012)^[32] found no association between ANA, anti-ds-DNA and vitamin D level. Antinuclear antibodies are autoantibodies directed against nuclear antigen such as DNA and considered as a serological hallmark in SLE. Pathogenic role of ANA is represented by multiple mechanisms including organ damage by immune complexes deposition (Wijaya and Bahrin, 2017).^[33]

Regarding the renal biopsy, there was a significant positive correlation between vitamin D level and both of renal biopsy indices (activity, chronicity). In addition, we found a significant negative correlation between vitamin D level and 24 h protein in urine while a nonsignificant inverse correlation with albumin/creatinine ratio. These negative correlations can be explained by the fact that vitamin D deficiency causes reduction in urine creatinine in patients with chronic kidney disease leading to magnitude proteinuria (Agarwal *et al.*, 2011).^[34] In addition, our results showed a significant positive correlation between vitamin D level and eGFR while a nonsignificant positive correlation with both of serum creatinine, blood urea. This explained that low level of vitamin D may affect the ability of nephron to filtrate blood. In addition, there was

Table 4: Correlation analysis between *CDK-1* and clinical, laboratory data

	CDK-1 gene expression			
	Before Ro-3306 Addition		After Ro-3306 Addition	
	r_s	P	r_s	P
SLEDAI	0.003	0.982	0.238	0.096
SLICC/ACR	0.229	0.110	0.004	0.981
Renal SLEDAI	0.047	0.746	0.153	0.288
Urinary alb./cr. Ratio	0.611*	<0.001*	0.392*	0.005*
24 h protein in urine	0.455*	0.001*	0.156	0.278
Serum creatinine	0.234	0.102	0.191	0.183
Blood urea	-0.020	0.891	0.076	0.599
eGFR	-0.183	0.204	-0.174	0.226
Chronicity index	-0.023	0.899	-0.128	0.470
Activity index	-0.039	0.826	-0.002	0.992
ANA	0.070	0.627	0.337*	0.017*
Anti-ds-DNA	0.421*	0.002*	0.126	0.382
C3	-0.233	0.103	-0.216	0.132
C4	-0.133	0.357	-0.211	0.142
CRP	0.331*	0.019*	0.566*	<0.001*

a nonsignificant inverse correlation between vitamin D and C3, C4, SLEDAI, SLIC/ACR, renal SLEDAI.

IFN type I production is mainly induced by viral infection but also can be secreted by most nucleated cell types in response to activation of host pattern recognition receptors. The main type I IFN producer is the plasmacytoid dendritic cell (Mackern-Oberti *et al.*, 2015).^[14] Type I IFN acts as a key regulator of SLE and LN pathogenesis. Wu *et al.*^[16] (2016) found that type I IFN was positively regulated by *CDK-1*, which is a member of the cyclin-dependent protein kinases that regulate cell cycle. Our results indicate that *CDK-1* gene was down-expressed in the majority of LN. In contrast to that, Wu *et al.* (2016)^[16] reported overexpression of *CDK-1* gene in LN patients. This can be explained by ethnicity, lifestyle, and types of drug administered.

CDK-1 gene expression in the group without RO-3306 was lower than the group with RO-3306. Statistical analysis of these data revealed that RO-3306 cause decrease of *CDK-1* gene expression as in accordance with Vassilev *et al.* (2006)^[35] Our correlation analysis showed a significant positive correlation between *CDK-1* expression (before and after RO-3306 addition) and urinary albumin/creatinine ratio. In addition, there was a significant positive correlation between *CDK-1* expression (before RO-3306 addition) and 24 h protein urine, while after RO-3306 addition, there was a nonsignificant positive correlation. In accordance with our findings, Castellano *et al.* (2015)^[36] showed that locally produced type I IFN acts with an autocrine effect on renal proximal tubular cells leading to amplification of the tubulo-interstitial damage in lupus nephritis. This tubulo-interstitial damage leads to increase in fluid delivery

to the macula densa. Subsequently, there is an exacerbation of glomerulosclerosis, leading to further filtrate leak and proteinuria (Hodgkins and Schnaper, 2012).^[37]

Another correlation analysis showed a significant positive correlation between *CDK-1* expression (before and after RO-3306 addition) and CRP. These findings can be explained by CRP as an inflammatory marker is associated with the disease activity, which is regulated by IFN type I. In addition, correlation analysis showed a nonsignificant positive correlation between *CDK-1* expression (before and after RO-3306 addition) and serum creatinine, SLEDAI, SLIC/ACR, renal SLEDAI, while a nonsignificant inverse correlation between *CDK-1* expression (before and after RO-3306 addition) and eGFR was found.

In addition, correlation analysis showed a nonsignificant inverse correlation between *CDK-1* expression (before and after RO-3306 addition) and both renal biopsy indices. This can be explained by the fact that only 34 out of the total 50 lupus nephritis patients underwent renal biopsy, and the mean of both renal biopsy indices was low. In accordance with our findings, Shi and Zhang (2009)^[38] reported that CDK1 was not related to the clinical stages and histological differentiation of all ovarian cancer tissues.

However, a nonsignificant positive correlation was found between *CDK-1* expression (before RO-3306 addition) and ANA, while after RO-3306 addition, there was a significant positive correlation. In addition, a significant positive correlation was found between *CDK-1* expression (before RO-3306 addition) and anti-dsDNA, while after (RO-3306 addition), there was a nonsignificant positive correlation. In accordance with our findings, Domeier *et al.* (2018)^[39] found that type I IFN signaling causes loss of B cells' tolerance and promoting auto-reactive B cell development into plasma cells with over production of autoantibodies in SLE. As well as, correlation analysis revealed a nonsignificant inverse correlation between *CDK-1* expression (before and after RO-3306 addition) and both C3, C4.

Finally, a nonsignificant inverse correlation was found between *CDK-1* expression before RO-3306 addition and vitamin D level but not to a significant level. However, after RO-3306 addition, there was a nonsignificant positive correlation. In accordance with our findings, Sharan *et al.* (2011)^[40] reported that vitamin D treatment has reduced *CDK-1* expression in human uterine leiomyoma cells when compared with untreated control cells.

From our study, we can conclude that vitamin D deficiency acts as a potential risk factor for developing SLE with its clinical consequences, especially lupus nephritis. In addition, vitamin D deficiency is highly correlated with inflammatory markers and autoantibodies that mediate immune complex deposition and organ damage. But *CDK-1* gene is not associated with renal biopsy indices

nor with activity indices of SLE but associated only with autoantibodies production, urinary proteins, and inflammatory markers in patients with lupus nephritis. Hence *CDK-1* may have an association with the diagnosis of LN but its association with the progression of staging of LN is still confusing and need further studies.

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Conflicts of interest

There are no conflicts of interest.

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