Vitamin D receptor genotype modulates the correlation between vitamin D and circulating levels of let-7a/b and vitamin D intake in an elderly cohort.

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Short Title: *VDR* genotype influences the correlation between vitamin D and let-7a/b.

Key Words: microRNA (miRNA), vitamin D, let-7, *VDR*, BsmI, ApaI, nutrigenomics.

Abstract

BACKGROUND AND AIMS: Circulating microRNA (miRNAs) are linked to disease and are potential biomarkers. Vitamin D may modulate miRNA profiles, and vitamin D status has been linked to risk of disease, including cardiovascular disease and cancers. We hypothesise that genotypic variance influences these relationships. We examined the correlations between vitamin D intake and circulating levels of the miRNAs let-7a/b, and the involvement of two common *VDR* polymorphisms, BsmI and ApaI. **METHODS:** 200 participants completed food frequency and supplement questionnaires, and were assayed for circulating let-7/b expression by qPCR. Polymorphisms were detected using RFLP-PCR. **RESULTS**: let-7b expression negatively correlated with vitamin D intake (r_s=-0.20, p=0.005). The magnitude and direction of correlation was maintained in the presence of the BsmI restriction site (r_s=-0.27, p=0.0005). However, in the absence of BsmI restriction site, the direction of the correlation was reversed (r_s=+0.319, p=0.0497). These correlations were significantly different (z-score=2.64, p=0.0085). The correlation between vitamin D intake and let-7a was only significant in those without the ApaI restriction site. **CONCLUSIONS:** The correlation between vitamin D intake and let-7a/b expression in this cohort varies with *VDR* genotype. This study highlights the importance of considering underlying genotypic variance in miRNA expression studies, and in nutritional epigenetics generally.

Introduction

miRNAs are a class of short non-coding RNAs that can regulate gene expression at the post transcriptional level, normally by blocking mRNA translation or triggering degradation [1-5]. miRNAs are estimated to participate in nearly all cellular processes [6]. To date, over 2500 mature miRNAs have been identified (miRBase, http://www.mirbase.org, release 21, June 2014). Disease specific miRNA profiles have been identified, including for those diseases with known dietary risk factors [7-9]. Evidence is mounting that miRNA profiles can be modulated by nutrients and bioactive compounds [10]. Traditionally, the focus has been on tissue expression of miRNAs, but circulating miRNAs have recently been identified in serum and plasma, which have the advantage of being easily accessible for use as biomarkers. These circulating miRNAs are now being linked to particular diseases [11].

let-7a and let-7b are well characterised members of the let-7 family of tumour suppressor miRNAs. They are highly expressed in the cardiovascular system [12] and are robustly detected in plasma samples [13-15]. Altered expression of let-7 has been implicated in a number of later life diseases; circulating let-7b has been suggested as a potential biomarker for cardiovascular diseases, such as atherosclerosis and myocardial infarction [12] and the let-7 family may be involved in regulation of glucose homeostasis in diabetes [16]. Furthermore, expression of let-7a/b is often reduced in malignant tissue, relative to healthy tissue[17] and in some cases these altered profiles are reflected in blood circulation[14,18]. For circulating miRNAs to be valuable as biomarkers for disease, it is important to understand how other environmental factors influence their expression levels.

Vitamin D is well known for the influence it has on gene expression. The active vitamin D metabolite, 1,25-dihydroxyvitamin D_3 (calcitriol), directly regulates gene transcription via the vitamin D receptor (VDR), which acts as a nuclear receptor transcription factor [19]. The potential for vitamin D to modulate gene expression via additional indirect pathways is now being investigated. Vitamin D is synthesised in the skin in response to sunlight, consumed in the diet as cholecalciferol (vitamin D_3) or ergocalciferol (vitamin D_2), or taken as a supplement. Although current recommended intakes are based only on its role in bone health [20], epidemiological evidence is now mounting for a potential role for suboptimal vitamin D levels in multiple conditions, including diabetes [21], cardiovascular disease [22], autoimmune disease [23] and cancers [24]; however, results to date remain inconsistent [20]. Vitamin D supplementation is now being investigated in the treatment and prevention of a number of these conditions [25-27]; in particular, its potential as a cancer chemo-preventative, due to the identified role of the VDR in cell-cycle regulation and differentiation [28].

Multiple *in vitro* models using malignant cell lines have demonstrated the anti-proliferative effect of vitamin D [29-32], which correlates with the altered expression of a number of miRNAs, including the let-7 family [33-36], suggesting that aberrant expression of these miRNAs may contribute to the malignant phenotype and that this can be moderated by vitamin D treatment [37,38]. In a model of serum starvation using a breast epithelial cell line (MCF12F), calcitriol treatment had a protective effect. Serum starvation led to a significant increase in the expression of multiple miRNAs, including the let-7 family, however, this was reversed in the presence of calcitriol, suggesting miRNA expression may be a mechanism protecting against cellular stress via a vitamin D related process [39], which may have

implications in multiple disease states including diabetes, cardiovascular disease, and cancers [40].

Limited studies investigating the correlation between serum levels of vitamin D and miRNA expression in humans have been conducted. In a study of 13 pregnant women, categorised by low (≤25.5ng/ml) or high (≥31.7ng/ml) plasma calcitriol level, eleven miRNAs were differentially regulated [41]. In a small study, 2 groups of 5 subjects were given high doses of vitamin D for 12 months, and their miRNA levels assessed relative to baseline, several miRNAs were differentially expressed after 12 months, including let-7a [42], an established target of VDR, with a Vitamin D Response Element (VDRE) in the regulatory region of the gene controlling its expression [43].

To further investigate the role of vitamin D in modulating levels of circulating miRNAs we studied the relationship between vitamin D intake and the expression of circulating let-7a/b, two well characterised tumour-suppressor miRNAs, in a large human cohort. To further investigate the role of the VDR in this relationship, two common polymorphisms in the *VDR* gene BsmI (rs1544410) and ApaI (rs7975232) were assayed in participants.

Methods

Subjects and sample collection

This study consisted of 200 participants, aged 65 year or more, living on the Central Coast of New South Wales, Australia, who completed a food frequency and supplement questionnaire, and donated blood for analysis. Blood was collected in heparin lined tubes (Greiner Bio-one, Frickenhausen, Germany) and plasma isolated by centrifugation. Informed consent was obtained prior to participation under University of Newcastle Human Research Ethics Committee approval number H-2008-0431.

Food Frequency and supplement questionnaires

Daily intake of vitamin D was estimated using a validated food frequency questionnaire, covering 225 food items and all food groups. Subjects also provided a list of all supplements and their frequency of intake. The food frequency questionnaires were analysed using Foodworks™ (Version 2.10.146, Xyris Software, Brisbane, QLD, Australia) [44,45]. The correlation between estimated intake and plasma concentrations of 25(OH) vitamin D has been

validated in a subset (n=80; adjusted r^2=0.46, p<0.001) of this cohort (see supplementary methods and figure S1).

Genotyping

Genomic DNA was isolated from whole blood and amplified using PCR. Restriction fragment length polymorphism assays and gel electrophoresis were used to genotype two diallelic polymorphisms of the *VDR* gene; BsmI G/A (rs1544410) and ApaI A/C (rs7975232) [44,46] both located on the last intron [47]. The presence of restriction sites for the BsmI and ApaI enzymes were coded as '*b*' and '*a*' and the absence of restriction sites as '*B*' and '*A*', respectively. See supplementary methods for additional details.

Circulating let-7a/b expression by qPCR

miRNAs were isolated from plasma using Trizol LS and Glycoblue [48]. Three synthetic *C. elegans* miRNAs (cel-238, -54, -38) were added to plasma samples as exogenous spike in controls to normalise data, as no appropriate endogenous house-keeping gene exists in plasma [49]. cDNA was synthesised using universal primers and qPCR primer design was conducted as per the rules described in Balcells *et al.* [50].

Statistics

Statistical analyses were performed using JMP (Version 11, SAS Institute Inc., Cary, NC, USA). Correlations between continuous variables were assessed using Spearman's correlation coefficient, due to the non-normal distribution of vitamin D intake in this cohort. z-scores were used to assess significant differences between correlations (http://vassarstats.net/rdiff.html). Analyses were performed correcting for age and sex, however this had no notable significant impact on results.

Categorical data were assessed using the Mann-Whitney test when comparing two categories and the Kruskal-Wallis test was used when comparing multiple categories. Differences between groups were considered to be statistically significant at $p \leq 0.05$.

Results

Participant characteristics and vitamin D intake

The mean age of participants was 75.0 ± 0.5 years (range: 65-94 years), and 57.5% were female. The median daily reported vitamin D intake was 6.8 µg/day (range: 0-65.6 µg /d). 57

(28.5%) participants reported taking a supplement containing vitamin D. The recommended adequate daily intake for vitamin D in Australia is 10 µg/day for 51-70 year olds, and 15 µg/day for those aged over 70. The recommended upper limit of consumption is 80 µg/day [51]. Based on these criteria, 177 participants had inadequate intake for their age group, and none had excess intake above the upper limit.

VDR Genetic Variants

The allelic and genotypic frequencies for both polymorphisms tested are given in table 1. The genotype frequency did not deviate from Hardy-Weinberg equilibrium expectations for either the BsmI or the ApaI polymorphism (p=0.19 and 0.85, respectively). These two polymorphisms exhibit strong linkage disequilibrium [52]. In this population, 91.7% of those with the BB genotype (BsmI restriction site absent) were also AA (ApaI restriction site absent), however the AA genotype was more than twice as frequent as the BB genotype.

Association between Vitamin D intake and let-7a/b expression

A weak, but significant negative association was found between let-b expression and vitamin D intake (Table 2; r_s=-0.20, p=0.005). The same trend was seen for let-7a, but this did not reach statistical significance (Table 2; r_s=-0.14, p=0.057). Adjustment for age and sex did not significantly alter these relationships.

Categorical analysis was conducted on the data, based on adequate daily intake (greater than or adequate daily intake vs. less than adequate daily intake), as these are clinically relevant categories. There was a significant difference between the mean let-7b expression levels between those who reported adequate vitamin D intake or above, and those with below adequate intake (Figure 1A; relative expression=-0.49 vs 0.14, p=0.013). Results for let-7a were not significant (Figure 1B; relative expression=-0.19 vs 0.10, p=0.1132). Comparison of let-7a/b levels between those who reported taking a supplement containing vitamin D and those who relied on diet alone for vitamin D intake found lower levels of relative expression of both let-7b (-0.49 vs 0.14, p=0.0014) and let-7a (-0.09 vs 0.15, p=0.039) in those consuming supplements.

Association by genotype

To determine the effect of BsmI and ApaI genotype on the relationship between let7a/b expression and vitamin D intake, analyses were repeated, stratified by genotype. Genotypes

were stratified as “restriction site absent” (AA, BB genotypes) or “restriction site present” (Aa/aa, Bb/bb genotypes). This revealed that the magnitude and direction of correlation between let-7b relative expression and vitamin D intake was maintained in carrying an allele with a restriction site (Table 2; n=176, r_s=-0.27, p=0.0005). However, the direction of the correlation was reversed (n=24, r_s=+0.319, p=0.0497) in those where the restriction site was absent. These correlations were significantly different (z-score=2.5, p=0.0124). Similar results for let-7b were obtained for the ApaI variant (Table 2). The only significant correlation observed between let-7a and vitamin D intake was in the subpopulation without ApaI restriction site (AA) (Table 2). Neither vitamin D intake nor miRNA expression levels alone differed significantly between genotype groups.

While no significant difference was found when let7a/b expression data was analysed by genotype alone (Figure S3), analysis by genotype and category of intake did reveal some interesting patterns. Reflective of the continuous analysis presented in Table 2, Figure 2A shows that inverse relationships of expression to intake exist between participants in which the BsmI restriction site polymorphism is absent and those in which it is present. A similar pattern is seen in the analysis stratified by presence or absence of the ApaI restriction site polymorphism, however not all relationships were statistically significant. For let-7a the only statistically significant relationships found were for those possessing the BsmI restriction site, and those possessing the ApaI restriction site (Figure 3).

Discussion

This study is the largest study to date investigating the relationship between vitamin D intake and circulating miRNA levels. We have demonstrated firstly, that there is a significant correlation between reported vitamin D intake and let-7b expression in circulation. Secondly, we have demonstrated that this relationship varies dependent on underlying *VDR* genotype. The relationship between let-7a expression and vitamin D was only significant in participants without the ApaI restriction site in the *VDR* gene.

While studies such as this can only demonstrate correlation, and not causation, the modulation of the relationship by genotype suggests the VDR may play a causative role. However, evidence of this interaction is greater for let-7b than let-7a, despite the presence of a VDRE in the let-7a-2 gene [43]. While vitamin D is able to modulate gene expression via the interaction with VDR and the VDRE, modulation of miRNA expression may also occur via indirect

mechanisms, such as vitamin D modulation of epigenetic mechanisms. Regulation of mRNA levels via miRNA signalling is now becoming recognised as a potential additional mechanism for the action of vitamin D and consequences of these interactions are now being investigated [53-55]. These additional pathways need to be considered when investigating the potential therapeutic and preventative use of vitamin D for chronic diseases. Additionally, understanding the molecular influence of current recommended daily intakes and supplementation regimens may shed light on the proposed links between vitamin D intake and later life diseases.

Evidence as to the links between vitamin D status and diseases such as cancer and cardiovascular disease has so far been inconsistent [20]. This study demonstrates that underlying genotype can influence molecular markers, and this may contribute to explaining some of these inconsistencies. BsmI and ApaI are both intronic polymorphisms, as such they do not influence the coding amino acids in the VDR protein. However, several studies have linked these polymorphisms to altered outcomes, including bone density, fracture risk and cancer risk [56-59]. This may be linked to these polymorphisms influencing mRNA levels or mRNA stability [60,61], however results have been mixed [62,63]. Furthermore, both BsmI and ApaI are in high linkage disequilibrium with a number of other *VDR* polymorphisms, including TaqI in the 3'UTR and the polyA variable number tandem repeat (VNTR) [52], which may alter expression levels, stability or transcriptional activity of the *VDR* [61,64,65]. The VNTR has at least 12 different alleles, but essentially follows bimodal distribution, with "b" found with long repeats (n=18-24) and "B" found with short repeats (n=13-17) [52]. Resolution of haplotypes with additional polymorphisms in this area of the *VDR* gene may provide additional information on the results observed here. Strong linkage disequilibrium may exist with additional polymorphisms in the regulatory or coding regions that may explain the observed associations.

An obvious short-coming of this work is that vitamin D status was only estimated by reported dietary and supplementary intake. However, a significant correlation between plasma 25(OH) vitamin D, and reported dietary and supplementary intake has been demonstrated in a subset of this cohort (Figure S1). Several other studies have shown reasonable correlations between reported intake using food frequency questionnaires and plasma levels [66-69]. Additionally, as this is an elderly cohort, relative contribution of endogenously synthesised and intake of exogenous vitamin D are altered, compared to the general population. Cutaneous production of vitamin D reduces with age due to decreased levels of provitamin D3 (7-dehydrocholesterol) [70] the precursor for vitamin D synthesis in the skin. Additionally, changes in lifestyle habits

that reduce sun exposure occur, therefore making dietary intake increasingly important [71-73]. In this cohort plasma 25(OH) vitamin D levels did not appear to be influenced by solar activity in the 6 weeks prior to collection (figure S2), suggesting intake is the major contributor to plasma 25(OH) status in this elderly cohort. Unfortunately, no information is available on skin pigmentation or estimated time spent outside for this cohort, which may explain the remaining variance between intake and blood levels of vitamin D. However, due to the health risks associated with excess sun exposure, any intervention looking to modulate vitamin levels in an "at risk" population is likely to attempt to do so using dietary and supplementary means [72].

In conclusion, the correlation between let-7a/b expression and vitamin D intake in this cohort varies with *VDR* genotype. This is the first time such a relationship has been described between vitamin D status, *VDR* genotype and the level of circulating miRNAs. While this is yet to be demonstrated for additional miRNAs, and the mechanisms behind these correlations are yet to be elucidated, this study highlights the importance of considering underlying genotypic variance in miRNA expression studies, and in nutritional epigenetics generally.

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Table 1: Allelic and genotypic frequencies of *VDR* SNPs BsmI (rs1544410) and ApaI (rs7975232)

	Frequencies (%)
rs1544410	
B allele	38.7
b allele	61.2
BB genotype	12.0
Bb genotype	53.5
bb genotype	34.5
rs7975232	
A allele	51.0
a allele	49.0
AA genotype	27.0
Aa genotype	48.0
aa genotype	25.0

Upper case = restriction site absent, lower case = restriction site present

Table 2: Correlation between vitamin D intake and let-7a/b expression, stratified absence/presence of by BsmI (rs1544410) and ApaI (rs7975232) restriction polymorphism

	Total	**BsmI**			**ApaI**	
r_s (p value)	n=200	**Restriction site present** n=176	**Restriction site absent** n=24	**z-score**	**Restriction site present** n=146	**Restriction site absent** n=54
let-7b	*-0.20 (0.005)*	*-0.27 (0.0005)*	*+0.319 (0.049)*	*2.64 (0.0085)*	*-0.2899 (0.0006)*	0.0614 (0.6621)
let-7a	-0.1367 (0.0566)	-0.0655 (0.3958)	0.0254 (0.9106)	1.14 (0.12)	-0.0209 (0.086)	*0.3132 (0.0224)*

Italics highlights those relationships with a p-value <0.05.

Figure legends

Figure 1: A) let-7b and **B)** let-7a expression in those who reported consuming adequate vs inadequate levels of vitamin D (means and standard error of means). n=200. * indicates $p<0.05$.

Figure 2: let-7b expression (means and standard error of means) in those who reported consuming adequate vs inadequate levels of vitamin D, stratified by presence/absence of **A)** BsmI or **B)** ApaI restriction site polymorphisms. $BsmI^-$ (restriction site absent, BB) n= 24; $BsmI^+$ (restriction site present, Bb/bb) n= 176; $Apa1^-$ (restriction site absent, AA) n=54; $Apa1^+$ (restriction site present, Aa/aa) n = 146. * indicates $p<0.05$, ** indicates $p<0.01$, *** indicate $p<0.001$.

Figure 3: let-7a expression (means and standard error of means) in those who reported consuming adequate vs inadequate levels of vitamin D, stratified by the absence or presence **A)** BsmI or **B)** ApaI restriction site polymorphisms. $BsmI^-$ (restriction site absent, BB) n= 24; $BsmI^+$ (restriction site present, Bb/bb) n= 176; $Apa1^-$ (restriction site absent, AA) n=54; $Apa1^+$ (restriction site present, Aa/aa) n = 146. *indicates $p<0.05$.