RESEARCH ARTICLE

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Sun Exposure Makes no Discrimination based on Vitamin D Status and VDR-Foki Polymorphisms for Non-Melanoma Skin Cancers Risk in Iranian Subjects: A Case-Control Study

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Abstract

Background and Objective: Sunlight exposure, the main source of endogenous vitamin D synthesis, may increase the risk of non-melanoma skin cancers (NMSC) development. Vitamin D receptor (VDR) polymorphisms are associated with various malignancies. This study aimed to examine the associations between vitamin D status and VDR FokI polymorphisms in Iranian subjects with NMSC. **Materials and Methods:** This case-control study included 73 diagnosed cases of NMSC and 72 healthy controls from dermatology clinics at Razi Hospital, Tehran, Iran. A questionnaire was used to assess sunlight exposure. The extracted DNA from whole blood samples was genotyped and serum concentrations of 25-hydroxycalciferol (25(OH)D)) and intact parathyroid hormone (iPTH) were measured. **Results:** We found a significant higher duration of cumulative sunlight exposure in cases compared with controls ($p<0.001$). However, 25(OH)D and iPTH concentrations were not significantly different between cases and controls (30±15 vs. 29±15 ng/mL, $p=0.78$ and 46.0±20 vs. 40.5±23 pg/mL, $p=0.14$, respectively). We did not observe any significant increased risk of NMSC due to f allele, as compared with FF (OR =2.33, 95% CI 0.81-6.75, $p=0.12$). **Conclusion:** Though sunlight exposure was associated with increased NMSC risk, there were no significant associations between vitamin D status or VDR FokI polymorphisms with NMSC development in our subjects.

Keywords: Vitamin D- non-melanoma skin cancers- VDR-FokI polymorphisms- sun exposure- case-control study

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Introduction

Skin cancers are among the most common human neoplasms (Bikle, 2004). In Iran, the incidence of skin cancers is on a rise (Razi et al., 2015) accounting for approximately 15% of all types of cancers (Keyghobadi et al., 2015). While solar ultra violet beam (UVB) is the major natural source of vitamin D by triggering its dermal biosynthesis (Pike and Christakos, 2017), direct exposure to the same wavelengths of UVB has been known as the main culprit in development of skin cancers (Pfeifer and Besaratinia, 2012; Wyatt et al., 2015). It is believed that UVB causes molecular damage to the skin whereby suppresses immune system and contributes to immune-based skin conditions including hyperkeratosis and malignancies (Halliday et al., 2008). On the contrary, melanoma, the deadliest skin cancer, is commonly seen on the sites of the body that are not exposed to sun and occupational sun exposure was reported to be inversely associated with this malignancy (Kennedy et al., 2003). Moreover, lower serum 25(OH)D concentrations are associated with a poorer prognosis of cutaneous melanoma (Wyatt et al., 2015). Indeed, a growing body of evidence shows the predisposing effect of vitamin D deficiency (VDD) in many human pathologies including cardiovascular disease, diabetes, autoimmune disorders and certain malignancies (Holick, 2010; Jamshidinaeini et al., 2016).

Exploration of vitamin D receptor (VDR) on a vast variety of tissues and cells including basal cell and squamous cell carcinomas indicated new roles for this vitamin (Bikle, 2012; Nikooyeh et al., 2018a; Vishlaghi and Lisse, 2020). The VDR gene has several known polymorphisms whose associations with non-melanoma

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skin cancers (NMSC) have been investigated (Lee and Gyu Song, 2015; Von Schuckmann et al., 2016; Morgado-Águila et al., 2020).

Regarding the possible role of vitamin D in skin cancers and more specifically NMSC, several issues could be raised: (i) do NMSC patients have longer duration of occupational direct sun exposure than unaffected people? and if yes, (ii) does it have any influence on their vitamin D status? in other words, do NMSC patients have higher vitamin D status compared with the unaffected people as reported by some studies (Asgari et al., 2010; Soares et al., 2018)? (iii) considering the reported link between VDR polymorphisms and risk of several cancers, including prostate, breast, bowel (Hama et al., 2011; Vaughan-Shaw et al., 2017) and skin malignancies (Lee and Gyu Song, 2015; Von Schuckmann et al., 2016), is there any association among VDR FokI polymorphisms, vitamin D status and NMSC risk? This study was a struggle to answer these questions.

Materials and Methods

Study design and subjects

This was a case-control study conducted from September 2016 to April 2018, the protocol of which has been fully described elsewhere (Rezaiian et al., 2020). Cases comprised subjects from both sexes aged 40 to 75 years with confirmed diagnosis of basal cell carcinoma (BCC) or squamous cell carcinoma (SCC) in skin biopsy examination from the Dermatology Clinics at Razi Hospital, which is a single-specialty referral dermatology center in Iran. We enrolled just those subjects whose NMSC diagnosis had been made within three months prior to the time of recruitment and the stage of their lesions was either T1 or T2 (Fahradyan et al., 2017), as reported by dermatopathologist. The controls were age- and sex-matched unrelated healthy volunteers (Table I). The following criteria rendered individuals ineligible to enter this study: taking any nutritional supplements containing vitamin D, calcium, omega-3 fatty acids or antioxidants for at least 3 months preceding the time of recruitment; medications with influence on vitamin D metabolism including but not confined to corticosteroids, estrogens and calcitonin for at least 3 months prior to the time of recruitment; history of any other cancers, renal or liver diseases. The study protocol and objectives (including reporting the results as publications) were fully described for all participants before they signed an informed written consent. Then, they completed a general questionnaire on marital status, education level, medication and supplement use history, disease history, sunscreen use and average duration of daily direct sun exposure. To evaluate sun exposure, participant's job (sedentary/indoor, field work/outdoor, agriculture, other), average duration of direct sun exposure (< 10, 10-60, 60-120 and > 120 minutes a day), usual time of day of sun exposure (before 11 AM, between 11 AM and 15 PM, after 15 PM), exposed body areas (only face, face and hands from wrists down to fingers, face and arms), sunscreen use habits and its SPF were asked. This questionnaire has been repeatedly used in previous works (Nikooyeh et al., 2011; Nikooyeh et al., 2016b). On the other day, an overnight fasting venous blood samples were obtained from all subjects for laboratory analyses. The study procedures were ethically approved by the Ethics Committee of the National Nutrition and Food Technology Research Institute (NNFTRI; Ethics code: ir.sbmu.nnftri.Rec.1395.69). Figure 1 shows the study protocol at a glance.

Anthropometric measures

Weight was measured with light clothing and without shoes using a digital scale (Seca 808; Seca, Hamburg, Germany) to the nearest of 0.1 kg. Height was measured using a stadiometer (Seca 216, Seca, Hamburg, Germany) to the nearest of 0.1 cm. Body mass index (BMI) was calculated using the equation body weight (kg)/height2 (m). Weight status was defined based on BMI as underweight (< 18.5), normal (18.5–24.9), overweight (25.0-29.9) and obese (>30) (Health et al., 2015).

Laboratory investigations

Blood sampling and handling

Ten milliliter of venous blood taken following an overnight fasting was divided in two tubes either with or without ethylenediamine tetra-acetic acid (EDTA). Blood samples in the tube without EDTA were kept at room temperature (RT) for 20-30 minutes and then centrifuged at 800 g at RT for 20 minutes. Sera were then separated and transferred to fresh microtubes for biochemical analyses. Blood glucose and lipids were determined on the same day of blood sampling while serum aliquots were kept at -80°C for further analyses. The anticoagulated whole blood samples were used for genomic DNA extraction.

Circulating 25(OH)D and iPTH

Serum 25(OH)D and iPTH were assayed using EIA kits (both from Euroimmun, Medizinische Labordiagnostika AG, Germany). Subjects were categorized as vitamin D deficient if 25(OH)D concentration was below 20 ng/mL, insufficient with concentrations 20-29 ng/mL and sufficient with concentrations of 30 ng/mL and above (Chapuy et al., 1997; Holick et al., 2005; Bischoff-Ferrari et al., 2006; Holick, 2007).

DNA extraction and genotyping

Genomic DNA was isolated from whole blood samples using PrimePrep Genomic DNA isolation kit (GeNet Bio, Daejeon, South Korea) according to the manufacturer's protocol. For VDR FokI polymorphism (rs2228570) the forward primer was 5′- GTCAAAGTCTCCAGGGTCAG -3′, and the reverse primer used was 5′- GCCTGCTTGCTGTTCTTAC -3′. Genotyping was done by high-resolution melting (HRM) assay using StepOnePlus system (Applied Biosystems, Foster City, USA). The PCR reactions were carried out in a final volume of 20 µL using the 5x Hot FIREPol HRM Mix (HRM PCR buffer, HotStarTaq Plus DNA Polymerase, nucleotides and EvaGreen dye), 0.3 nM of forward and reverse primers each (final concentration) and 30 ng DNA under the following conditions: initial denaturation-activation step at 95°C for 15 min, followed by a 40-cycle program (denaturation at 95°C for 15 s,

annealing at 61°C for 20 seconds, 72°C for 20 seconds) and HRM step from 60 to 95°C rising at 0.1°C per second. Curves for each duplicate were checked on the shape and peak height to meet reproducibility. Normalized and temperature-shifted melting curves from HRM, suggestive of SNP, were distinguished, and direct Sanger sequencing was used to confirm genotyping results from the samples.

Statistical analyses

Data were expressed as mean±SD and frequencies for continuous and categorical variables, respectively. Normality of data was checked using Kolmogrov-Smirnov test. For between-group comparisons, independent sample t test, Mann–Whitney, or χ^2 tests were used when appropriate. Means of variables were compared among different polymorphism groups using analysis of variance (ANOVA). The associations between VDR FokI polymorphism and risk of NMSC were estimated by computing odds ratios (ORs) and 95% confidence intervals (CIs) using logistic regression analyses. The Hardy-Weinberg equilibrium (HWE) was tested by a goodness-of-fit chi-squared test to compare the observed genotype frequencies with the expected frequencies among controls. All statistical analyses were done using SPSS software IBM SPSS Statistics version 23. A two-tailed value of less than 0.05 was considered statistically significant.

Results

Characteristics of the study participants

Overall, 145 participants including 73 NMSC patients and 72 healthy controls were enrolled (Table 1). The mean age of the patients and controls was 56±9 and 58±8 years, respectively. Men consisted the greater proportion of both study groups. Compared with the healthy controls, NMSC subjects were significantly less educated (p=0.006). However, BMI did not statistically differ between two groups (p=0.8).

Table 1. Comparison of Age, Gender, duration of Sun Exposure and BMI of NMSC Subjects and Healthy Controls

Characteristic	NMSC (n=73)	Controls (n=72)	P value
Age (years), Mean ± SD	56±9	58±8	0.36
Sex			
Male	50 (68.5%)	43 (59.7%)	0.3
Female	23 (31.5%)	29 (40.3%)	
Sun exposure			
Negligible	8 (11.0%)	8 (11.1%)	
10-60 min	13 (17.8%)	38 (52.8%)	<0.001
60 min to 2 h	8 (11.0%)	9 (12.5%)	
> 2 h	44 (60.3%)	17 (23.6%)	
BMI (kg /m^2)	28.04±4.3	27.88±3.8	0.8

BMI, Body mass index

Table 2. Comparison of Vitamin D Status between NMSC Subjects and Healthy Controls

Variable	NMSC (n_1=73)	Controls (n_2=72)	p value
25(OH)D (ng/mL)	30±15	29±15	0.78
Deficient (<20ng/mL), n(%)	20 (27.4)	22 (30.6)	
Insufficient (20-29.9 ng/mL), n(%)	23 (31.5)	21 (29.2)	0.9
Sufficient (>30ng/mL), n(%)	30 (41.1)	29 (40.3)	
iPTH (pg/mL)	46.0±20	40.5±23	0.14

iPTH, Intact parathyroid hormone

Vitamin D status

Despite significantly longer duration of sun exposure (including occupational exposure) in cases than in controls (p<0.001), there was no significant between-group difference in concentrations of circulating 25(OH) D and distribution of vitamin D status. About 60% of the subjects in both groups had suboptimal circulating 25(OH) D concentrations (Table 2).

VDR FokI polimorphisms

Our analyses revealed that the frequencies of VDR variants in our study population were in HW equilibrium (chi2 value = 5.094, p=0.07 for control). The genotypes of FF, Ff and ff were 57%, 26%, 16% in NMSC cases and 68.1%, 23%, 8% in healthy controls, respectively.

The results of the comparison of distribution of different VDR Fok-I SNPs, including recessive genotype (Ff+ff), in NMSC patients and healthy controls (rs2228570) are demonstrated in Table 3. Logistic regression analysis did not show any significant increased risk of NMSC due to f allele (ACG codon), as compared with FF (ATG codon). Also, there were no significant differences in BMI, serum 25(OH)D and iPTH concentrations among different VDR FokI polymorphisms or between dominant (FF) and recessive (Ff+ff) genotypes (Table 4).

Distribution of different vitamin D status among VDR FokI genotypes in NMSC subjects and healthy controls did not show any significant difference (Table 5). Though logistic regression analysis revealed a higher risk of NMSCs for those subjects with VDR FokI-ff (33%) and undesirable vitamin D status (51%), these associations were not statistically significant (OR: 1.33, 95% CI: 0.60-2.9, p = 0.47 and OR: 1.51, 95% CI: 0.70-3.23, p = 0.28, respectively). The recessive genotype (Ff+ff), as compared with dominant one (FF), also showed 65% increased risk of NMSCs. However, this association was statistically insignificant (Table 6).

Table 3. Comparison of Distribution of Different VDR Fok-I SNPs in NMSC Subjects and Healthy Controls

Fok-I SNP	NMSC (n_1=73)	Controls (n_2=72)	OR	95% CI	p value
FF	42 (57%)	49 (68%)	-		
Ff	19 (26%)	17 (23%)	2.33	0.806-6.75	0.12
ff	12 (16%)	6 (8%)	1.3	0.602-2.82	0.5
Ff+ff	31 (42%)	23 (31%)	1.57	0.798-3.100	0.19

CI, Confidence interval; SNP, Single nucleotide polymorphism

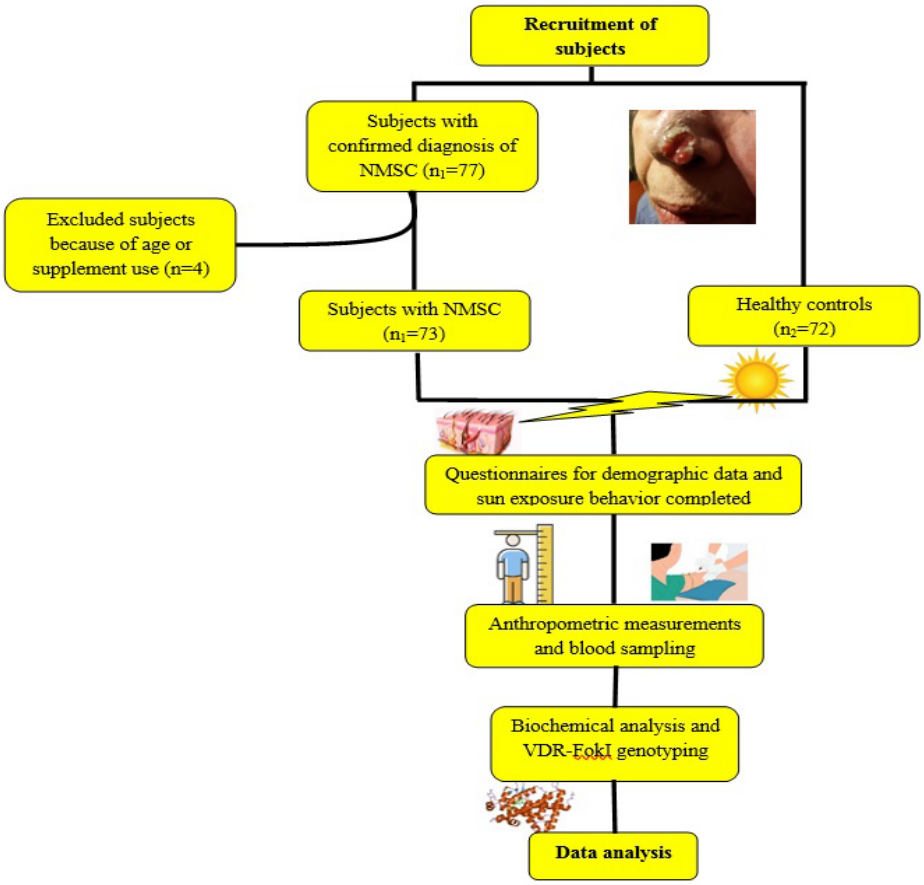

Figure 1. The Study Protocol at a Glance

Table 4. Comparison of Body Mass Index (BMI), Circulating 25(OH)D and iPTH Concentrations among VDR FokI Genotypes

Variable	Genotype (n=145)				p value[a]	p value[b]
	FF (n=90)	Ff (n=35)	Ff (n=16)	Ff+ff (n=51)		
BMI (kg/m^2)	27.6±4	28.3±3	28.8±3	28.4±3	0.48	0.26
25(OH)D (ng/mL)	30.6±16	28.0±14	30.1±15	28.7±14	0.69	0.46
iPTH (pg/mL)	41.3±20	48.3±24	42.7±25	46.4±24	0.29	0.18

[a], p values stands for difference between VDR FokI genotypes (FF, Ff, ff); p values were obtained from ANOVA; [b], p values are for difference between VDR FokI FF and Ff+ff genotypes.

Discussion

We found a significant association between cumulative (including occupational) sunlight exposure and increased risk of NMSC. However, no significant between-group difference in circulating concentrations of 25(OH)D was detected. A great body of evidence indicates that chronic sunlight exposure may induce NMSC (Holick, 2004). It is roughly estimated that only 25% of life span UV exposure occurs before the age of 18 (Iannacone et al., 2012). Despite the potential role of sun exposure in skin cancer development and also the potential protective effect of vitamin D against various malignancies, it is still the matter of debate that how much sunlight is needed to provide adequate concentrations of circulating 25(OH) D without exerting carcinogenicity. Some case-control studies indicated an association between higher pre-diagnostic concentrations of circulating 25(OH)D and an increased risk of BCC development (Asgari et al., 2010; Soares et al., 2018). Along the same line of evidence, some prospective cohort studies reported an increased risk of non-melanoma and melanoma skin cancers with increasing concentrations of serum 25(OH)D (Eide et al.,

Table 5. Distribution of Different Vitamin D Status among VDR FokI Genotypes in NMSC Subjects and Healthy Controls

Genotype-Vitamin D status*	NMSC (n_1=73) n (%)	Controls, (n_2=72) n (%)
FF-sufficient	31 (42.5)	35 (48.6)
FF-deficient	11 (15.1)	14 (19.4)
Ff-sufficient	13 (17.8)	11 (15.3)
Ff-deficient	6 (8.2)	6 (8.3)
ff-sufficient	9 (12.3)	3 (4.2)
ff-deficient	3 (4.1)	3 (4.2)

* All differences were not statistically significant (chi square, p > 0.05)

Table 6. Logistic Regression Analyses for the Associations between VDR FokI Polymorphism and Vitamin D Status with NMSC Risk

			OR	95% CI	p value
Model 1	Fok-I SNP	FF	---		
		Ff	2.51	0.874-7.67	0.082
		ff	1.33	0.606-2.94	0.473
	Vitamin D status	Sufficient	---		
		Deficient/insufficient	1.514	0.709-3.234	0.284
Model 2	Fok-I SNP	FF	-	-	-
		Ff+ff	1.65	0.827-3.31	0.154
	Vitamin D status	Sufficient	-	-	-
		Deficient/insufficient	1.49	0.703-3.18	0.296

Models are adjusted for age and sex

2011; Afzal et al., 2013). Similarly, a very recent meta-analysis of 13 prospective studies reported a significant association between circulating 25(OH)D concentrations and NMSC risk (Mahamat-Saleh et al., 2020). In contrast, a case-control study from Iran reported high prevalence of VDD in both BCC patients and healthy people (Darjani et al., 2017). One of the reasons of these discrepancies may be the very high prevalence of suboptimal serum 25(OH)D concentrations in Iran (Neyestani et al., 2012; Nikooyeh et al., 2016a; Nikooyeh et al., 2017; Nikooyeh et al., 2018b) which may veil any possible effect of vitamin D status on NMSC risk.

We found no significant association between VDR FokI polymorphisms (rs2228570) and NMSC risk. Accordingly, in a nested case-control study within Nurses' Health Study, there was no significant association between FokI ff genotype and skin cancers whereas BsmI BB variant was associated with increased risk of SCC (Han et al., 2007). In contrast with this finding, some meta-analysis studies suggested a possible predisposing role for polymorphisms of VDR FokI ff in NMSC development whereas carriers of BsmI Bb and BB might have a lower risk for skin cancer development (Gandini et al., 2009; Raimondi et al., 2009). Notwithstanding, the results of a systematic review did not show any significant association between VDR variants of TaqI, BsmI and FokI and risk of NMSC (Denzer et al., 2011). The possible role of other VDR polymorphisms, including BsmI, in development of NMSCs needs further prospective studies.

In conclusion, there is an association between duration of sun exposure and increased NMSC risk which is independent of vitamin D status and VDR FokI polymorphisms in Iranian subjects.

Limitations

In case–control studies recall bias occurs because cases tend to recall past exposures more accurately than controls. We did not examine any possible association between NMSC and other VDR polymorphisms, including BsmI and ApaI whose associations with NMSC have been reported recently (Morgado-Águila et al., 2020). Our data on vitamin D status were limited to a single measurement of 25(OH)D in the blood sample obtained at the enrolment time and this measurement does not necessarily reflect vitamin D status of the subjects in critical periods of life mainly childhood and young adult.

Abbreviations

BCC: Basal cell carcinoma
BMI: Body Mass Index
CI: Confidence interval
EIA: Enzyme immunoassay
HWE: Hardy-Weinberg equilibrium
HRM: high-resolution melting
25(OH)D: 25-hydroxycalciferol
IU: International unit
NMSC: Non-melanoma skin cancer
OR: Odds ratio
SCC: Squamous cell carcinoma
SNP: Single nucleotide polymorphism
UV: Ultraviolet
VDD: Vitamin D deficiency
VDR: Vitamin D receptor
WHO: World Health Organization

Author Contribution Statement

This study was designed by TN with the intellectual aids of BN and FR. Statistical analyses were done by FR under the guidance and supervision of BN. All field works were performed by FR. Laboratory bench works were done by FR with the aids of AK, MZ, NS and TN. SHD and AHE helped in clinical assessments and interpretation of findings. The preliminary manuscript was written by FR and revised and finalized by TN. This study represents part of Ph.D. dissertation of FR.

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Ethical Statement

The study protocol and objectives were fully described for all participants before they signed an informed written consent. The study procedures were ethically approved by the Ethics Committee of the NNFTRI (Ethics code: ir.sbmu.nnftri.Rec.1395.69).

Data Availability Statement

The datasets generated during and/or analyzed during the current study are not publicly available due to ethical consideration but are available from the corresponding author on reasonable request.

Declaration of Competing Interest

The authors declare no potential conflicts of interest.

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