The Correlation Between Plasma Levels of Vitamin D and Epigenetic Alterations of Treg-Specific Demethylated Region (TSDR) in Rheumatoid Arthritis Patients

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Abstract- Rheumatoid arthritis (RA) is the most common autoimmune inflammatory disease of joints among adults. Regulatory T cells (Treg) control immune responses in this illness. Through the expression of FoxP3, a Treg transcription factor, Vitamin D keeps autoimmune diseases in check. Yet, the molecular mechanism of FoxP3 expression by vitamin D is not well-inspected. It may influence FoxP3 expression via epigenetic changes. This study aimed to investigate the correlation between plasma levels of vitamin D and the demethylation of the TSDR region in Foxp3 promoter in patients with RA. Twenty untreated RA patients and 41 healthy controls participated in this study. Plasma levels of 25-OH vitamin D were measured by competitive ELISA method. The demethylation of TSDR regions in Foxp3 gene was also assessed using the quantitative Methylation Specific PCR (qMSP) method. The demethylation of TSDR region was significantly lower in RA patients compared with healthy controls (P=0.006). Vitamin D plasma levels were significantly higher in RA patients rather than healthy people (P=0.034). There was no statistically significant correlation between vitamin D plasma levels and demethylation of TSDR region. As expected, epigenetic alternation showed considerable difference between RA patients and healthy controls, but about vitamin D correlation with methylation modification, more studies are needed.

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Keywords: Vitamin D; Epigenetics; DNA methylation; TSDR; Rheumatoid arthritis

Introduction

Regulatory T lymphocytes (Treg) adjust the immunological responses of the body in the way that a decrease in their numbers or function could commence various autoimmune disorders, including Rheumatoid Arthritis (RA), Systemic Lupus Erythematosus (SLE), type I diabetes, and multiple sclerosis (MS) (1). FoxP3, the transcription factor related to Tregs, is crucial for the development and function of this cell lineage (2). There have been reports suggesting a decrease in FoxP3 expression in RA (3-6). RA is the most commonplace autoimmune disorder of the joints in the adults with symptoms such as pain, hardened joints, and inflammation of the lining of the joints that interfere with the function and movement of the body. This ailment is the result of B- and T-lymphocytes and macrophages responding against self-antigens. Several factors, like genetic or biomechanical agents and environmental components, are involved in RA (7). One of the environmental elements is vitamin D. Low levels of this vitamin are reported in the serum of autoimmune-inflicted patients, like RA, MS, type I diabetes, and SLE. It is believed that vitamin D has an important part in the pathogenesis of the aforementioned diseases (8-11). 1,25-Dihydroxyvitamin D3 (VD3), the most active form of this vitamin, after traversing the cell membrane binds to its receptor (VDR) in the cytoplasm. Afterward, it attaches

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to an intronic conserved noncoding sequence (CNS) region +1714 to +2554 of FoxP3 gene in the nucleus called vitamin D response element (VDRE). This causes a spike in FoxP3 gene promoter activity and the subsequent increase of FoxP3 expression. Thus, by means of increasing FoxP3 expression and then increasing the inhibitory effect of Tregs, this vitamin represses the immune response (12). So far, the molecular mechanism by which vitamin D regulates FoxP3 expression has not been vividly explained, and more experiments are needed on this subject (13). Recent studies show that epigenetic modifications, like gene promoter methylation, are indispensable in regulating biological activities, including cell cycle and cell differentiation (14). These epigenetic modifications are also involved in regulating FoxP3 gene expression. A CpG-rich region, known as Treg-specific demethylated region (TSDR), has been discovered at 5'UTR of FoxP3 gene that its methylation stops FoxP3 expression. So, TSDR methylation status controls FoxP3 expression activities, and cells with demethylated TSDR regions indefinitely express FoxP3 (15,16). On another note, the role of vitamin D in altering the methylation status of various gene promoters has been verified (17). Furthermore, through methylation, other vitamins and nutrients, like folate, polyphenols in tea, and soy isoflavones are important in the pathogenesis of other diseases like cancer. For this reason, it is safe to say lifestyle and food choices are essential in epigenetic alterations and eventually disease incidence or prevention (18) .

This study aimed to elucidate the correlation between plasma levels of vitamin D in RA patients and the demethylation of TSDR regions in FoxP3 in comparison with a healthy control group. This was to demonstrate whether this vitamin has any effect on FoxP3 epigenetic alterations. If there is indeed a pattern, this vitamin can be used as an effective agent in regulating RA.

Materials and Methods

Study groups

Twenty patients who referred to the Helal-Ahmar Clinic of Kermanshah University of Medical Sciences (KUMS) in the summer and fall of 2015 were enrolled in our study after being diagnosed by a rheumatologist according to EULAR/ACR criteria and other supplementary tests. Informed consent was obtained from all individual participants included in the study. Forty-one people of the medical school staff of KUMS, whose age and sex was matched with the patient group, were chosen as the control group. The demographic status of each group is represented in table 1. Only recently-diagnosed RA patients who had not received any medication were allowed to enter our study, and people with a history of previous rheumatic disease or consumers of vitamin D were excluded from our study.

Table 1. Demographics status of patient and control groups

Studied population	Patient group (20 patients)	The control group (41 healthy)	*P*
Age (year-old)	43±11.24	44±9.11	0.55
Sex (male)	3 (15%)	6 (14.6%)	0.80

Measuring plasma levels of vitamin D

Plasma 25-OH vitamin D in the patient and control groups was assessed by enzyme-linked immunosorbent assay (ELISA) using the competitive method according to the Ratio Diagnostics (RD) kit instructions. Plasma levels less than 10 ng/ml were considered as vitamin D deficiency, levels at 10-30 ng/ml as insufficiency, levels at 30-100 ng/ml as sufficient, and levels above 100 ng/ml as intoxication.

DNA extraction and bisulfite treatment

The DNA was extracted from whole blood of patient and control groups using standard salting-out method (19). The quality and quantity of the extracted DNA were determined with 1% agarose gel electrophoresis and spectrophotometry techniques, respectively. DNA was frozen and kept at -20° C until further use. Samples were treated with bisulfite using EpiTect Bisulfite Kit (Qiagen) according to the manufacturer's instruction and preparation for the methylation-specific PCR (MSP) assay. The bisulfite treatment converts unmethylated cytosine bases to uracil, while 5-methylcytosine bases remain intact during the process. This was accomplished by Thermal Cycler 96 (Roche Life Science). PCR settings were as follows: 95° C for 5 minutes, 60° C for 25 minutes, 95° C for 5 minutes, 60° C for 85 minutes, 95° C for 5 minutes, and eventually 60° C for 175 minutes. The concentration of treated DNA was measured by

NanoDrop device.

Quantitative methylation-specific PCR (qMSP)

Syber green master mix (Takara) was used for this reaction, and the two pairs of primers specific for the methylated (M) and unmethylated (U) TSDR regions were crafted using MethPrimer software (20). Target DNA is a 117-base pair sequence that is located in the CpG islands of the first FoxP3 intron. There are 10 CpG islands in this sequence. The final volume of real-time PCR reaction, done by LightCycler 96 System (Roche Life Science), was 20 µl. Twenty-five pmol of each M and U primers and 50-100 ng of treated DNA were used. A reaction containing a fully-methylated DNA was used as positive control for M primers, and a reaction with an untreated DNA was considered as the negative control for the M and U primers. Cycling conditions and TSDR primers are listed in table 2. TSDR demethylation levels were calculated with an equation explained previously (21): $100/ [1 + 2^{(CtTG-CtCG)}]\times100\%$. Briefly, Ct_{TG} and Ct_{CG} indicate the Ct of U and M primers, respectively. Because of the fact that TSDR is located on the X chromosome, and only one out of two TSDR alleles are methylated, because of X-inactivation in women, demethylation of female samples was corrected with a coefficient of two.

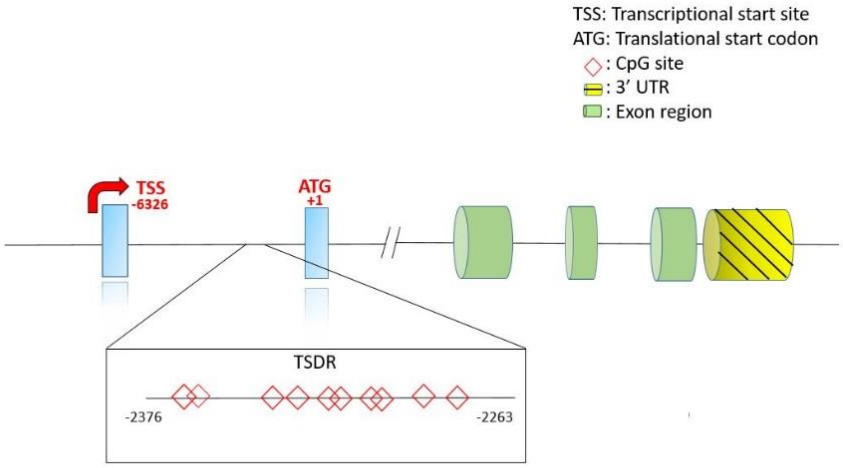

Figure 1. Schematic illustration of the TSDR in the first intron of the Foxp3 gene. The DNA target for Q-MSP amplification was composed of a 117 bp segment that covered 10 CpG dinucleotides (16).

Table 2. Primer sequences utilized in assessing the TSDR demethylation of FoxP3 gene

Array	Primer sets	Primer sequence	PCR Product size	Cycle condition
Q-MSP	Demethylated primer	Forward: GGATAGGGTAGTTAGTTTTTGGAATG Reverse: CCACCATTAACATCATAACAACCA	117	Preincubation:95° C for 30 sec; 45 cycles of 95°C for 5 sec followed by 30 sec at 60° C.
	Methylated primer	Forward: GATAGGGTAGTTAGTTTTCGGAAC Reverse: CCGCCATTAACGTCATAACG	116	

Statistical analysis

The independent sample unpaired *t*-test or its non-parametric equivalent, Mann-Whitney test, helped with the analyses. Spearman's rank correlation coefficient was also used. The obtained results were analyzed using SPSS version 16 and GraphPad Prism version 6. A *P* of <0.05 was considered significant.

Results

A significant difference in vitamin D of patient and control groups

25-OH vitamin D levels of plasma were shown to be different in the examined groups, with the mean 25-OH

vitamin D of patients being higher than the control group. This difference was statistically significant (*P*=0.034) (Figure 2). The mean plasma levels of this vitamin are shown in table 3.

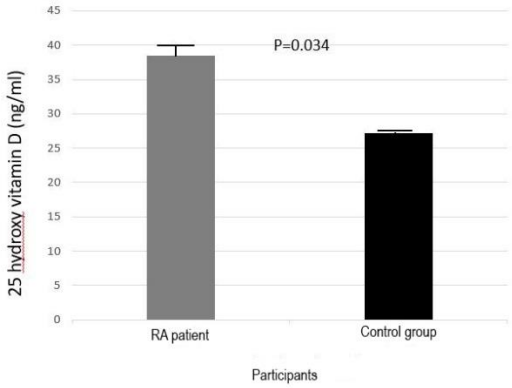

Figure 2. A visual comparison of plasma vitamin D of patient and control groups.

Table 3. Comparison between plasma vitamin D and TSDR demethylation of the FoxP3 gene in the patient and control groups.

Expression level	RA Cases (N=18)	Healthy Controls (N=41)	*P*	Adjusted *P**
Vit.D**	38.43±6.828	27.22±3.551	0.034	0.034
TSDR	8.55±1.472	64.78±15.144	0.006	0.012

* FDR correction for multiple comparisons by Benjamini-Hochberg method.
** Vit.D unit = ng/ml; cut off = 30 ng/ml

Demethylation level of TSDR in PBMCs

Bisulfate-treated genomic DNA was used in this study. The demethylation rate of TSDR was significantly lower in RA patients rather than healthy controls (8.55% vs. 64.78%; *P*=0.006) (Table 3). So the more expression of Foxp3 is expected in healthy subjects compared to RA patients since elevated methylation of TSDR decreases FoxP3 expression.

The correlation of TSDR demethylation with vitamin D

There was not a statistically significant correlation between 25-OH vitamin D plasma levels and TSDR demethylation (r=0.03) (Figure 3).

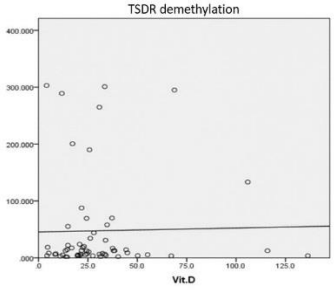

Figure 3. The relationship between plasma levels of vitamin D and TSDR demethylation for FoxP3 in the RA patients and control group

Discussion

This study was conducted in an effort to examine the correlation between plasma levels of 25-OH vitamin D and epigenetic alterations of Treg cells. We found that the 25-OH vitamin D of plasma in RA patients was higher than the control group. In some experiments, much like the results of our study, higher vitamin D of RA patients compared with the control group or non-statistically significant difference was reported (11,22-24). Due to the fact that the main source of vitamin D, in most people, is exposure to sunlight, and vitamin D levels fluctuate in different seasons, and diet (fish, milk, etc.) is a known source of vitamin D (25), the higher vitamin D levels of patients can be attributed to different sampling condition of both groups. In contrast, some studies reached results contrary to ours reporting a lower vitamin D in patients compared with controls (26-28). Consumption of some prescriptive drugs by RA patients, like hydroxychloroquine that reduces plasma vitamin D, can justify this discrepancy (8). Patients in our study were all new-case and had not received any medication. All in all, more clinical studies are needed to illuminate the role of

vitamin D deficiency in the pathogenesis of autoimmune diseases (12). In a study by Marinho *et al*., prescription of vitamin D for a 6-month period alleviated the symptoms and increased the $CD4^{+}FoxP3^{+}$ cell (29). Although various studies have concluded the heightened production of $CD25^{-}CD4^{+}$ T cells by vitamin D, its exact mechanism is still unclear (12,13). This may be due to the changes in the methylation status of FoxP3 because evidence indicates that vitamin D has a demethylation effect on gene promoters. Florath *et al*., found no connection between vitamin D levels and methylation of leukocytes' genome (30). Tapp *et al.,* claimed that there is an inverse correlation between vitamin D and CGI gene methylation in ulcerative colitis patients (31). Also, by studying patients afflicted by colon malignancy, Rawson *et al*., concluded that people with higher vitamin D have less methylation in WNT5A and DKK1 genes (32). And, Lopes *et al*., observed a diminished E-cadherin promoter methylation following vitamin D induction (33,34). In the present study, no statistically significant correlation between plasma levels of vitamin D and TSDR demethylation was found. This could be attributed to VDRE mutations. There are three response elements in the CNS region of FoxP3, designated as VDRE1, VDRE 2, and VDRE3 (located at +2380 ~ +2397, +2504 ~ +2521, and +2527 ~ +2544), and a mutation in any of these regions will diminish the proper response to this vitamin and FoxP3 gene promoter activity even in extreme levels of vitamin D. On a related note, studies indicate that in the presence of IL-2 and TCR stimulation vitamin D can induce FoxP3 expression in T cell. Thus, absence of any of these factors could hinder the influence of this vitamin on methylation status of gene promoter (12). Furthermore, another study evaluated the correlation of vitamin D with FoxP3 and concluded that insufficient plasma levels of vitamin D cannot affect FoxP3 gene expression (35). Finally, in order to clarify the impact vitamin D might have on FoxP3 promoter methylation status, we suggest preclinical and clinical studies to be conducted, and mutation and polymorphism of VDRE in RA can also be helpful. In addition, retrospective studies to examine any history of vitamin D deficiency in RA patients can help for a better deduction.

As expected, epigenetic alternation showed a considerable difference between RA patients and healthy control but about vitamin D correlation with methylation modification, more studies with large sample size are needed.

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