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Effects of Vitamin D3 level on the gene expression of Immune checkpoint Cytotoxic T-lymphocytes antigen-4 in Iraqi patients with rheumatoid arthritis

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Abstract

Background: Rheumatoid arthritis is chronic autoimmune inflammatory disease that affects synovium, rheumatoid arthritis. Antigen presentation cells (APCs) link B7-1 and B7-2 with cytotoxic T-lymphocyte related protein-4 (CTLA-4), the member of the immunoglobulin superfamily, that causes T-regulatory cells to cause APCs to produce an inhibitory signal to autoreactive T cells (Treg). This study looked at the effects of vitamin D3(Vit-D3) on the expression of the CTLA-4 gene in whole blood taken from RA patients.

Methods: The case control study was conducted on 100RA patients acute and remission stages of the disease depending on DAS-28 and CDAI with respect to the level of vitamin D3. The cases and samples were obtained from Baghdad Teaching Hospital, Baghdad, Iraq from Jun 2022 to January 2023. In addition,45 healthy subjects were enrolled in this study. Standardized gene expression at a level of a reference gene (GAPDH) and enumerated by the ΔCt value and fold change ($2^{-\Delta\Delta Ct}$) method using qPCR. The ESR was measured in all subjects and serum CTLA-4, anti-cyclic citrullinated peptide (ACCP), RF was measured using ELISA techniques. The D3 level was assessed using immunofluorescent technique.

Results: Significantly increased ESR and serum CTLA-4 levels, ACCP, CRP, RF in RA patients compared to controls ($p \leq 0.05$), respectively. The vitamin D3 was significantly reduced in acute cases and substantial raised in remission case compared to the controls ($p \leq 0.05$) fold change of gene expression was dramatically elevated in patients excepts in acute male cases compared to 1. There was a significant positive correlation between vitamin D3 and CTLA-4 expression.

Conclusion: The current study showed that Vit-D3 up-regulates CTLA-4 gene expression as the defensive mechanism in contradiction of a severity of a disease and possible therapeutic targets through the action of Treg population and immunotolerance.

Introduction

Rheumatoid arthritis (RA) is a complex autoimmune disease, considered by an ongoing T-cell response that had avoided normal control mechanism in both groups of rheumatoid arthritis, the inflammatory stage includes initial clinical outcomes of arthralgia, swelling, redness, and uniform restrictive variety of motion. And the remission stage in response to anti-inflammatory treatments and sub-clinical and chronic synovitis [1]. Immune system dysfunction is one of the important topographies in the progress of autoimmunity. The CTLA-4 is the leader immune-checkpoint theatre, the vital outcome in the regulation of immune tolerance and suppression [2]. The T-cell response-regulating genes might be the main factor of the vulnerability to RA, it generates two major isoforms of CTLA-4 including, membrane-bound (m-CTLA-4) and soluble form (sCTLA-4) [3]. CTLA-4 is a crucial negative controller of T-cell initiation and co-inhibitory receptor, it is expressed soon after T cells are activated on their surface and is a member of the CD28 family [4]. Numerous studies reported the role of Vit-D3 in autoimmunity, the Vit-D3 had been exposed to combat the repressive effect of inflammatory cytokines on CTLA-4 and improve CTLA4-promoted conquering of inflammation [5]. A key critical role in RA is the activation of T lymphocytes, which requires more than just the T-cell receptor (TCR) recognizing specific antigenic peptides to activate them, nonetheless similarly the costimulatory signs providing by additional surface molecules on T cells [6]. the gene expression of CTLA-4 is up-regulated to prevent the proliferation of activated T-cells and reduce cytokines mediated inflammation including, IL-1 subfamily, IL-2, IL-4, IL-17, and TNF-Alpha production through the action of regulatory and anti-inflammatory aspects of proinflammatory cytokines includes IL-10 [7]. By interacting with CD28, B7 fragments' primary purpose is to increase and tolerate T-cell responses. They also offer inhibitory signals when they bind to CTLA-4 [8]. Numerous research has previously linked higher CTLA-4 protein levels in the sera of auto-immune disease patients [9]. The current study was aimed to detect the soluble form of CTLA4, ACCP, autoantibodies against the Fc portion of IgG (RF- Factor), Vitamin D3 and the gene expression of CTLA-4 isoform in the inflammation and remitting stage among Iraqi patients with Rheumatoid arthritis, as well as to investigate the correlation between serum level of vitamin D3 and CTLA4 gene expression.

Methods

Subjects

One hundred of Iraqi RA patients were enrolled in this study, they were sub-grouped into inflammation stages with low serum D3 level (21/55) and relapsing stage with moderate and optimum vitamin D3 level (34/55). The samples were collected from Baghdad teaching hospital, Baghdad, Iraq from Jun 2022 to January 2023. their oldness was ranged from 36 to 65 years and their age coordinated to 45deceptively healthy individuals (18 male, 27 female) and their age reached was 32- 57 years. Based on laboratory investigations and medical inspection for both patients and control.

Measuring of sCTLA-4, ESR, CRP, VIT D3, RF and Anti-CCP level

A quantitative sandwich ELISA kit was used to measure soluble CTLA-4 rendering to the instructions provided by producer My BioSource, USA. The Rf, CRP, ESR were measured for all individuals. The ACCP-Ab was measured using SUNLONG sandwich ELISA kit, Zhejiang, Hangzhou, China. The serum vitamin D3 was measures using BioMerieux SA, France.

Primers were used in this study

The sequences of the primers used in this study designed using the free site https://www.ncbi.nlm.nih.gov/tools/primer-blast/primertool. The forward primer for Homo sapiens CTLA-45'-CTACCTGGGCATAGGCAACG-3', reverse: 5'-CCCCGAACTAACTGCTGCAA -3'. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as endogenous control, the forward primer was 5'GACAGTCAGCCGCATCTTCT3', and the reverse primer was 5'GCGCCCAATACGACCAAATC3'. According to the producer Takara Bio Inc, Shiga, Japan, the primers were created and used.

Preforming the Expression of CTLA-4

Blood samples were used to extract and purify RNA according to the manufacturer's instructions used Relia-Prep™ RNA Miniprep, Promega, USA. The Reverse transcriptase, RT mix reagent, England Kit used to convert total RNA to cDNA using Superscript IV-VILO Master Mix, Invitrogen, USA. The reaction was performed using a SaCycler-48 thermal cycler, Sacace, Italy. For quantitative PCR (qPCR), the reaction mixture was prepared using KAPA-SYBR® Fast qPCR master mix, USA. The GAPDH housekeeping gene was used as an endogenous control. The melting-curve analysis was attained the separation topographies of dsDNA during cycles with increasing denaturing temperature.

Statistical analysis

GRAPH Pad Prism v-8 and IBM SPSS Statistics v-27 were used to calculate the mean and mean, standard error, and correlation; P0.05 was deemed non-significant. Using the equations CT = CT of target gene - CT of U gene, CT = CT of each sample - average

control C, and Fold change = 2-Ct, the fold change was calculated. It was noticed that the control value was regarded to be 1, samples with values less than 1 are considered down-regulated, while samples with values greater than 1 are considered up-regulated. Clinical Disease Activity Index (CDAI) for RA was assessed using https://www.mdcalc.com/clinical-disease-activity-index-cdai-rheumatoid-arthritis. Disease Activity score-28 (DAS-28) was calculated used https://www.das-score.nl/nl-nl.

Results

The overall mean age in RA patents was 51.6±7.66 years and 48.3±8.17 years, p= 0.06 in the control group with probability <0.05, the RA patients age was distributed according to their gender in to 12 males (2; with 40-50 age, 12; > 50 years) and 43 females (3; <40, 16; 40-50, 22 >50 years), p 0.270. Approximately the patients were sub-grouped according to DAS into Severe Inflammation with low D3 level 21 (38%), and Remission (Low-Moderate) with optimum level of D3 34 (62%) with p= 0.084 (Table 1).

Result showed that most of clinical cases according to DAS-28 and CDI, was in remission stage p<0.05, due to many factors including lifestyle, NSAID therapy, steroids, and other anti-inflammatory therapies (table 2, 3).

Serum level of ACCP, D3, RF and CRP

Analysis of variants illustrates significant differences p≤0.05=*, p≤0.03=** in RA patients ESR, CRP, RF and ACCP compared to controls, respectively. There were non-significant differences in vitamin D3 and soluble CTLA-4 in severe cases compared to controls p-value >0.05, while a dramatically rose in D3 and sCTLA4 level in remission cases compared to controls, p-value <0.05 compared to controls, respectively (Table 4). There was positive correlation between vitamin D3 and CTLA-4, in which serum level of CTLA-4 increased with the increase of D3 in RA patients with *Pearson R* 0.8421.

Quantitative expression of CTLA-4 was established by qRT-PCR. The gene expression was standardized to the level of a house-keeping gene GAPDH and enumerated by the folding 2−ΔΔCt method.

Case type	Gender			*p*-value
	Male		Female	
RA	12 (22%)		43 (78%)	0.130
Control	9 (20%)		36 (80%)	
Gender	Age Groups			P-value
	<40 years	40-50 years	>50 years	
Female	3 (100%)	16 (88%)	22 (64.7%)	0.270
Male	0 (0%)	2 (12%)	12 (35.7%)	
Case type	Age (years)			*p*-value
RA	51.6±7.66			0.06
Control	48.3±8.17			

Table 1: The Distribution of age and gender in patients and control

Case type	Cases and Percentage	Gender	*p*-value
Severe Inflammation with low D3 level.	21 (38%)	Male; 4 (19%) Female; 17 (81%)	0.001
Remission (Low-Moderate) with optimum level.	34 (62%)	Male; 8 (23.5%) Female; 26 (77.5%)	

Table 2: Distribution of RA patients according to Clinical Disease Activity score (DAS)

Score	Severe cases with low D3 level.	Remission (Low-Moderate) with optimum D3 level.	*p*-value
DAS-28- ESR	6.8 ±0.44	6.1±0.69	0.166
DAS-28- CRP	5.33±0.12	4.81±0.36	0.21
CDAI	23.66 ±0.12	18.66±0.23	0.089

DAS28 = Disease Activity Score with 28 joint amounts, CDAI = Clinical Disease Activity Index, CRP = C-Reactive Protein; ESR = erythrocyte sedimentation rate.

Table 3: DAS-28 and CDAIin RA patients

RA markers	Gender	N	ESR (mm/1h)	*p*	CRP (mg/L)	*p*	RF (IU/ml)	*p*	ACCP (U/ml)	*p*	D3 (ng/ml)	*p*	CTLA-4 (ng/ml)	*p*
C	M	18	5.24±1.33		2.44±1.31		2.13±0.7		1.33±0.88		33.12±3.2		1.13±0.21	
	F	27	9.33±2.46		5.34±1.06		6.45±1.3		2.01±0.89		32.6±7.6		1.89±0.63	
S Cases	M	4	42.24±6.33	**	43.33±4.6	**	92.8±8.9	**	213.7±6.8	**	13.66±2.1	ns	2.03±1.42	ns
	F	17	48.66±5.42	**	52.68± 2.33	**	108.9±9.6	**	247.6± 8.3	**	11.64±4.3	ns	2.24±1.04	ns
R-Cases	M	8	18.33±1.28	*	16.18±1.3	*	25.48±1.2	*	62.3± 4.2	*	47.6±4.55	*	6.87±2.34	*
	F	26	24.66± 3.22	*	21.69±4.1	*	38.87±2.0	*	71.2±5.1	**	43.8± 7.33	*	7.24±3.02	*
r	-	-	-0.3612		-0.3916	-	-0.4420	-	-0.4486	-	0.8421	-	-	-
95% CI	-	-	-0.8663 to 0.7376	-	-0.8760 to 0.7188	-	-0.8893 to 0.6883	-	-0.8920 to 0.6813	-	0.007868 to 0.9789	-	-	-
r^2	-	-	0.03399	-	0.04960	-	0.07798	-	0.08492	-	0.7630	-	-	-
P	-	-	0.727	-	0.671	-	0.592	-	0.575	-	0.0398*	-	-	-

Data presented as Mean± standard Error, *= p value≤0.05, **= *p*-value ≤ 0.03, ns= p-value ≥0.05, C= controls, S = severe, R= Remission, r= Pearson r, r2= R squared, M= male, F= Female, CI= 95% confidence interval

Table 4: Difference ESR, CRP, RF, ACCP, D3 and CTLA-4 between RA patients and control and the correlation between CTLA-4 and RA markers.

RA Group	Gender	mean of ΔCt± SE	ΔΔCt	P value	Folding
Control	Male	7.66± 0.24	-	-	-
	Female	7.84±0.44	-	-	-
Severe cases with low D3	Male	8.292±1.02	0.629	0.124	0.6464
	Female	7.5±1.37	-0.34	0.089	1.26576
Remission cases with optimum D3 level	Male	1.75±0.46	-5.91	0.12	60.1295*
	Female	1.3±0.31	-6.54	0.062	93.0542*

*= significant difference, *p*-value<0.05

Table 5: Fold change expression of CTLA-4in RA patients and control

The RT-qPCR summary plot is assumed in figure (1). The results showed that CTLA-4 was over-expressed in RA remission patients (males and females) 60.1295, 93.0542, respectively. And the expression of CLLA-4 was slightly increase in acute RA females and downregulated in males,1.26576, 0.6464, respectively. (Table 5).

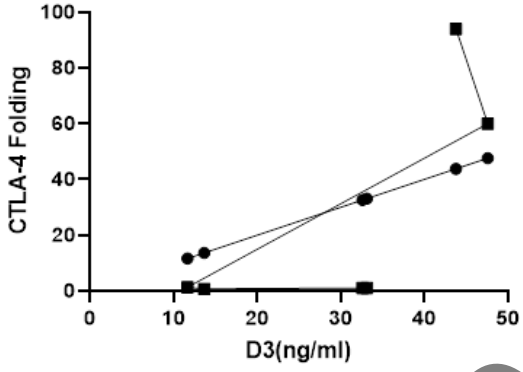

Figure 1: The correlation between Vit D3 and CTLA-4 folding, +correlation, r= 0.941, p<0.05.

There was positive correlation between vitamin D3 and CTLA-4, in which the gene expression of CTLA-4 increases with the increase of D3 in RA patients with Pearson R0.7422, p-value <0.05.

Discussion

Regulatory T cells (Treg) play a crucial role in immunological tolerance, and numerous studies have demonstrated Treg abnormalities in a variety of autoimmune disorders, including rheumatoid arthritis. CD28 and CTLA-4 are members of the immunoglobulin family that have been demonstrated to have a crucial role in the activation of Treg and immunological tolerance [11]. In the acute group, ACCP, RF, CRP, and ESR were shown to be greater than in the remission group, despite no correlation with age, gender at analysis, or disease duration. In accordance with Cavalcanti et al. [12,22], it was also associated with DAS-28 and CDAI. CTLA-4 immunoregulation has been thoroughly investigated. In current study, we discovered abnormality in RA patients' CTLA-4 expression in response to their vitamin D3 levels, and as a result, we reported that there was a positive correlation between the vitamin D3 level and CTLA-4 expression, in which the expression of CTLA-4 increases with the increase in vitamin D3 level, thus explaining the shift from severe disease to remission by immunomodulatory of Treg, as many previous studies [13] have reported. In addition, the downregulation of CTLA-4 may be responsible for Treg abnormalities in RA, which in turn results in aberrant T cell activation and development. However, the expression of CTLA-4 on traditional T cells prevents perversely activated T cells from penetrating and severely harming non-lymphoid tissues [14]. and induces immunological tolerance [15]. The effects of CTLA-4 cross-linking diminished the TCR- response in healthy Treg. Through phosphorylation of several TCR-related signaling markers and tyrosine phosphorylation [16], the Treg from RA patients remained resistant to the effects of CTLA-4 cross-linking. We hypothesized that decreased CTLA-4 in acute RA with low D3 levels contributes to defective protein phosphorylation regulation by CTLA-4 in RA Treg [17]. As numerous studies have suggested, decreased phosphorylation of AKT is associated with Treg [18] activity. Similarly, our data reveal a reduction in Akt phosphorylation in Treg due to the dual role of CTLA-4 in healthy individuals in response to vitamin D3, but not in RA patients. The effect of 25(OH)2 D3 on the proliferation of Th1 and Th17 cells, as well as its anti-inflammatory effects, while stimulating the proliferation of Treg cells, suggests a correlation between vit-D3 deficiency and the severity of rheumatoid arthritis, as indicated by Nemours studies on other autoimmune diseases [19,21]. These findings highlight the importance of vit-D3 in suppressing immune responses in order to treat autoimmune disorders such as ulcerative colitis, rheumatoid arthritis, autoimmune thyroiditis, and type 1 diabetes. Numerous prior research validated our conclusion that vit-D3supplement had a large effect on gene expression of CTLA-4 and a potential therapeutic target [20]. In contrast to the severity of the condition, the current investigation revealed that vitamin D upregulates CTLA-4 gene expression as the protective strategy. In addition, there were several limitations, such as screening for baseline vit-D3 insufficiency in individuals with mild to moderate RA and dose-response analysis of vitamin D3. Therefore, it is recommended to conduct larger trials with varying

vitamin D concentrations and longer observation periods.

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Competing Interest

The authors declare that there is no conflict of interest.

Authors' contributions

Aseel S. Mahmood: Design the review article Contributed to article writing and participation in molecular working methods.
Ahmed Sabah Kadhim: Samples collection.
Yasir W. Issa: Contributed data and analysis tools and immunological action methods.

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