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Relationship between methylation status of vitamin D-related genes, vitamin D levels, and methyl-donor biochemistry

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ABSTRACT

Vitamin D is known for its role in the regulation of gene expression via the vitamin D receptor, a nuclear transcription factor. More recently, a role for vitamin D in regulating DNA methylation has been identified as an additional mechanism of modulation of gene expression. How methylation status influences vitamin D metabolism and response pathways is not yet clear. Therefore, we aimed to assess the relationship between plasma 25-hydroxycholecalciferol (25(OH)D) and the methylation status of vitamin D metabolism enzyme genes (*CYP2R1, CYP27B1* and *CYP24A1*) and the vitamin D receptor gene (*VDR*). This analysis was conducted in the context of dietary vitamin D, and background methyl donor related biochemistry, with adjustment for several dietary and lifestyle variables. Percentage methylation at CpG sites was assessed in peripheral blood cells using methylation sensitive and dependent enzymes and qPCR. Standard analytical techniques were used to determine plasma 25(OH)D and homocysteine, and serum folate and B12, with the relationship to methylation status assessed using multi-variable regression analysis. *CYP2R1* and *VDR* methylation were found to be independent predictors of plasma 25(OH)D, when adjusted for vitamin D intake and other lifestyle variables. *CYP24A1* was related to plasma 25(OH)D directly, but not in the context of vitamin D intake. Methyl-group donor biochemistry was associated with the methylation status of some genes, but did not alter the relationship between methylation and plasma 25(OH)D. Modulation of methylation status of *CYP2R1, CYP24A1* and *VDR* in response to plasma 25(OH)D may be part of feedback loops involved in maintaining vitamin D homeostasis, and may explain a portion of the variance in plasma 25(OH)D levels in response to intake and sun exposure. Methyl-group donor biochemistry, while a potential independent modulator, did not alter this effect.

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1. Introduction

The active vitamin D metabolite, 1,25-dihydroxycholecalciferol (1,25(OH)D; calcitriol), is well known for its influence on gene regulation via its action on the vitamin D receptor (VDR), which acts as a nuclear transcription factor [1]. However, the potential for vitamin D to modulate gene expression indirectly, through modulation of epigenetic marks is now also being investigated [2]. A better understanding of the relationship between vitamin D status and modulation of DNA methylation may help to explain some of

Abbreviations: 1,25(OH)D, 1,25 dihydroxycholecalciferol; 25(OH)D, 25-hydroxycholecalciferol; PBCs, peripheral blood cells; TOMS, Total Ozone Mapping Spectrometer; VDR, vitamin D receptor.

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the variance that exists in vitamin D metabolism [3–5] and the influence of vitamin D status on disease risk. This may be particularly important in elderly populations, in which vitamin D deficiency is common and who may be at higher risk of diseases linked to suboptimal vitamin D status including osteoporosis [6], diabetes [7], cardiovascular disease [8], autoimmune disease [9] and some cancers [10–13].

The metabolism of vitamin D is a multistep process involving several enzymes. Ergocalciferol (vitamin D2; from dietary sources) and cholecalciferol (vitamin D3; from dietary sources and endogenous synthesis in the skin) are converted by calciol-25-hydroxylase (coded for by the gene *CYP2R1*) into 25-hydroxycholecaliferol (25(OH)D; calcidiol), which is the circulating storage form of the vitamin. Calcidiol-1α-hydroxylase (*CYP27B1* gene) then converts 25(OH)D into 1,25(OH)D, the biologically active ligand for the VDR [14,15]. A 24-hydroxylase enzyme (*CYP24A1* gene) is responsible for the inactivation of both 25(OH)D and 1,25(OH)D via hydroxylation [15] (Fig. 1).

Each of the genes encoding enzymes involved in the vitamin D metabolism pathway is potentially regulated by DNA methylation. DNA methylation occurs at CpG sites (cytosine residues followed by guanine residues), and clusters of CpGs are referred to as CpG islands. Differential methylation of these islands in promotor regions of genes may modulate gene expression, with hyper-methylation often associated with decreased expression, and hypo-methylation associated with increased expression [16]. *VDR*, *CYP2R1* and *CYP24A1* each have CpG islands spanning their promotor regions, and *CYP27B1* has a CpG island in the gene body (http://genome.ucsc.edu/) [2,17]. Vitamin D stimulation has also been shown to influence global methylation markers [18], and to alter the methylation status of multiple genes in multiple pathways, including those related to cell cycle regulation [19–22]. Therefore, DNA methylation and vitamin D metabolism may have a complex and bidirectional relationship.

In a small (22 African American male youths) genome-wide association study using DNA from blood leukocytes Zhu and colleagues identified a small differential methylation in *CYP2R1* and *CYP24A1* between those with severe vitamin D deficiency, compared to vitamin D sufficient individuals [19]. However, it should be noted that the statistical significance of these results did not survive corrections for multiple testing. Vitamin D deficient participants had higher methylation of *CYP2R1* and lower methylation of *CYP24A1*. This study was purely observational, and the direction of effect cannot be determined, however, following the standard paradigm of increased methylation leading to reduced

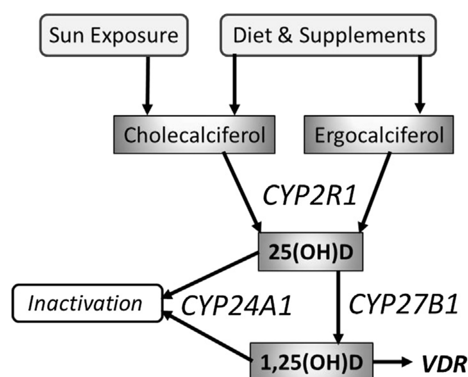

Fig. 1. Simplified flow chart of vitamin D metabolism. CYP2R1 is the gene for the enzyme calciol-25-hydroxylase; CYP27B1 is the gene for the enzyme Calcidiol-1α-hydroxylase; CYP24A1 is the gene for the 24-hydroxylase enzyme; VDR is the gene for the vitamin D receptor.

gene expression, this pattern of methylation may lead to a situation where methylation status contributes to vitamin D deficiency, by decreasing conversion into the active metabolite, and increasing inactivation [19]. However an important limitation of this study was the lack of adjustment for sun exposure or vitamin D intake.

More recently, Zhou and colleagues, in a study of vitamin D supplementation in post-menopausal women, investigated methylation status of *CYP2R1*, *CYP24A1* and *CYP27B1* DNA isolated from serum [23]. In a preliminary binary analysis of 36 subjects, methylation status of responders (those who had the highest rise in serum 25(OH)D in response to supplementation) were compared to non-responders (those with a limited increase in serum 25(OH)D in response to supplementation), using methyl specific PCR. Supporting the observations of Zhu et al., non-responders had higher methylation of *CYP2R1* at baseline and after supplementation, and this may explain the reduced response to supplementation. Following supplementation both responders and non-responders exhibited reduced methylation levels of *CYP24A1*, suggesting higher *CYP24A1* expression may be a response to degrade the additional supplemental vitamin D and maintain homeostasis [23]. This suggests that there is modification of methylation status, and that this may be both a cause and a response phenomenon, depending on the situation. In the same study no differential methylation was found for *CYP27B1*. In a validation study treating plasma 25(OH)D levels as a continuous variable in independent samples ($n = 117$ and 145), and using pyrosequencing to interrogate individual CpGs, only a limited number of CpG sites upheld the preliminary results following corrections for multiple testing. Importantly, the validation cohort excluded the participants of the two extremes (previously defined as responders and non-responders) used in the pilot study [23]. Additionally, relevance of the methylation status at individual CpG sites is not yet fully clear, compared to the broader methylation status across the CpG island.

A limitation of these studies is minimal correction for other dietary and lifestyle variables in the former study [19], and the use of DNA found in serum in the latter [23]. Both cohorts are also of limited size [19,23]. Vitamin D status may be modulated by a number of variables including BMI, age, sex, and calcium intake in addition to vitamin D intake and sun exposure [24]. Furthermore DNA methylation status may be sensitive to cigarette smoking status, alcohol intake, age, sex, and sun exposure [25,26]. Another important consideration is that methylation potential may be related to the availability of methyl donors resulting from the one carbon metabolism cycle, and as such vitamin D metabolism may be influenced by levels of methyl donor nutrients, such as folate [27]. Therefore, in the current study we examined the relationship between methylation status of vitamin D metabolism genes (*CYP2R1, CYP27B1* and *CYP24A1*) and *VDR*, plasma 25(OH)D status and a number of dietary and lifestyle variables, including folate status, in a mixed gender elderly cohort.

2. Methods

2.1. Subjects and sample collection

Participants for this study were 80 subjects drawn from a completed cross-sectional study of 831 elderly participants (aged 65 years or more, 58.5% female) living on the Central Coast of NSW, Australia (the Retirement Health and Lifestyle Study, RHLS). Details of the selection and randomisation process are included in the supplementary methods.

Whole blood was collected from participants and stored at −20 °C. Serum and plasma were stored at −80 °C. DNA was isolated from peripheral blood cells (PBCs) using Qiagen QIAmp

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