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Narrow band UVB effects on cutaneous vitamin D receptor expression and serum 25-hydroxyvitamin D in generalized vitiligo

Short title: NB-UVB and vitamin D in vitiligo

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Abstract

Background/purpose: Vitamin D has a role in variety of autoimmune diseases including vitiligo. Narrow band UVB (NB-UVB) treatment of vitiligo might act through its effects on vitamin D and its receptor. **Methods:** The present study is the first to elucidate NB-UVB effects on immunohistochemical vitamin D receptor (VDR) expression in generalized vitiligo and correlate it with serum vitamin D and repigmentation response. Using immunohistochemistry, VDR expression was estimated in skin biopsies of 30 controls and 30 vitiligo patients; from vitiligo lesion, perilesional skin at baseline and from repigmented and non responding skin after 24 NB-UVB sessions. Baseline serum 25-hydroxyvitamin D [25(OH)D] was investigated and repeated after 24 NB-UVB sessions. **Results:** VDR expression and serum 25(OH)D in controls were significantly higher compared to vitiligo patients. After NB-UVB therapy, there was a significant rise in VDR expression and serum 25(OH)D. VDR expression was significantly higher in repigmented skin compared to non responding lesion. Improvement in the clinical outcome score was associated with higher baseline VDR expression and higher serum 25(OH)D. **Conclusions:** NB-UVB phototherapy is associated with improved cutaneous VDR expression and vitamin D synthesis. Better repigmentation response to NB-UVB may be related to higher baseline VDR expression and its upregulation after phototherapy.

Keywords: NB-UVB, vitiligo, 25-hydroxyvitamin D, vitamin D receptor

Introduction:

Vitamin D is a steroid hormone with immunomodulatory actions [1]. It works through a nuclear hormone receptor; vitamin D receptor (VDR). [2]. Vitamin D has multiple effects on innate and adaptive immune responses [3]. The regulation of VDR content is an important mechanism for modulating the responsiveness of target tissues to vitamin D3 (cholecalciferol). In addition, the biological activity of vitamin D3 in cells is directly proportional to the tissue concentration of VDRs [4] which are expressed on melanocytes and may share in the regulation of melanin synthesis [5],

Vitamin D was also found to preserve the epidermal melanin unit and gain melanocyte integrity through several ways including regulating the activity, proliferation, migration of melanocytes and pigmentation pathways by modulating T cell activation [6].

Vitiligo being an autoimmune acquired pigmentary disorder is caused by destruction of functioning melanocytes in the affected epidermis [7]. It was found that deficient vitamin D levels might enhance vitiligo development through its immunomodulatory effects, and other effects on melanogenesis [8].

Since narrow-band ultraviolet B (NB-UVB) therapy is safe and effective modality, it is favored by most dermatologists for treatment of vitiligo [9]. Thus, in the present study, we aimed to evaluate the cutaneous VDR expression and serum 25(OH)D in generalized vitiligo vulgaris patients before and after NB-UVB therapy and correlate them together and with the repigmentation response.

Subjects and Methods:

Studied population:

This case-control study included 30 patients with generalized vitiligo and 30 age, sex and skin type matched healthy controls. Both patients and controls were selected from attendants of the Dermatology clinic, Menoufia University Hospital in the period between (June 2016 and September 2016).

Inclusion criteria:

Patients: Adult generalized vitiligo patients (age > 16 years) of Fitzpatrick skin type IV having depigmented patches on the trunk.

Controls: Adult healthy subjects (age > 16 years) of Fitzpatrick skin type IV with no family history of vitiligo.

All subjects signed a written informed consent prior to participation in the study and prior to photography, skin biopsies and blood sampling in accordance with Helsinki Declaration of 1975 (revised in 2000).

Exclusion criteria:

Subjects using sunscreens or receiving drugs that could alter the outcome of the study (topical or systemic vitamin D, calcium supplementation, systemic steroids and cholesterol lowering drugs) in the last 6 months were excluded from the study. Patients with chronic diseases (diabetes, liver or renal), thyroid or parathyroid disease, autoimmune disorders, and congenital or acquired errors of metabolism were not included, Known contraindications of NB-UVB were considered (light sensitive or aggravated dermatoses and patients with aphakia or cataract). Pregnant, lactating females and subjects receiving phototherapy within the last 6 months were also excluded from the study.

Clinical assessment

Data of patients were collected including age, gender, family history of vitiligo, and disease duration. All patients were examined by one dermatologist (demonstrating the extent of depigmentation according to rule of nines) and photographs were taken before and after phototherapy to be evaluated by two different dermatologists to document the extent of repigmentation.

Treatment Protocol:

Patients received two NB-UVB sessions per week. Since all patients were of skin type IV, the starting dose was 0.574 J/cm2, according to manufacture instructions with 25% dose increment every other session guided by the patients' response, for 3 months. Response to treatment was assessed objectively by comparison of photographs taken before and at 12 weeks (24 sessions of NB-UVB) after the initiation of therapy.

Clinical scoring:

Repigmentation percentage was calculated relatively to the previously assessed baseline status. The clinical outcome score was defined to be 4 if repigmentation was 75%-100%, 3 if repigmentation was 50%-75%, 2 if repigmentation was 25%-50%, 1 if repigmentation was less than 25%, and 0 when no repigmentation. According to the extent of repigmentation relative to baseline status, patients who responded to NB-UVB therapy were classified into group A (good responder; ≥50% repigmentation, score 3 and 4), group B (moderate responder; 25%-50% repigmentation, score 2) and group C (poor responder; ＜25% repigmentation, score 0 and 1) [10].

Estimation of serum 25(OH)D level:

One 3 ml venous blood sample was taken from each subject of the control group and 2 samples from each patient (at baseline and after 12 weeks of NB-UVB sessions). According to manufacturer instructions, samples were centrifuged at 2,000 rpm for 5 min then serum vitamin D was measured utilizing a competitive ELISA technique with a selected monoclonal antibody recognizing 25(OH) vitamin D_3/ D_2 (ORG 570 ELISA kit, Orgentec Diagnostika GmbH, Mainz, Germany) [11].

Serum levels of 25(OH) D were classified according to manufacturer instructions into sufficient if >20-160 ng/ml (>50-375 nmol/l); insufficient if 12-20 ng/ml (30-50 nmol/l) and deficient if <12 ng/ml (< 30 nmol/l) levels [12].

Skin biopsies and immunohistochemistry:

Four millimeter punch skin biopsies were harvested from sun unexposed skin (trunk) of each patient before NB-UVB therapy (one from the center of the vitiliginous lesion and another one from perilesional pigmented skin) and after 3 months of NB-UVB therapy (2 biopsies; one from the repigmented (responding) areas and the other biopsy from non-responding vitiliginous area). Another single skin biopsy was taken from the trunk of 30 subjects attending the clinic for cosmetic purposes (control group). Biopsies were fixed in 10% formalin and embedded in paraffin blocks and sectioned by microtome into 5 μm thick sections. The resulting sections were mounted on glass slides and subjected to routine histopathological examination using Hematoxyline-Eosin staining and immunohistochemical staining using anti-vitamin D receptor antibody (VDR polyclonal rabbit antibody [NBP1-51322] Novus Biologicals (USA)).

Sections were immunostained using automated Ventana system (Bench mark) and representative supersensitive Ventana antibodies. Diaminobenzidine tetrhychochloride (D.A.B.) was used as a chromogen. Sections were recut to 4 μm and dried at 60° C for 60 min in the oven. Anti-VDR antibody was diluted at 1:200 and fill into a Ventana antibody dispenser. The Ventana staining procedure includes pretreatment with Cell Conditioner 2 (pH 6) for 60 min (standard), followed by incubation with 1:250 diluted antibody at 37 °C for 32 min. Upon antibody incubation perform Ventana standard signal amplification, ultra wash, counter- staining with one drop of Hematoxylin for 4 min and one drop of bluing reagent for 4 min. For chromogenic detection we used ultra View Universal DAB Detection Kit (Ventana). Slides were removed from stainer; washed in water with a drop of detergent. [12]

Positive and negative controls were done and showed appropriate reactivity.

Using a light microscope at 400 magnifications, the number of positive keratinocytes (with VDR nuclear staining) was counted in six microscopic fields and given a percentage [14].

Statistical analysis:

Using SPSS version 15, data was summarized and tabulated. Comparisons between groups were done using Chi-square test for qualitative variables, independent sample-t-test for quantitative variables which are normally distributed while Mann-Whitney test was used for quantitative variables which are not normally distributed. Regarding comparing quantitative variables before and after therapy; paired sample-t-test was used for normally distributed variables, while Wilcoxon Signed Rank test was used for variables which are not normally distributed. Correlations were done to test linear relation between quantitative variables. *P* value $\leq$ 0.5 was considered significant. Least sample size estimated to be 31using the following assumption; power of study 80%, confidence interval 95%, effect size 0.5%, and standard deviation of the mean difference of VDR expression level is one.

Results:

Studied population

The study included two age and sex matched groups. Patients' group included 30 patients (16 males and 14 females), with age ranged between 18 and 45 years with a mean of 29.53 years (±8.53 SD). Vitiligo duration ranged between 5 and 32 months with a mean of 14.87 months (±8.67 SD). Eight patients (26.7%) had positive family history of vitiligo. Control group included 30 subjects (16 males and 14 females) with age ranged between 19 and 43 years and a mean of 29.53 years (±7.69 SD).

Clinical outcome score:

After NBUVB, patients' clinical outcome score mean percentage was 45.93 (±31.87 SD). Patients' response showed equal distribution within cases; ten (33.3%) patients were good responders, ten (33.3%) were moderate responders and ten (33.3%) were poor responders.

Serum 25-hydroxyvitamin D in control subjects and vitiligo patients (before and after NB-UVB):

All control subjects had sufficient 25(OH)D serum level which ranged between 45.20 and 113.30 ng/ml with a mean value of 74.41 (±18.76 SD). This level was significantly higher compared to mean±SD serum 25(OH)D level in vitiligo patients either before (14.69±14.05) or after (20.87±15.08) NB-UVB ($P<0.001$ for both) (Table 1).

Serum 25(OH)D level among vitiligo patients is shown in (Table 1). There was a significant improvement among vitiligo patients following NB-UVB therapy regarding both mean 25(OH)D serum level ($P=0.001$) and its sufficiency state ($P<0.001$) (Table 1)

Cutaneous VDR expression in control subjects and vitiligo patients (before and after NB-UVB):

Mean VDR expression percentage in control skin (Fig 1) was found to be (80.13±3.34 SD) which was significantly higher than that in lesional vitiligo (36.73±9.64 SD) (Fig 2a), perilesional skin (64.73±4.32 SD) (Fig 2b) before NB-UVB (P<0.001 for both), repigmented skin (Fig 2c) (P=0.05) and non responding skin (Fig 2d) after NB-UVB (P<0.001) (Fig 3a).

Among vitiligo patients, lesional VDR mean expression percentage was significantly lower when compared to perilesional skin at baseline (P<0.001) (Fig 3b) with a significant positive correlation between lesional and perilesional expression percentage (P<0.001),

VDR mean expression percentage in repigmented and non responding skin after NB-UVB were significantly higher than baseline lesional VDR mean expression (P<0.001 for both). In addition, after NB-UVB, VDR mean expression was significantly higher in repigmented lesions compared to non responding lesions (P<0.001) (Fig 3b).

Correlations between 25(OH)D serum values and VDR expression percentages:

Among vitiligo patients, there was a significant positive correlation between mean 25(OH)D serum levels before and after NB-UVB therapy (P=0.002) (Fig 4a).

Both baseline lesional and perilesional VDR expression showed significant positive correlation with serum 25(OH)D mean values after NB-UVB (P<0.001 for both) (Fig 4b, c).

Relationship of vitamin D serum categories and VDR expression:

Vitamin D receptor expression in vitiliginous skin was not associated with serum vitamin D categories before NB-UVB (P>0.05) (Figure 5a). However, baseline lesional and perilesional VDR expression was significantly associated with serum vitamin D sufficiency categories after NB-UVB being higher in sufficient versus insufficient and deficient groups in lesional skin (P<0.001) and in perilesional skin (P=0.001) (Figure 5b). In addition, VDR expression mean values in repigmented skin were significantly higher in sufficient when compared to deficient serum vitamin D group after NB-UVB (P=0.05) (Figure 5b).

Correlations of serum 25(OH)D values and VDR expression with clinical data of patients:

Both age of patients and disease duration didn't show any significant correlation with serum 25(OH)D mean values either before or after NB-UVB (P>0.05). However, both were negatively correlated with VDR expression mean percentage in non pigmented skin after NB-UVB (P<0.001, P=0.001 respectively).

Relationship between mean serum 25(OH)D, VDR expression, and clinical outcome score:

Clinical outcome score mean percentage was positively correlated with mean 25(OH)D serum level after NB-UVB (r= 0.778, *P*=0.001). It was also significantly higher in good responders (group A) compared to moderate responders (group B) and poor responders (group C) both before and after NB-UVB (P=0.03, 0.001 respectively) (Fig 5c).

In addition, clinical outcome score mean percentage was significantly associated with VDR expression percentage in both lesional (r= 0.969) and perilesional skin (r=0.812) before NB-UVB (P<0.001 for both) with significant positive correlation (*P*<0.001 for both).

Good responders (group A) showed higher expression percentage compared to moderate (group B) and poor responders (group C). However, no relationship has been found between VDR expression in repigmented and non pigmented skin and clinical outcome score (P>0.05) (Fig 5d).

Discussion:

Vitiligo is an acquired depigmentation of skin and hair. Exact cause of vitiligo is unknown, [15].Vitamin D deficiency is considered a risk factor for autoimmune diseases (which includes vitiligo). Furthermore, vitamin D and its analogues have been proposed as therapeutic tools in autoimmunity [16].

As known, two forms of vitamin D are dominantly found in humans, vitamin D2 and vitamin D3. Vitamin D3 is formed mainly in the skin in response to UVB radiation from sunlight or to lesser extent taken in diet or from supplements and fortified foods. Both forms of vitamin D are changed to 25(OH)D in the liver. Serum levels of 25(OH)D are the best determinant for vitamin D status since it has long half life (3 weeks) compared to the active form 1,25(OH_2)D and also measures the summation of cutaneous synthesis and dietary sources. In the present study, we measured serum 25(OH2)D_3/$_2$ by ELISA investigating the actual vitamin D status [17].

The association of low 25(OH)D serum level and vitiligo was proven in the current study by the significantly lower serum 25(OH)D level in vitiligo patients when compared to healthy controls. This agreed with some previous studies [8, 18-20]. Other studies [21-23] showed insufficient or very low vitamin D levels in most of vitiligo patients but without significant difference when compared to controls. They owed that indifference to seasonal,

nutritional, geographical and skin type factors. However, all of the studies including ours didn't prove causation, Furthermore, Karagün et al., [24] study didn't prove the role of vitamin D in vitiligo and recommended further research.

That controversy among studies may be related to the debate concerning the best method for measuring 25(OH)D levels. The accuracy of measurement varies greatly among different laboratories and among different assay methods. Antibodies utilized by some assays do not recognize both 25(OH)D2 and 25(OH)D3. In addition, the use of standard values and reference ranges for adequate vitamin D status is problematic; if unified to all laboratories and different assays. The same serum sample could show sufficient vitamin D value in one laboratory and an inadequate level in another (up to 17 ng/mL difference) [25].

Low serum vitamin D in vitiligo patients could be secondary to the disease process. Vitiligo patients prefer not to show their skin to people, and also protect their skin from sun using sunscreens, long sleeves and hats which could affect vitamin D synthesis.

Melanocytes, like keratinocytes, are capable of producing vitamin D and harbor VDR [26]. Locally produced vitamin D modulates skin innate and acquired immunity [27] and enhances maturation of melanocytes, through tyrosinase activity stimulation [28]. It also prevents their apoptosis [29] and upregulates VDR expression [30]. So in another point of view, deficient vitamin D levels might enhance vitiligo through its effect on melanogenesis [8].

The functional activity of vitamin D3 in tissues is directly proportional to VDR concentration which modulates tissue response to vitamin D. Thus, we measured not only serum 25(OH)D but also VDR expression in vitiliginous skin. This was done immunohistochemically to be the first VDR immunohistological study in vitiligo.

Vitamin D receptor immunohistochemical expression was significantly lower in vitiliginous skin (lesional and perilesional) when compared to control skin. In addition, there was a significantly higher VDR expression in perilesional skin than lesional vitiligo with a significant positive correlation. This was in agreement with Doss et al., [8] who investigated VDR expression in vitiligo using real time polymerase chain reaction (RT-PCR). They explained that the low serum vitamin D could affect VDR expression on keratinocytes in addition to the absence of melanocytes, which are one of the cells that express VDR in the skin. Furthermore, patients with vitiligo may be photoprotecting more than controls and this may affect VDR expression.

In addition, the present study showed absence of significant correlation between baseline VDR expression and serum 25(OH)D. This agrees with Doss et al., [8] study which found indifferent VDR expression between patients with sufficient or insufficient 25(OH)D serum level. This could be attributed to some factors which might influence VDR skin level; for instance, the genetic factor (VDR gene polymorphism) which has a growing evidence nowadays to affect VDR expression and function [18, 31].

Inspite that in vitro, vitamin D upregulates VDR expression [30], Curiel-Lewandrowski and colleagues [32] did not report any change in VDR mRNA expression following high dose oral vitamin D and this underscores our result.

Narrow band UVB, as a treatment for vitiligo, has many mechanisms to induce repigmentation. UVB portion of the sunlight (290–320 nm) induces transformation of 7-dehydrocholesterol to previtamin D3 in the epidermis.

In the present study NB-UVB was associated with improved serum vitamin D status. This was in accordance with Sehrawat et al., [22] and Lim et al., studies [33]. However, it was still significantly lower than control group. This may be attributed to the genetic

differences between vitiligo patients and healthy subjects which may affect the ability of skin to synthesize vitamin D in response to UVB. Moreover, this could be owed to the short duration of the study (12 weeks). Longer duration of NB-UVB phototherapy might be associated with further improvement of the vitamin D status as suggested by Sehrawat and colleagues [22] study which reported that the correlation between repigmentation of vitiligo lesions and vitamin D levels increases with increase in phototherapy duration.

NB-UVB induced vitamin D3 synthesis may be one of the mechanisms for photo-induced melanogenesis [21]. This was translated clinically, in the present study, by the improvement in the clinical outcome score which was positively correlated with serum 25(OH)D.

Few studies investigated UVB effect on VDR expression in normal skin. Mallbris et al. [34] reported that one erythmogenic UVB exposure significantly enhanced VDR expression at 24–28 hours in normal skin. Furthermore, Hong and colleagues [35] showed that mRNA VDR expression in mice is significantly upregulated by exposure to half the minimal erythema dose of UVB for 3 consecutive days. However, Lesiak et al. [36] observed that the impact of repeated UVB suberythemal doses on VDR expression is not cumulative and hypothesized that a homeostatic photomechanism controls UVB induced VDR expression in healthy skin.

In the current study, following NB-UVB, VDR expression improved in vitiliginous (both responding and non responding) skin. This reflects the up regulating effect of UVB on VDR expression. Although VDR expression improved in non responding skin after NB-UVB, it was still significantly lower than repigmented and control skin and this may be the cause of the clinical unresponsiveness. Furthermore, good and moderate response to

phototherapy in vitiligo patients was found to be associated with higher baseline serum 25(OH)D level and VDR expression percentage both at baseline and after NB-UVB.

Both age of patients and disease duration didn't show any significant correlation with serum 25(OH)D mean values. This was in agreement with the insignificant difference that was seen in Beheshti and colleagues [37] study among different age groups. However, VDR expression in non responding skin following NB-UVB was negatively correlated with both age of patients and vitiligo duration. So, older age of patients and longer disease duration may limit the response to phototherapy with poorer VDR expression. However in Makdani and Thakor [38] study, patients' age and vitiligo duration was found to have minimal effect on the targeted NB-UVB repigmentation response. Thus, this result is in need for larger scaled studies for confirmation.

In conclusion, NB-UVB is associated with improved vitamin D synthesis and cutaneous VDR expression. Better repigmentation response may be related to both higher baseline VDR expression and the upregulated VDR after NB-UVB. Furthermore, the improvement in the clinical outcome could be associated with higher serum 25(OH)D and higher baseline VDR expression in vitiliginous skin. Older patients and longer disease duration showed poor VDR expression with limited response to phototherapy.

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Table (1): 25(OH)D serum level in vitiligo patients and controls:

Serum 25(OH)D	Cases (n=30)		Controls (n=30)	P^1 value P^2 value P^3 value
	Before NB-UVB	After NB-UVB		
Mean ±SD	14.69±14.05	20.87±15.08	74.41±18.76	$<0.001^{1**}$
Median	10.10	18.40	76.40	$<0.001^{2**}$
Range	4.90-63.30	8.40–70.10	45.20–113..30	0.001^{3**}
	N (%)	N (%)	N (%)	
Deficient	20 (66.7)	8 (26.7)	0 (0.0)	$<0.001^{1**}$
Insufficient	8 (26.7)	12(40.0)	0 (0.0)	$<0.001^{2**}$
Sufficient	2 (6.7)	10 (33.3)	30 (100.0)	$<0.001^{3**}$

25(OH)D: 25-hydroxyvitamin D; NB-UVB: narrow band ultraviolet B; P^1 comparison between cases before NB-UVB and controls, P^2 comparison between cases after NB-UVB and controls, P^3 comparison among cases before and after NB-UVB

Figure legends:

1- Immunohistochemical staining of VDR in normal control showing nuclear staining in most of keratinocytes (a×200) (b×400).

2- VDR expression (×200) in (a) lesional skin showing sparse nuclear staining, and (b) perilesional skin before NB-UVB, (c) repigmented skin after NB-UVB from good responder patient showing nuclear staining in most of keratinocytes and (d) non responding skin after NB-UVB.

3- VDR expression in (a) vitiligo patients and controls, (b) among cases before and after NB-UVB.

4- Correlations between (a) serum 25(OH)D mean level before and after NB-UVB, (b) VDR expression in vitiligo lesion and serum 25(OH)D after NB-UVB, (d) Perilesional VDR expression and serum vitamin D after NB-UVB.

5- Association between VDR expression and vitamin D serum categories (a) before and (b) after NB-UVB. Relationship of the clinical outcome score and (c) serum 25(OH)D mean level (d) VDR expression in vitiligo patients.