ORIGINAL ARTICLE

DNA methylation of vitamin D genes: A connecting link between vitamin D levels and grade of SARS-CoV-2 infection

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Abstract

Background: The mechanistic relationship between the methylation process of the vitamin D gene and its impact on vitamin D levels and COVID-19 infection severity remains a subject of ongoing investigation. However, the lack of vaccines and targeted treatment in the initial phase of the pandemic had stimulated considerations regarding the potential use of vitamin D as a strategy for managing the disease. Vitamin D genes regulate vitamin D synthesis through epigenetic changes such as methylation, which affect various gene expressions. *Aim and Objectives:* The present study aimed to establish the relationship between vitamin D gene methylation in COVID-19 patients and vitamin D deficiency and the severity of COVID-19 symptoms. *Material and Methods:* Fifty RT-PCR confirmed SARS-CoV-2 patients were cross-sectionally evaluated, after ethical approval and informed consent, for severity of covid infection, serum levels of vitamin D along with other blood parameters and percentage of vitamin D gene methylation. ANOVA and correlation coefficient were used for statistical analyses with '*p*' value <0.05 being considered significant. *Results:* There was no significant correlation between plasma vitamin D level and vitamin D gene methylation (CYP27B1 and CYP24A1) although there was significant correlation among methylation status of Vitamin D genes. This study revealed that the SARS-CoV-2 infection grades were significantly correlated with the degree of methylation of the vitamin D genes. *Conclusion:* Our study concludes that the percent methylation of the vitamin D genes and the interplay between these genes may determine vitamin D levels and COVID severity. In future, confounding factors that could influence the methylation (e.g., age, pre-existing health conditions, vitamin D supplementation) also should be considered

Keywords: COVID-19, Vitamin D deficiency, Vitamin D genes, Methylation

Introduction

The COVID-19 pandemic had been tough and posed a multitude of challenges. However, it gave scientists the scope to trigger research in new directions to understand the pathophysiology of the disease [1-2]. A significant body of research had found that low vitamin D levels were common in severe cases of the SARS-CoV-2 infection. However, the nonexistence of vaccines and targeted treatment in the initial phase of the pandemic had stimulated considerations regarding the potential use of vitamin D as a strategy for managing the disease [3-4]. Many studies with different mechanisms had explained that active vitamin D (encoded by the gene CYP2R1) served as an immunomodulator and antimicrobial mediator. But how vitamin D prevents or improves disease prognosis in

COVID-19 infection is still a mystery. It is a known fact that the active vitamin D metabolite 1,25-dihydroxycholecalciferol (1,25(OH)D; calcitriol) regulates gene expression through the Vitamin D Receptor (VDR) and a nuclear transcription factor. However, recent literature reported that the DNA methylation of vitamin D genes modulated the expression of vitamin D and was responsible for vitamin D levels in disease prognosis [5-9].

The enzymatic processes are subject to regulation via DNA methylation within the vitamin D metabolic pathway. CYP2R1 (25-hydroxylase), CYP27B1 (1-hydroxylase), CYP24A1 (24-hydroxylase), and VDR are the genes involved in vitamin D regulation and production. The calciol-25-hydroxylase gene encodes the enzyme that converts vitamin D2 (ergocalciferol) and vitamin D3 (cholecalciferol) into 25-hydroxy cholecalciferol (25(OH)D; calcidiol). Following that, the enzyme Calcidiol-1a-hydroxylase, which is encoded by the CYP27B1 gene, plays a critical role in converting 25(OH)D into 1,25(OH)D, which binds to the VDR.

Simultaneously, the 24-hydroxylase enzyme, encoded by CYP24A1, deactivates both 25 (OH)D and 1,25 (OH)D through hydroxylation, and the body starts experiencing vitamin D depletion. In short, higher the methylation of CYP2R1, lower the vitamin D (OH)D production [6-9]. Therefore, this raises the question: can this be a reason for the increase in disease severity? [10-12]. Vitamin D, in particular, exerts various influences on the immune system and has the capacity to epigenetically modulate the immune system's response to SARS-CoV-2. Notably, a recent clinical study demonstrated the advantages of vitamin D supplementation in individuals afflicted with COVID-19 [4].

Hence, the present study aimed to establish the relationship between vitamin D gene methylation in COVID-19 patients and vitamin D deficiency and the severity of COVID-19 symptoms.

Although recent data on vitamin D deficiency and COVID-19 infection are limited, DNA methylation can potentially assist in identifying vitamin D deficiency and its association with COVID-19 infection.

Material and Methods

Present study was a cross-sectional study of six months approved by the Institutional Ethics Committee. Patients were recruited after taking written informed consent. Only clinically diagnosed COVID-19-positive patients confirmed by reverse transcription polymerase chain reaction (RT-PCR) were evaluated for triage before admission. According to WHO standards, the COVID-19-positive patients were categorised into mild, moderate, and severe symptoms [13].

Patients with a history of severe illnesses such as cancer, respiratory disease, gastrointestinal disease, and kidney disease were excluded from the study. Additionally, pregnant women, patients with missing data, and patients on vitamin D supplementation were excluded from the study. The samples were collected and tested at the Biochemistry Centre Clinical Laboratory at B. J. Government Medical College and Sassoon General Hospital, Pune.

The isolation of DNA from Peripheral Blood Cells (PBC) was done using the Qiagen QIAmp DNA Mini Kit (company name) according to standard protocol, including RNAse treatment. The percentage analysis of vitamin D gene methylation was performed according to the EpiTect II qPCR assay

method. It utilised Methylation-sensitive (Ms) and Methylation-dependent (Md) restriction enzymes for differential digestion of unmethylated and methylated DNA. CYP2R1, CYP27B1, CYP24-A1, and VDR gene expressions were analysed by EpiTect II qPCR assays as per the manufacturer's protocol. Plasma 25 (OH)D levels were measured with ELISA. Other laboratory parameters, including D-dimer, complete blood count, liver function test, kidney function test, and determination of serum IL-6 and ferritin levels, were also observed for this study. A serum 25(OH)D level < 20 ng/ml in the sample was considered as vitamin D-deficient according to the guidelines. The data analysis was done using the Statistical Package for the Social Sciences (SPSS) software V-23. The ANOVA test was performed to understand the significant differences in vitamin D and methylation genes (CYP2-R1, CYP27B1, CYP24A1, and VDR) according to the severity of the SARS-CoV-2 infection. The correlation between vitamin D and gene methylation was performed. Moreover, the correlation between the methylation status of SARS-CoV-2 infection and CpG island genes was also established. A probability value of 0.05 was accepted as the level of statistical significance.

Results

A total of 50 RT-PCR confirmed COVID-19 patients were included in the study. There was a male preponderance with a gender ratio of 2.1:1. A total of 38% (n = 19) patients were classified in the mild symptomatic category of COVID-19 infection; 30% (n = 15) patients were found to be moderately infected; and 32% (n = 16) of patients were severely ill. Vitamin D deficiency was detected in 74% (n =37, < 20 ng/ml) of the total number of patients. Eight percent of patients had inadequate plasma vitamin D levels ranging between 20-30 ng/ml, and 18% had adequate vitamin D ranges between 31-40 ng/ml.

There was negative correlation between plasma vitamin D level and vitamin D gene methylation (CYP27B1 and CYP24A1) observed although it was not significant. Here we interpret that as vitamin D levels decrease due to increased methylation of the CYP27B1 gene, simultaneously it reduces activity and decreases the methylation of CYP24A1, increasing its catabolic expression. CYP27B1 is involved in the hydroxylation of 25 (OH)D, while simultaneously CYP24A1 acts as a catabolic enzyme inactivating both 25 (OH)D and 1,25 vitamin D3 (Table 1).

Moreover, there was no correlation between plasma vitamin D level and CYP2R1. Vitamin D Receptor (VDR) was found as CYP2R1 was controlled by cholesterol substrate, and VDR must rely on CPR27 B1 and CYP24A1. However, we found a highly significant positive correlation between methylation of CYP27B1 and CYP2R1, CYP27B1 and CYP24A1, and CYP24A1 and VDR, as the calculated p value was less than 0.0001 (Table 2). We observed that the methylation of the CYP27B1 gene increased with decreased expression, which was again affected simultaneously by methylation and the expression of CYP2-R1 and CYP24A1.

The methylation status of the vitamin D gene revealed that the SARS-CoV-2 infection grades were significantly correlated with the degree of methylation of the vitamin D genes (Table 3).

Table 1: Correlations of vitamin D levels with vitamin D gene methylation interpretation

Correlation between vitamin D and vitamin D gene	r_s	*p*
CYP2R1	0.02	0.97
CYP27B1	-0.189	0.19
CYP24A1	-0.22	0.13
VDR	-0.004	0.98

Table 2: Correlation among methylation: of various vitamin D genes

Correlation between	r_s	*p*
CYP2R1 & CYP27B1	0.532	<0.0001
CYP2R1 & CYP24A1	0.465	0.001
CYP2R1 & VDR	0.293	0.039
CYP27B1 & CYP24A1	0.606	<0.0001
CYP27B1 & VDR	0.435	0.002
CYP24A1 & VDR	0.672	<0.0001

Table 3: Comparison of percentage of methylation according to COVID severity in study group

Methylation (%)	COVID severity			F	*p*
	Grade I (n=19)	Grade II (n=15)	Grade III (n=16)		
CYP2R1	35.769 ± 28.58	19.543 ± 21.11	7.931 ± 9.56	7.23	0.002
CYP27B1	36.397 ± 26.72	26.513 ± 25.62	14.320 ± 18.76	3.64	0.034
CYP24A1	37.797 ± 28.85	38.105 ± 19.88	26.578 ± 27.38	1.04	0.36
VDR	32.572 ± 30.68	43.290 ± 23.88	32.692 ± 32.65	0.69	0.51

By post hoc Bonferroni test

Parameter	Grade I vs Grade II	Grade I vs Grade III	Grade II vs Grade III
CYP2R1	0.10	0.001	0.43
CYP27B1	0.72	0.029	0.50
CYP24A1	1	0.63	0.67
VDR	0.89	1	0.97

Discussion

To the best of our knowledge, this is the first study to test the association between modulation of DNA methylation of vitamin D genes (CYP2R1, CYP27B1, CYP24A1, and VDR) and vitamin D deficiency and COVID-19 infection severity.

Every enzyme encoded in the gene in the vitamin D metabolic pathway is expressed by the DNA methylation process. Deaton and Bird (2011) demonstrated that changes in the methylation status at the promoter regions can regulate gene expression; higher methylation, decreased expression, and vice versa. This status of hypomethylation and hypermethylation affects the vitamin D levels in the body. Therefore, these affected levels of vitamin D contribute to the immune response trigger against the infection [6-8]. Thus, the production of vitamin D in the body was signifi-cantly blocked leading to deficiency and ultimately helping virus replication.

Vitamin D exerts various influences on the immune system and has the capacity to epigenetically modulate the immune system's response to SARS-CoV-2. The active vitamin D metabolite 1,25-dihydroxy vitamin D (1,25(OH)2D) triggers good innate defence mechanisms and modulates inflammation in response to diseases such as COVID-19. However, many studies have reported that vitamin D deficiency was common among COVID-19 patients and needed medical attention [14-16]. Similarly, our results also found that 74% of the total studied population who were positive for SARS-CoV-2 infection were vitamin D deficient.

In our study, no significant negative correlation between plasma vitamin D level and vitamin D gene methylation (CYP27B1 and CYP24A1) was observed. Thus, we interpreted that vitamin D status can be a result of a balance between CYP27B1 and CYP24A1 expression.

Moreover, there was no correlation between plasma vitamin D level and CYP2R1, VDR found. Zhou *et al*., (2014) and Zhu *et al*., (2013) had established the association between CYP2R1 and CYP24A1 with respect to VDR expression [8, 9]. However, our study found a highly significant positive correlation between CYP27B1 and CYP2R1, CYP27B1 and CYP24A1, and CYP24A1 and VDR. We observed that as the expression of CYP27B1 increased, the expression of CYP2R1 and CYP24A1 also significantly increased, and as CYP24A1 increased, VDR also significantly increased. As methylation of CYP24-A1 increased, its catabolic activity decreased, and more vitamin D was available for VDR.

This was the first ever observation, to the best of our knowledge. Zhu *et al.,* (2013) found that vitamin D deficiency was associated with methylation modulation in vitamin D gene expression, which is in support of our findings. Therefore, CYP2R1 and CYP27B1 genes modulating the expression via DNA methylation, reflecting vitamin D deficiency, were responsible for increasing the disease severity.

Therefore, 74% vitamin D deficient COVID-19 positive patients ring the bell [8-9]. Additionally, Moran-Auth *et al*., (2013) found that the expression of CYP2R1, CYP27B1, CYP24A1, and VDR were demonstrated in immune cells, indicating that vitamin D genes regulate the status of vitamin D modulating immune response [17]. This study also supports our hypothesis that vitamin D deficiency might be the responsive parameter in COVID-19 severity. As per the results, an active vitamin D modulator had been blocked by the DNA methylation of the vitamin D gene, producing an insufficient amount of vitamin D. Therefore, the disease severity was higher in vitamin D-deficient patients than in patients with sufficient vitamin D levels [6-9, 17]. The constraints of this research may include the limited sample size and the cross-sectional design, as well as the absence of adjustments for variables such as diet, sun exposure, pollution, and infections that influence the regulation of the VDR by modulating vitamin D levels.

Conclusion

This study reveals a significant deficiency in vitamin D among COVID-19 patients. It also sheds light on the DNA methylation of genes responsible for influencing the severity of the condition rather than vitamin D deficiency. The degree of methylation may also play a crucial role, as it negatively impacts interplay between methylation of Vitamin D genes which disrupts vitamin synthesis. Future investigations should delve into whether anomalies in vitamin D metabolism exist, potentially reshaping our approaches to COVID-19 research and treatment in longitudinal studies with larger sample size. Any deviations in vitamin D metabolism could have profound implications, significantly altering our strategies in the study and management of COVID-19 infection considering confounding factors.

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