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Research Article
Vitamin D Receptor Gene Polymorphism Among Indonesian Women in North Sumatera

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Abstract

Background: Women were found to be at particularly high risk vitamin D deficiency. High prevalence of vitamin D deficiency was reported in tropical countries. **Objective:** This work aimed to investigate range level of 25(OH)D serum in women with single nucleotide polymorphisms of Vitamin D Receptor (VDR) gene who lived in North Sumatera, Indonesia. **Materials and Methods:** Vitamin D levels were determined in women displaying *Taq*I and *Bsm*I single nucleotide polymorphic variants in the Vitamin D Receptor (VDR) gene. A cross-sectional design was adopted and a total of 292 subjects were included over the period of four years (2012-2016). Sun exposure duration, occupation, Vitamin D intake, physical activity level and body mass index were also recorded. **Results:** Serum vitamin D concentrations were found to be deficient in 122, insufficient in 158 and sufficient in only 12 subjects, but none were in the standard normal range for sunny countries (54-90 ng mL^{-1}). Moreover, all subjects were found to be heterozygous for *Taq*I and *Bsm*I variants with TC and AG genotype, respectively. Mean serum 25(OH)D levels fell in the range 20.8±6.4 ng mL^{-1}. The median was 21.2 ng mL^{-1} and the interquartile range was 16.45-24.59 ng mL^{-1}. **Conclusion:** Vitamin D deficiency/insufficiency was found to be common in healthy women with *Taq*I and *Bsm*I single nucleotide polymorphisms in the VDR gene and a normal range for 25(OH)D serum levels was posited.

Key words: Genetic, Indonesian, obese, single nucleotide polymorphism, VDR gene, vitamin D, 25(OH)D serum level

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

High prevalence of vitamin D deficiency has been reported in many parts of the world, including Indonesia. Women in North Sumatera, Indonesia were found to be at particularly high risk, as they, obese and non-obese, tended to avoid sun exposure[1,2]. Numerous studies have linked vitamin D deficiency to obesity, lifestyle variables and single nucleotide polymorphisms, namely *Taq*I and *Bsm*I, of Vitamin D Receptor (VDR) gene[3-6].

According to Khor *et al.*[3] and Engelsen *et al.*[7], avoiding sunlight exposure and routine use of sunscreen preparations contribute to lower 25(OH)D serum levels in women and children. A preference for white skin in dark skin communities may explain why some women would opt for such life-style choices. Sunscreens significantly decrease the synthesis of vitamin D3. Complete cloud cover and shade were shown to decrease the energy received from ultraviolet light by 50 and 60%, respectively. Moreover, the skin is unable to make vitamin D using sun rays at latitudes higher than 37° S[4,8].

Due to limited mobility and lower levels of physical activity, obese individuals may not get enough sun exposure. Moreover, storage in body fat compartments makes it harder for cells to release vitamin D, which is counter intuitive as these individuals have greater demands for the vitamin to support greater weight with healthy bones. Unfortunately, decreased bioavailability of 25(OH)D and increased levels of the active vitamin D metabolite result in a negative feedback mechanism that further decreases serum 25(OH)D levels by inhibiting its hepatic synthesis. However, according to Sari *et al.*[1], vitamin D deficiency in the Indonesian women of North Sumatera was not correlated with obesity, but rather due to single nucleotide polymorphisms.

Studies have shown that low vitamin D levels may result from lifestyle factors, obesity and polymorphisms in VDR gene[5,9]. Moreover, according to Al-Daghri *et al.*[10], *Taq*I (rs731236) and *Bsm*I (rs1544410), single nucleotide polymorphisms in the VDR gene, could lead to the development of type-2 diabetes mellitus due to increased susceptibility to inflammation and metabolic reactions. Vupputuri *et al.*[6] estimated that up to 94.3% of Asian Indians suffered from vitamin D deficiency. Surprisingly, a much higher prevalence was reported in those who displayed *Taq*I (T>C genotype) polymorphism in the VDR gene (*Taq*I T>C = 82.98%, TT = 12.77% and CC = 4.25%). Having observed a sample of 156 women, Sari *et al.*[1] concluded that the heterozygous TC genotype was a major factor to low levels of vitamin D.

Arguably, the normal range for serum vitamin D in sunny countries is 54-90 ng mL^{-1}. In general, vitamin D levels below 20 ng mL^{-1} indicate a deficiency; in the range 20-29 ng mL^{-1} denote insufficiency and at 30 ng mL^{-1} or above imply sufficiency i.e., they fall within the normal range[11].

This study aimed to determine the normal range for vitamin D in the Indonesian women in North Sumatera, where sunlight is abundant. Moreover, we sought to elucidate the correlations between vitamin D levels and single nucleotide polymorphisms (*Taq*I and *Bsm*I), obesity and lifestyle variables.

MATERIALS AND METHODS

Subjects: This cross-sectional study was carried out amongst women aged 20-50 years, working indoors as office staff, doctors, nurses and teachers and outdoors as street sweepers and farmers. The subjects included 292 healthy women belonging to different ethnic groups and residing in different parts of North Sumatera. The study was conducted in the dry seasons (April to October) between May, 2012 and May, 2016 to ensure abundant sunlight. Recruitment location was in Sumatera Island, North Sumatera, Medan, Indonesia at latitude 3.57 N and longitude 98.65 E. The temperature was in the range of ±32°C (90°F).

The subjects'occupations were classified into two categories: indoor jobs (e.g., office staff, doctors, nurses and teachers) and outdoor jobs (e.g., street sweepers and farmers). Over nine ethnic groups reside in North Sumatera. In the present study, the subjects were grouped into three ethnic groups as follows, depending on the area from which they hailed: (1) Javanese, (2) Bataknese and (3) Others (Malay, Nias, Minang, Acehnese). Ethnicity was recorded based on the father's ethnic background. Sunlight exposure was defined as the cumulative sunlight exposure per day. Subjects were grouped into two sunlight exposure groups, which respectively included those with (1) Less than 1 h and (2) Those with more than 1 h of sunlight exposure per day.

Subjects were recruited after careful background checks, physical examination and standard baseline investigations. Those with history of diabetes mellitus, myocardial infarction and/or renal and liver dysfunction were excluded. Exclusion criteria also included pregnancy, lactation and being on medications that influenced lipid serum levels. All participants were presented with written informed consent forms approved by the Ethics Committee of Medical Faculty and Haji Adam Malik Hospital of Sumatera Utara University, Indonesia Number 171/KOMET/FK USU/2012 and Number 120/KOMET/FK USU/2015.

Anthropometry and nutrient intake: Anthropometric indices included height (to the nearest 0.5 cm), weight (to the nearest 0.1 kg) and BMI = weight (kg)/height (m)/height (m). Categorized BMI was based on Asia Pacific, <18.5 classified as underweight, 18.5-22.9 classified as normal weight, 23-24.9 classified as overweight/at risk, 25-29.9 classified as obese I and >30 classified as obese II. Assessment of vitamin D intake was based on food recall for two days (one weekday and one weekend day). Calculations for Indonesian foods were performed using Nutrisurvey 2005.

Laboratory analysis: Serum 25(OH)D concentrations were measured by using a chemiluminescent immunoassay (CLIA) apparatus (Diasorin, Stillwater, MN). Measurements were between 4.0 and 150 ng mL^{-1} with an inter-assay precision of 3.90% CV. Levels below 20 ng mL^{-1} implied deficiency, within 20-32 ng mL^{-1} insuficiency; between 32-100 ng mL^{-1} sufficiency; above 100 ng mL^{-1} excess and above 150 ng mL^{-1} intoxication. The range 54-90 ng mL^{-1} is considered to be the normal reference range in sunny countries. The classification was above was based on the work of Grant and Hollick[12], however, according to Hollick[13], while levels below 20 ng mL^{-1} indicated deficiency, insufficiency occurred with levels between 21-29 ng mL^{-1} and levels above 30 ng mL^{-1} inferred sufficiency. To convert ng mL^{-1} to nmol L^{-1}, the value is multiplied by 2.496.

Analysis of single nucleotide polymorphism in VDR gene: Detection of Single Nucteotide Polymorphisms (SNP) in VDR gene was performed in three steps: (1) DNA isolation through the salting out method, (2) DNA purity validation and (3) SNP genotyping using Applied Biosystem Step One Plus Real-Time PCR Systems.

To isolate DNA, 3 mL of whole blood were collected in an EDTA collection tube (BD Vacutainer, New Jersey, USA). The blood was centrifuged at 3000 rpm for 10-15 min to precipitate leucocyte sediments. Next, 300 µL of leucocyte sediments were added to 900 µL of an Eritrosit Lysis Solution in a 1.5 Eppendorf tube. The tube was inverted twice or thrice and incubated for 10 min at 4°C. Then, the tube was centrifuged at 13000 rpm for 3 min and the supernatant was discarded leaving a leucocyte pellet. The process was repeated up to 5 times until the solution was clear. The collected pellet was dispersed by vortex and 300 µL of the nuclei lysis solution (Reagent:Promega) were added. After inverting 2-3 times, 100 µL of protein precipitant were added to the solution. The tube was vortexed for 20 sec and centrifuged at 13000 rpm for 3 min at room temperature. Next, the supernatant was injected into a 1.5 Eppendorf sterile tube filled with isopropanolol (300 µL). After vortexing for 3 sec, the tube wascentrifuged at 13000 rpm for 1 min. The DNA was then observable as a pellet and the supernatant was carefully removed. Washing was done using 300 µL of 70% ethanol and 1 min of centrifugation at 13000 rpm. Lastly, the supernatant was removed and the pellet was air dried inside a laminar-flow hood for one night. It was then diluted with 100 µL of a DNA rehydration solution and stored at 4°C for one night before being transferred to a -20°C freezer.

The DNA purity was checked using a nanophotometer (IMPLEN; P360, CA, USA) based on the 260/280 ratio. The ratio ranged from 1.8 to 2.2, indicating good purity. Hence, the third step, SNP genotyping, was initiated using 1-10 ng of DNA. Both of VDR gene polymorphisms, *Taq*I and *Bsm*I, were tested by allele discrimination using a StepOnePlus™ Real Time PCR instrument (Applied Biosystems, Foster City, CA, USA). TaqMan probes were obtained from Applied Biosystems (Foster City, CA, USA). The PCR was run on 'Fast' mode. The protocol began with the activation of DNA polymerase at 95°C for 20 sec, which was succeeded by denaturation at 95°C for 3 sec and annealing at 60°C for 30 sec. The process comprised forty cycles. Strand fluorescence was detected at 60°C. The assay was performed for a 10 mL reaction solution using TaqMan genotyping master mix and 96-well reaction plates. A MicroAmp Fast Optical 96-well reaction plate covered with MicroAmp Optical Adhesive Film was obtained from Applied Biosystem (Foster City, CA, USA).

Genotyping was conducted using probes for two alleles. Probes' tips had been labelled with high energy fluorescent stain (FAM and VIC) at one side only using a reporter located at the 5' end and a quencher located atthe 3' end of the probe.

Statistical analysis: Data was analyzed using version 11.5 of the IBM-SPSS statistical program (IBM Corp., Chicago, IL). Categorical variables were expressed as percentages. Continuous, normally distributed variables were expressed as Mean±SD and non-normally distributed continuous variables were expressed as median (minimum-maximum).

RESULTS

The survey included 292 subjects overall, all subjects completed the assessments. Demographic and anthropometric data of studied are demonstrated in Table 1. The highest percentage were in age range 30-40 years old and

the lowest were in age range 40-50 years old, with ratio for each age group were 1:1.3:1. The lowest age was 20 years old, the highest age was 50 years old and the median age was 35 years old.

The most prevalent ethnic found in the study was Javanese, followed with Bataknese, this was interesting because even the study done in Sumatera island not in Java island but still found most prevalent ethnic was Javanese (Table 1). Occupation of the subjects categorized in two occupation which were indoors and outdoors. This study reported that indoors and outdoors occupation had almost equal percentage (50.7 and 49.3%, respectively) (Table 1).

Table 1 shows that in this study, the most highest BMI categorized percentage was normal category and the lowest was underweight category. The lowest BMI was 16.8 kg m^{-2}, the highest BMI was 47.7 kg m^{-2} and the median was 24.1 kg m^{-2}.

All subjects had normal range daily food intake except fiber and vitamin D intake. Fiber intake was very low than daily recommendation (25-30 g day^{-1}). Daily vitamin D intake was lower than Indonesian nutrient daily allowance that recommend 15 mcg vitamin D day^{-1}. The lowest vitamin D intake was 0.5 mcg day^{-1}, the highest was 9.5 mcg day^{-1} and the median was 2.7 mcg day^{-1} (Table 2).

Daily sun exposure have an equal percentage between less than 1 h and more than 1 h. Physical activity shows most of the subjects had low physical activity, none of the subjects had high physical activity and Table 2 shows calcium range level, the lowest serum calcium level was 5.1 mg dL^{-1}, the highest was 10.5 mg dL^{-1} and the median was 9.2 mg dL^{-1}.

Table 3 shows the single nucleotide polymorphisms detected in the VDR gene in the present work. Most of the subjects were homozygous mutant and heterozygote, none of them was homozygous wildtype that indicated normal genotype.

Mean serum 25-hydroxyvitamin D concentrations, along with the percentile values are listed in Table 4. Mean serum 25(OH)D concentrations were in the range 20.8±6.4 ng mL^{-1}, with the lowest level was 7.1 ng mL^{-1}, the highest was 42.5 ng mL^{-1} and median was 21.2 ng mL^{-1}. Based on Grant and Hollick's classification of 2005[12], most of the subjects had low 25(OH)D serum concentrations manifesting in vitamin D deficiency and insufficiency. Only 12 subjects (4.1%) had sufficient 25(OH)D levels, but none were in the normal range for sunny countries (54-90 ng mL^{-1}). About 122 subjects were found to suffer deficiency (41.8%) and 158 (53.8%) suffered from insufficiency. Similarly, according to Hollick's classification of 2007[13], most of the subjects had low 25(OH)D serum concentrations, with only 19 (6.5%) reaching sufficient 25(OH)D levels. About 122 subjects were presumed to have deficiency (41.8%) and 151 (51.7%) were presumed to have insufficiency.

Table 4 also showed 25, 50 and 75% percentiles were marked at 16.5, 21.2 and 24.5 ng mL^{-1}, respectively. Hence, the interquartile range, the normal range in our sample

Table 1: Demographic and anthropometric variables in polymorphic Indonesian women for vitamin D receptor genes

Variables	Mean±SD/n (%)
Age (years)	35.8±8.6
20-30	92 (31.5%)
30-40	112 (38.4%)
40-50	40-50 (30.1%)
Ethnic	
Javanese	161 (55%)
Bataknese	117 (40%)
Others	14 (10)
Occupation	
Indoors	148 (50.7%)
Outdoors	144 (49.3%)
Anthropometry	
BMI (kg m^{-2})	24.8±4.3
Underweight	6 (2.1%)
Normal	107 (36.6%)
Overweight	49 (15.8%)
Obese I	95 (32.5%)
Obese II	35 (12%)

Continues variable: Mean±SD, Categorical variable: n (%), SD: Standard deviation

Table 2: Lifestyle variables in polymorphic Indonesian women for vitamin D receptor genes

Variables	Mean±SD/n (%)
Daily food intake	
Energy (kcal)	1513.2±54.2
Carbohydrates (g)	188.3±85.1
Proteins (g)	44.5±18.4
Fats (g)	46.2±38.7
Cholesterol	238.7±210.7
Fibers	5.8±6.9
Vitamin D intake (μg)	5.2±6.9
Vitamin D intake	
Low	241 (82.7%)
Moderate	51 (17.3%)
Daily sun ray exposure	
≤1	155 (53.2%)
>1	137 (46.8%)
Physical activity	
Low	102 (65.4%)
Moderate	54 (34.6%)
Biochemical biomarkers	
Serum calcium (mg dL^{-1})	9.19±0.54
Low	2 (0.7%)
Normal	290 (99.3%)

Continues variable: Mean±SD, Categorical variable: n (%), SD: Standard deviation

Table 3: Single nucleotide polymorphisms in Indonesian women in North Sumatera

Genotypes	n (%)
rs731236 (*Taq*I)	
C	289
T	367
CC	-
TT	39
CT	289
rs1544410 (*Bsm*I)	
A	168
G	416
AA	-
GG	124
AG	168

Genotype AA/CC: Homozygous wildtype, Genotype TT/GG: Homozygous mutant, Genotype CT/AG: Heterozygous

Table 4: Serum levels of 25-hydroxyvitamin D in polymorphic Indonesian women for vitamin D receptor genes

Variables	Mean±SD
25-hydroxyvitamin D serum levels (ng mL^{-1})#	20.8±6.4
Vitamin D status[12]	
Deficiency	122 (41.8%)
Insufficiency	158 (54.1%)
Sufficiency	12 (4.1%)
Normal in sunny countries	-
Vitamin D status[13]	
Deficiency	122 (41.8%)
Insufficiency	151 (51.7%)
Sufficiency	19 (6.5%)
Percentile values for vitamin D (ng mL^{-1})	
Minimum	7.1
5% Percentile	10.1
25% Percentile	16.5
50% Percentile	21.2
75% Percentile	24.6
95% Percentile	31.9
Maximum	42.5

population, was estimated to be 16.5-24.6 ng mL^{-1} which may be conveniently rounded to 15-25 ng mL^{-1}.

DISCUSSION

The present investigation was conducted to posit a new, more accurate range for serumvitamin D levels in the women of North Sumatera. We focused on women living in abundant sunlight areas and having single nucleotide polymorphisms (*Taq*I and *Bsm*I) in the VDR gene. The study also sought to assess the association between serum vitamin D levels, obesity and lifestyle variables.

Vitamin D deficiency was reported to occur only in countries having four seasons in the year and some studies showed that the deficiency occurred mainly in obese individuals[11,14-16]. It is demonstrated that high vitamin D deficiency prevelance could occur even in those parts of the world where exposure to the sun could be higher. Indonesia is a tropical country with two seasons. Yet, vitamin D deficiency was detectable not only in Indonesian obese women, but also in women with normal adiposity (non-obese subjects). This study showed that significant differences in adiposity and BMI had no significant influence on 25-hydroxyvitamin D serum concentrations.

According to Chiu *et al.*[17], serum 25(OH)D concentrations are positively correlated with insulin sensitivity and negatively correlated with hypovitaminosis affecting β cell function. Serum 25-hydroxyvitamin D concentrations are largely determined by vitamin D intake and sunlight exposure[18]. Alvarez and Ashraf[19], reported that 25(OH)D levels were influenced by ethnic difference, especially in African Americans, who attained significantly lower insulin sensitivity than European Americans. This study involved three different ethnic groups in North Sumatera: Javanese, Bataknese and others. Low 25(OH)D serum concentration levels were observed in all equally.

Avoiding sunlight exposure was found to be a key factor reducing serum 25(OH)D concentrations in all subjects. Indonesia is a tropical country with two dry and wet seasons. Data was collected in the dry season, which is characterized by higher levels of sunlight (UV-B). Yet, exposure to sunlight was found to be very low in all subjects, most of who seemed to prefer no exposure. Some of the subjects had cumulative sunlight exposure of more than 1 h but tended to hide and avoid sunlight. For example, when walking to work, women in North Sumatera wear sunscreen preparations, hold umbrellas, wear clothes covering the entire body and walk under the shade of buildings.

The majority of the women tested worked indoors, which made them rather prone to low sunlight exposure and physical activity. Lower physical activity levels are related to the nature of the job. Spending long hours in the work place leaves little time to exercise. Moreover, limited movement while working also contributes to low physical activity. Although exposure to UV-B rays may raise serum 25(OH)D levels, excessive UV radiation is neither recommendable nor necessary to maintain adequate vitamin D production. To convert previtamin D3 to vitamin D, the skin requires more than direct sunlight exposure. Higher skin temperatures are also needed. Probably, higher physical activity could help generate higher skin temperatures from the inside[20].

Vitamin D intake was shown to be below the recommended dietary allowance in all subjects. Vitamin D3 has 5 times the activity of vitamin D2 and dietary food sources may not supply enough for adequate health. Cholecalciferol (D3) is found mainly in salmon, sardine, mackerel, tuna and

cod fish oil. It is also found in limited quantities in milk, eggyolk, butter and margarine. Supplements commonly contain ergocalciferol (D2) extracted from mushroom or D3 extracted from lanolin. Ordinary dietary sources of vitamin D3 evidently do not supply enough for adequate health (around 250-300 IU day^{-1} in USA). Individuals with low vitamin D intake are advised to take supplements that are safe and reliable sources of vitamin D3. However, according to Hollis[21], neither vitamin D supplements nor food sources of vitamin D are consumed on daily basis. Alarmingly, this study revealed that working women consumed very limited amounts of vitamin D food sources (egg yolk, fish, meat and mushroom). Women also seldom consume vitamin D supplements. They tend to consume vitamin C or E supplements because of their antioxidant effects on the skin, even though low dietary intake of vitamin D and low sunlight exposure can have detrimental effects on health.

Deficiency of vitamin D was previously linked to VDR gene polymorphisms. However, different prevalence rates were reported in different populations for each of the single nucleotide polymorphisms of the VDR gene, like *Taq*1, *Bsm*1, *Fok*1 and *Apa*1. Research examining the correlations between these polymorphic variants and vitamin D deficiency in Indonesia has been lacking. Previous studies also reported race, obesity and lifestyle that affecting vitamin D deficiency[22-26]. Moreover, the factors contributing to deficiency in tropical countries have not been fully elucidated so far[6,8,10].

There is no consensus on the optimal serum levels of 25(OH)D. Using Hollick's 2007 classification of vitamin D deficiency-insufficiency, it may be plausible that all women with single nucleotide polymorphisms in the VDR gene suffered from vitamin D deficiency or insufficiency[13]. However, in the present work, it is demonstrated that normal 25(OH)D serum levels in these women rather fall anywhere between 16.45 and 24.59 ng mL^{-1} (or 15-25 ng mL^{-1}).

Further research is need to assess whether adults having 25(OH)D serum levels in this range are at high risk of any disease, such as diabetes mellitus, cardiovascular disease or cancer. In the present study, all subjects appeared to be living normally and presented with normal serum calcium levels. It is predicted that micro evolution was responsible for lowering vitamin D levels in these women, yet did not subject them to the adverse effects of vitamin D deficiency. A main limitation of the present study was that it failed to assess other parameters, such as the parathyroid hormone and serum phosphate levels. It also did not assess osteoporotic parameters, such as CTx and bone mineral density. A more comprehensive analysis seems to be warranted to confim and further elucidate the present findings.

Overall, the present work provided ample and compelling evidence to suggestthat serum 25(OH)Dlevels had to be maintained within a range of 15-25 ng mL^{-1} to ensure optimal health in women with single nucleotide polymorphism in the VDR gene.

CONCLUSION

Vitamin D deficiency/insufficiency was found to occur in healthy women with *Taq*I and *Bsm*I single nucleotide polymorphisms in the VDR gene. A new normal range for serum 25(OH)D levels was proposed for North Sumatera. Further research is warranted, however, to assess the physiological effects of vitamin D deficiency in tropical countries and to further explain this deficiency in terms of microevolution.

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