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Original article

Relationship between vitamin D receptor gene polymorphisms and anemia in postmenopausal Vietnamese women

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***Background*:** Both in vitro and in vivo studies have shown that calcitriol, the active form of vitamin D, is involved in hematopoiesis. Vitamin D receptor (*VDR*) gene has been suggested as one of the candidate genes for anemia.
Objective: Investigate relationship between anemia and the commonly studied polymorphisms of *VDR* gene (*Fok*I, *Bsm*I, *Apa*I and *Taq*I) in terms of genotype and haplotype in Vietnamese.
***Methods*:** A case-control study including 132 postmenopausal women without chronic kidney diseases was designed to investigate the relationship between *VDR* polymorphism and anemia. Four single nucleotide polymorphisms (SNPs) *Fok*I (rs2228570), *Bsm*I (rs1544410), *Apa*I (rs7975232), and *Taq*I (rs731236) were typed by polymerase chain reaction and restriction fragment length polymorphism method.
***Results*:** Genotype distributions of four SNPs were in Hardy-Weinberg equilibrium in both anemia and control groups. The SNPs at the 3'end of the *VDR* gene (*Bsm*I, *Apa*I and *Taq*I) exhibited a strong linkage disequilibrium. There was no significant association between anemia and *VDR* polymorphism in terms of allele, genotype, and haplotype in the analyses unadjusted or adjusted for the covariates (age, body mass index, educational level, serum ferritin, iron and albumin).
***Conclusion*:** *VDR* gene did not influence anemia in postmenopausal women without chronic kidney disease. For further study on the association between *VDR* gene and anemia, the use of larger sample size, a prospective study design, and additional markers would enhance the reliability and validity of findings.

***Keywords*:** Anemia, association, haplotype, postmenopausal Vietnamese women, vitamin D receptor

Vitamin D is a prohormone with several active metabolites that act as hormones. The vitamin D endocrine system is involved in a wide variety of biological processes including bone metabolism, modulation of the immune response, and regulation of cell proliferation and differentiation [1]. Both in vitro and in vivo studies have shown that calcitriol, the active form of vitamin D, is involved in hematopoiesis [2]. The significant association between Vitamin D deficiency and anemia has been reported [3]. The effects of this steroid-like molecule are essentially exerted via its cognate, the vitamin D receptor (*VDR*) [4]. Moreover, there was a correlation between *VDR Taq*I polymorphism and vitamin D3 level [5]. Thus, *VDR* gene has been suggested as one of the candidate genes for anemia.

After menopause, women are likely to have lower risk of iron deficiency anemia, as compared to childbearing women. However, postmenopausal women have reached an age when the incidence of

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chronic health conditions becomes more prevalent. In addition, there is a strong relation between anemia and chronic disorders including chronic kidney disease, inflammation, and osteoporosis. Thus, they stand at a crossroads between living the remainder of their lives in essentially good health or facing the likely onset of chronic diseases including anemia that might have been prevented. Although several studies focus on the role of *VDR* in anemia in hemodialysis and chronic kidney patients, there is overall paucity of studies investigating whether *VDR* polymorphism affects blood hemoglobin (Hb) levels in the postmenopausal women without kidney diseases.

The *VDR* gene, located on chromosome 12q12–q14, has at least five promoter regions [6, 7], eight protein-coding exons, and six untranslated exons, which are alternatively spliced [8]. Allelic variants of the gene encoding *VDR*, recognized by restriction endonucleases including *Fok*I in exon 2 (rs2228570), *Bsm*I (rs1544410) in intron 8, *Apa*I (rs7975232) and *Taq*I in exon 9 (rs731236), have been most often investigated [6, 9]. However, genetic profile of *VDR* and its association with anemia have been lacking from Vietnam to date.

In this study, we investigated the relationship between anemia and the commonly studied polymorphisms of *VDR* gene (*Fok*I, *Bsm*I, *Apa*I, and *Taq*I) in terms of genotype and haplotype in Vietnamese cohort.

Methods

Subjects

A case-control study including 132 (69 cases and 63 controls) postmenopausal women was designed to investigate the relationship between *VDR* polymorphism and anemia. The subjects were recruited from a district of a rural area in the Northern Vietnam, where malaria is unlikely to have been a cause of anemia in the target population as it has been largely eliminated. Most of them (97.7%) were farmers and manual workers and the others were office clerks. Women who had one or more of the following factors were excluded from the study: 1) acute infectious diseases or hookworm infection; 2) current cancer or hyperparathyroidism; 3) a kidney stone in the past 5 years and renal disease. This study was approved by the Ethics Committee of the National Institute of Nutrition, Vietnam and the Ethics Committee of Tokushima University, Japan. All participants provided written informed consent before entering the study.

Measurements

All participants completed a structured questionnaire. Data were collected on current age, age at menopause, ethnicity, educational level, occupation, medical and reproductive history, smoking, and drinking history. Educational level was categorized in three groups, by number of years of schooling: low level (≤5 years), medium level (6-8 years), and high level (≥9 years). Body weight and height were measured in light clothing and without shoes to the nearest 0.1 kg and 0.1 cm respectively. Body mass index (BMI) was calculated as weight per square of height (kg/m^2).

Serum concentration of hemoglobin (Hb) and ferritin (SF) were measured using the cyanomethemoglobin method (Sigma diagnostic kits) and a two-site enzyme-linked immunosorbent assay (Ramco, Huston, USA), respectively. Serum iron and albumin concentration were also determined in the Micronutrient Laboratory of National Institute of Nutrition, Vietnam. Anemia was defined as a hemoglobin concentration <120 g/L and iron deficiency as a serum ferritin <15 ng/mL, in accordance with recommendations of World Health Organization for non-pregnant women [10].

Genotyping

Peripheral blood samples were obtained from each woman and genomic DNA was extracted from peripheral blood leukocytes, using QIA amp DNA blood kit (Qiagen GmbH, Hilden, Germany). Vitamin D receptor genotypes were determined with a PCR-RFLP method as previously described [11], with some modification (**Table 1**). The *VDR* gene loci with C-T polymorphism in exon 2 detected by *Fok*I restriction enzyme, G-A, C-A polymorphisms in intron 8 detected by restriction enzymes *Bsm*I and *Apa*I, respectively and T to C in exon 9 detected by restriction enzyme *Taq*I (NEB). By convention, the small f, b, a and t denote the presence of *Fok*I, *Bsm*I, *Apa*I and *Taq*I restriction sites, respectively; and the capital F, B, A and T indicate the absence of *Fok*I, *Bsm*I, *Apa*I and *Taq*I restriction sites, respectively.

Statistical analysis

Genotype frequencies were compared and tested for Hardy-Weinberg Equilibrium (HWE) by Pearson's χ^2 test or Fisher's exact test when appropriate. A pairwise |D'| value (the absolute value for the disequilibrium parameter) that ranges from 0 (complete

Table 1. PCR protocols and primers for typing the polymorphisms of VDR gene.

Polymorphism	dbSNP[a]	Primers	Tm	Restriction enzyme	Allele size (bp)
*Fok*I	rs2228570	5'-agctggccctggcactgactctgctct-3'	60°C	*Fok*I	C(F): 265bp
	C/T	5'-atggaaacaccttgcttcttctccc-3'			T(f): 196bp+69bp
*Bsm*I	rs1544410	5'-caacaaagactacaagtaccgcgtcagtga-3'	54°C	*Bsm*I	A(B): 822 bp
	G/A	5'-aaccagcggaagaggtcaaggg-3'			G(b):646bp+176bp
*Apa*I	rs7975232C/A	5'-cagagcatggacagggagcaag-3'	62°C	*Apa*I	A(A): 746bp
		5'-gcaactcctcatggctgaggtctca-3'			C(a):532bp+214bp
*Taq*I	rs731236T/C	5'-cagagcatggacagggagcaag-3'	62°C	*Taq*I	T(T): 746bp
		5'-gcaactcctcatggctgaggtctca-3'			C(t): 497bp+249bp

[a]Accession number of each polymorphism to dbSNP at http://www.ncbi.nlm.nih.gov/.

linkage equilibrium status) to 1.0 (complete LD status) among SNPs was measured. Haplotype frequencies for multiple loci were estimated by the expectation maximization method and association of haplotype with anemia was assessed by use of the software program SNPstats [12].

Quantitative variables were checked for normal distribution and compared using Independent-Sample T test or Mann-Whitney U test. Distribution of allele and genotype of *VDR* in anemia and control groups was determined by Pearson's χ^2 test. Logistic regression analysis was used to evaluate association of *VDR* polymorphism with anemia in the models unadjusted or adjusted for covariates (age, BMI, educational level, serum ferritin, iron and albumin). Here, data are presented as odds ratio (OR) with 95 percent confidence intervals (95% CIs). A p-value under 0.05 was considered statistically significant. The above statistical procedures were performed using SPSS version 16.0 (SPSS, Chicago, USA).

Results

The characteristics of subjects in anemia and control groups are shown in **Table 2**. All subjects were women not smoking or drinking alcohol. We found no statistically significant differences between the two groups in age, years since menopause, weight, height, BMI, and serum concentration of transferrin, iron, and albumin ($p > 0.05$). There was a significant difference of serum ferritin between anemia and control groups ($p < 0.05$). However, no subjects suffered from iron deficiency (serum ferritin >15 ng/mL).

Table 2. Characteristics of subjects in anaemia and control groups.

Characteristics	Anaemia (N=69)	Control (N=63)	P-value
Age (year)[a]	55.8 ± 3.2	55.3 ± 4.4	0.434
Years since menopause[b]	7.0 (4.0-16)	7.0 (4.2-14)	0.939
Weight (kg)[a]	45.3 ± 6.6	45.8 ± 6.4	0.652
Height (cm)[a]	149.4 ± 5.5	149.1 ± 4.7	0.758
Body mass index (kg/m^2)[a]	20.2 ± 2.4	20.6 ±2.7	0.439
Serum albumin (g/dL)[a]	4.6 ± 0.36	4.6 ± 0.25	0.638
Serum iron (μg/dL)[a]	82.6 ± 28.6	91.1 ± 30.3	0.102
Serum ferritin (ng/mL)[b]	76 (32.5-240)	99 (42-286)	0.013
Serum transferrin (ng/mL)[b]	27 (20.2-46.4)	28 (21.5-44.9)	0.404

[a]Data shown as means ± SD and p-value by Independent-Samples T test. [b]Data shown as median (95% percentiles) and p-value by Mann-Whitney U test.

The allele and genotype frequency of each *VDR* SNPs is shown in **Table 3**. The most frequent genotypes of the *Fok*I, *Bsm*I *Apa*I, and *Taq*I polymorphisms were Ff, bb, Aa, and TT, respectively. The BB genotype of *Bsm*I and tt genotypes of *Taq*I polymorphisms were very rare in the studied cohort. Genotype distributions of four polymorphisms were in Hardy-Weinberg Equilibrium in both anemia and control groups (all p-value >0.37). From four polymorphisms of the *VDR* gene, the polymorphisms at the 3'end of the gene (*Bsm*I, *Apa*I and *Taq*I) exhibited a strong linkage disequilibrium with D' ranged from 0.83 to 0.99 (p <0.001). They formed three frequent haplotypes in control group: baT (67.8%), bAT (23.3%), Bat (7.1%), and in anaemia group: baT (64.5%), bAT (26.8%), Bat (4.4 %). The SNP rs2228570 was separated from the others with D' ranged from 0.074 to 0.202 in the anemia and control groups.

Table 3 shows that the allele and genotype frequencies of *VDR* SNPs were not different between anemia and control groups, indicating no association between individual *VDR* SNP and anemia. We did not find any significant association of *VDR* SNP with anemia when all SNPs were entered in the logistic regression models unadjusted or adjusted for age, BMI, educational level, serum ferritin, iron and albumin (**Table 4**). Moreover, the similar observation was found when we performed analysis of haplotype in the relation to anemia (**Table 5**). Taken together, our data did not support the influence of *VDR* polymorphism in anemia in the selected cohort.

Discussion

The hypothesis that *VDR* may be involved in pathogenesis of anemia derives from the observation that vitamin D deficiency had significant association with anemia and calcitriol, the active form of vitamin D, was involved in hematopoiesis [2, 3]. Several studies supported the link between *VDR* and anemia in hemodialysis and chronic kidney patients. For instance, the *VDR Bsm*I gene polymorphism might predict both Hb level and erythropoietin need in hemodialysis patients [13], and the BB variant of *Bsm*I was related to decrease recombinant human erythropoietin requirements to achieve higher hemoglobin levels in maintenance hemodialysis patients without chronic inflammation [14]. Our results do not support the hypothesis that *VDR* influences anaemia in postmenopausal women without chronic kidney disease. We have reported a lack of statistical significance for such an association in terms of allele,

Table 3. The distribution of allele and genotype of VDR gene in anaemia and control groups.

VDR		Anaemia Number (%)	Control Number (%)	P-value by χ^2 test
Alleles				
*Fok*I	F	77 (55.8)	66 (52.4)	0.578
	f	61 (44.2)	60 (47.6)	
*Bsm*I	B	12 (8.7)	11 (8.7)	0.992
	b	126 (91.3)	115 (91.3)	
*Apa*I	A	49 (35.5)	39 (31.0)	0.433
	a	89 (64.5)	87 (69.0)	
*Taq*I	T	132 (95.7)	117 (92.9)	0.327
	t	6 (4.3)	9 (7.1)	
Genotypes				
*Fok*I	FF	20 (29)	18 (28.6)	0.638
	Ff	37 (53.6)	30 (47.6)	
	ff	12 (17.4)	15 (23.8)	
*Bsm*I	BB,Bb	12 (17.4)	10 (15.9)	0.815
	bb	57 (82.6)	53 (84.1)	
*Apa*I	AA	7 (10.1)	5 (7.9)	0.706
	Aa	35 (50.7)	29 (46.0)	
	aa	27 (39.1)	29 (46.0)	
*Taq*I	TT	63 (91.3)	54 (85.7)	0.312
	Tt	6 (8.7)	9 (14.3)	

Table 4. Logistic regression analysis of VDR polymorphisms with anaemia.

Polymorphism	Genotype	Unadjusted model OR (95%CI)	P-value	Adjusted model[a] OR (95%CI)	P-value
*Fok*I	ff	1.0	-	1.0	-
	Ff	1.43 (0.57-3.59)	0.441	1.56 (0.59-4.08)	0.370
	FF	1.51 (0.55-4.13)	0.423	1.72 (0.59-4.99)	0.322
*Bsm*1	BB+Bb	1.0	-	1.0	-
	bb	0.21 (0.02-1.89)	0.164	0.19 (0.02-1.83)	0.151
*Apa*I	AA	1.0	-	1.0	-
	Aa	0.80 (0.22-2.9)	0.734	0.83 (0.21-3.27)	0.789
	aa	0.62 (0.16-2.35)	0.478	0.63 (0.16-2.53)	0.519
*Taq*I	Tt	1.0	-	1.0	-
	TT	9.15 (0.84-99.2)	0.069	8.46 (0.71-101)	0.092

[a]Model adjusted for age, BMI, educational level, serum ferritin, iron, and albumin.

Table 5. Analysis of association of VDR haplotypes with anaemia (N=132).

Haplotype[a]	Frequency	Unadjusted model OR (95%CI)	P-value	Adjusted model[b] OR (95%CI)	P-value
FbaT	0.3652	1.00	-	1.00	-
fbaT	0.2958	0.91 (0.45 - 1.83)	0.79	0.77 (0.36 - 1.63)	0.5
FbAT	0.1293	1.26 (0.46 - 3.40)	0.65	1.03 (0.37 - 2.90)	0.95
fbAT	0.1225	1.19 (0.47 - 2.97)	0.72	1.24 (0.46 - 3.35)	0.67
FBAt	0.0415	1.48 (0.35 - 6.22)	0.59	1.86 (0.39 - 8.96)	0.44
fBAT	0.0247	11.2 (0.13 - 940)	0.29	11.4 (0.10 - 1375)	0.32
Global test	-	-	0.29	-	0.29

[a]Haplotype consists of SNPs: *Fok*I in exon 2 (rs2228570), *Bsm*I (rs1544410) in intron 8, *Apa*I (rs7975232) and *Taq*I in exon 9 (rs731236). Haplotype frequency <0.02 is not included in the table. [b]Model adjusted for age, BMI, educational level, serum ferritin, iron, and albumin.

genotype and haplotype in the analyses unadjusted or adjusted for the covariates (age, BMI, educational level, serum ferritin, iron and albumin). *VDR* gene was associated with anemia in hemodialysis and chronic kidney patients, but not in postmenopausal women without these diseases, suggesting the possibility of interaction between *VDR* gene and erythropoietin regulating genes in the pathogenesis of anemia. Further study needs to investigate the interaction.

In this paper, we are the first to analyze genotype and haplotype of the common SNPs of *VDR* in postmenopausal Vietnamese women. The findings show that the three SNPs *Bsm*I, *Apa*I, and *Taq*I are located in a strong LD block, consistent with those in Caucasian and Asiatic populations. Furthermore, according to the International HapMap Project (http://www.hapmap.org/), this LD block also appears to be present in various populations. This LD block formed two most frequent haplotypes baT and bAT in our cohort, which jointly represented more than 91% of all haplotypes. It is in line with the observation of other studies in Asians that the haplotypes baT and bAT are the most frequent, while haplotypes baT, Bat, and bAT are abundant in Caucasians [15]. The *Fok*I SNP is located separately from this LD block. The previous study have indicated that *Fok*I SNP locating in the 1.3 kb LD-breaking spot between the two adjacent LD blocks and this SNP is the only SNP out of the 68 that has no detectable LD with any other SNP and cannot be assigned to any block [16].

Because the activated *VDR* plays a fundamental role in the body by regulating numerous primary target genes [4, 6], *VDR* gene has been suggested as one of the candidate genes for many disorders and diseases. For instance, in terms of relationship between *VDR* and cancer risk, the recent meta-analysis [17] in

Caucasian populations have shown that a significant increase in skin cancer [OR; 95% confidence intervals (CIs): 1.30; 1.04-1.61] and breast cancer (OR = 1.14; 95% CI: 1.03-1.27) risk were found when comparing *Fok*I ff with FF carriers. A significant reduction in prostate cancer risk was observed for carriers of *Bsm*I Bb compared with bb genotype (OR = 0.83; 95%CI: 0.69-0.99). Both Bb and BB carriers had a significant reduced risk of cancer at any site. It is necessary to conduct studies to replicate such associations in Vietnamese populations.

The inconsistent association between *VDR* polymorphisms and diseases of interest is often observed. For example, *Apa*I, *Bsm*I, and *Fok*I polymorphisms in the *VDR* gene were associated with susceptibility to Graves' disease in Asian populations, but not in Caucasian populations [18]. Thus, many factors should be taken into account when an association study is conducted, such as population differences in allele frequencies, or variation in intake and/or conversion of vitamin D. Additionally, *Bsm*I, *Apa*I, or *Taq*I are 3' non-functional polymorphisms that may only be associated with risk of disease in some populations through linkage disequilibrium with another functional variant. The *Fok*I polymorphism is a more compelling candidate for association studies given the data demonstrating that the CC genotype results in a more active form of *VDR* [4, 15].

In conclusion, our study shows characteristics of four common SNPs of *VDR* gene in terms of genotype and haplotype, and suggests no significant association between the individual *VDR* gene and anemia in the postmenopausal women without chronic kidney disease. For further study on the association between *VDR* and anemia, use of larger sample size, prospective study design, and additional markers would enhance the findings.

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