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Association Between Vitamin-D Receptor Gene FokI Polymorphism and Graves' Disease Among Taiwanese Chinese

Rong-Hsing Chen,[1] Chwen-Tzuei Chang,[1] Huey-Yi Chen,[2,3] Wen-Chi Chen,[2,4] Chang-Hai Tsai,[5,6] and Fuu-Jen Tsai[2,5]*

[1]*Department of Medicine, China Medical University Hospital, China Medical University, Taichung, Taiwan*
[2]*Department of Medical Genetics, China Medical University Hospital, China Medical University, Taichung, Taiwan*
[3]*Department of Gynecology & Obstetrics, China Medical University Hospital, China Medical University, Taichung, Taiwan*
[4]*Department of Urology, China Medical University Hospital, China Medical University, Taichung, Taiwan*
[5]*Department of Pediatrics, China Medical University Hospital, China Medical University, Taichung, Taiwan*
[6]*Department of Bioinformatics, Asia University, Taichung, Taiwan*

$1,25(OH)_2D_3$, exerting its biological effects through the vitamin-D receptor (VDR), plays a role in the modulation of the human immune system. The aim of this study was to test for the presence of an association between *VDR* gene polymorphism and the susceptibility to Graves' disease (GD) for Taiwanese Chinese. Using a polymerase chain reaction (PCR)-based restriction analysis, we screened the *VDR* exon 2 start codon T/C (*VDR-Fok*I) polymorphism to determine the genotypes for 88 GD patients and 90 normal controls. From the genotype analysis, GD patients featured a greater proportion of the CC genotype (44.3%) and a smaller proportion of the TT genotype (12.5%) than was the case for normal controls (CC: 23.3% and TT: 28.9%; chi-squared test, $P = 0.003$). The odds ratios (ORs) for the risk of the CC genotype's appearance compared with the corresponding values for the TT and TC genotypes, for the GD patient group, were, 4.39 (95% confidence interval [CI]: 1.82–10.61) and 2.10 (95% CI: 1.06–4.18), respectively. With respect to the allelic analysis, we observed significantly increased C-allele (65.9%) and decreased T-allele (34.1%) frequencies among GD patients compared to normal controls (C: 47.2% and T: 52.8%; chi-squared test, $P = 0.002$). The OR for the risk of appearance of the C allele in the GD-patient group was 1.93 (95% CI: 1.27–2.95). In conclusion, the *VDR-Fok*I T/C polymorphism might be able to be used as a genetic marker to predict the likelihood of GD development. J. Clin. Lab. Anal. 21:173–177, 2007.

Key words: Graves' disease; vitamin-D receptor; FokI; polymorphism

INTRODUCTION

Graves' disease (GD) is a common autoimmune thyroid disease and, reportedly, is the major cause of hyperthyroidism. Clinically, GD is characterized by diffuse goiter, thyrotoxicosis, and infiltrative ophthalmopathy. The pathogenesis of GD involves action upon thyroid gland by thyrotropin-receptor antibodies (TRAbs) against thyrotropin (or thyroid-stimulating hormone [TSH]) receptors (1). These antibodies feature not only TSH-displacing activity but also thyroid-stimulating activity, such that subsequent to binding to TSH receptors, TRAbs activate adenylate cyclase, thereby initiating a chain of reactions that leads to abnormal thyroid growth and hypersecretion of thyroid hormones (2).

TRAbs are a group of IgG_1 antibodies produced via a concatenation of immunological processes (3). In

*Correspondence to: Fuu-Jen Tsai, M.D., Ph.D., 2, Yuh-Der Road, Taichung, 404, Taiwan. E-mail: d0704@www.cmuh.org.tw

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general, stimulation of human $CD4^+$ T helper (Th) lymphocytes by specific antigens results in the development of both Th1 and Th2 patterns of cytokine production (4,5). Th1 cells produce interferon (INF)-γ, IL-2, and tumor necrosis factor (TNF)-α, and promote cellular immune activity, whereas Th2 cells produce IL-4, IL-5, and IL-10, and provide optimal help to B cells for IgG_1 secretion (4,5). The Th1 cytokines can directly suppress the proliferation of Th2 cells and thus indirectly inhibit IgG_1 formation by B cells (6).

$1,25(OH)_2D_3$, a most-potent natural vitamin-D metabolite, plays a role not only in the regulation of calcium homeostasis but also in the overall modulation of the immune system (7). This secosteroid, exerting its biological effects through the interaction with the vitamin-D receptor (VDR), inhibits the proliferation of activated T cells, reduces the production of IL-2, INF-γ and TNF-α, and preferentially inhibits Th1 functions (8–10). It would therefore be expected that alteration to $1,25(OH)_2D_3$ activity could interfere with its biological effect upon the immune system and thus influence the development of certain autoimmune disease. In practice, $1,25(OH)_2D_3$ has been demonstrated to prevent autoimmune thyroiditis in animal models and to ameliorate hyperthyroidism for humans (11,12); however, it has been previously reported that the serum TRAb levels of GD patients are not influenced by the administration of $1,25(OH)_2D_3$ (12).

The human *VDR* gene has been reported to have many different polymorphisms at various different sites (13). Among these polymorphisms, the exon 2 start codon T/C (or f/F) variation, as detected by the restriction enzyme *Fok*I, has been demonstrated to feature an association with GD susceptibility for Japanese, German, and Polish populations (14,15), although a related study involving individuals from the UK (Caucasian) has revealed no such result (16). In order to test whether this gene could constitute a susceptibility gene for GD for Chinese individuals, we screened the *VDR* exon 2 start codon T (A<u>T</u>G)/C (A<u>C</u>G) (*VDR-Fok*I) polymorphism, using polymerase chain reaction (PCR)-based restriction analysis, to compare the genotype for GD patients and normal controls deriving from a selected Taiwanese study population.

PATIENTS AND METHODS

Patient Selection

A total of 88 unrelated GD patients (70 women) aged between 17 and 71 years (average: 35.5 ± 10.6 years) were enrolled into this study, which ran from January 2003 to June 2003 inclusively. All study-participating patients were of the Han race and resided in central Taiwan. None of the female patients were pregnant at the time of the study, and none had delivered within the entire 1-year period prior to study enrolment. The presence of hyperthyroidism, diffuse goiter, and a positive serum TRAb titer (>10%), supported by infiltrative ophthalmopathy and a positive serum antimicrosomal Ab (AMiA) and/or antithyroglobulin Ab (ATA) titer, were used to define GD. Thyroid ultrasonographic examination revealed a general diffuse hypoechogenic pattern and no evidence of nodular lesions for all study patients. The control group consisted of 90 (80 women) ethnically and residentially matched healthy volunteers over the age of 40 years who featured neither goiter nor any evidence of thyroid dysfunction. None of these individuals revealed the presence of any antithyroid Abs in their sera. Further, these individuals also exhibited no previous personal or family history of thyroid disease or of any autoimmune disease. This study was approved by the institutional ethics committee, and informed consent was requested of, and obtained from, each study subject prior to their enrolment in this study.

PCR

Experimental genomic DNA was prepared from peripheral blood using a genomic DNA-isolation reagent kit (Blossom, Taipei, Taiwan). Conventional PCR analysis for the *VDR-Fok*I polymorphism was carried out in a total volume of 25 μL, containing genomic DNA (2–6 pmol of each primer); 1 × Taq-polymerase buffer (1.5 mM $MgCl_2$); and 0.25 units of AmpliTaq DNA polymerase (PerkinElmer, Foster City, CA). The forward and backward primers used were, 5′-AGCTGGCCCTGGCACTGACTCTGCTCT-3′ and 5′-ATGGAAACACCTTGCTTCTTCTCCCTC-3′, respectively, according to the 1997 report by Harris et al. (17). PCR amplification was performed in a programmable thermal cycler GeneAmp PCR System 2400 (PerkinElmer). The cycling conditions were set as follows: one cycle at 94°C for 5 min, 35 cycles at 94°C for 30 sec, 58°C for 30 sec, and 72°C for 20 sec, and one final cycle of extension at 72°C for 7 min.

The PCR product of the 265-bp band was mixed with two units of *Fok*I (Novel, Beverly, MA) and the reaction buffer according to the manufacturer's instructions. The restriction site was designed to be located at the recognizable allele of A<u>T</u>G (allele f) to form an appropriate excision site. Two fragments, one 169-bp and the other 96-bp in size, will be present if the product is excisable (17). The reaction was incubated at 37°C overnight, following which, 10 μL of the product was loaded into a 3% agarose gel containing ethidium bromide and electrophoresed under standard condi-

tions. This polymorphism was thus divided into three specific genotypes: an excisable homozygote (TT), an unexcisable homozygote (CC), and a heterozygote.

The laboratory work for all samples from both patients and controls was performed uniformly by a single, well-trained professional technician contemporaneously, and the work was subsequently repeated in a similar fashion by another equally-well-trained technician at a different time. All gels were inspected by investigators who were blinded to the clinical phenotypes of the individuals being studied.

Statistical Analysis

The genotype and allelic frequencies of the polymorphism for the GD and control groups were statistically compared using the chi-squared test performed with the Statistical Package for Social Sciences (SPSS), version 8.01, software (SPSS for Windows; SPSS Inc.; Chicago, IL) following the examination of allelic frequencies incorporating the application of Hardy-Weinberg equilibrium (HWE) test for genotype frequencies. Results were considered to have achieved statistical significance when the probability of a finding of intergroup difference occurring simply by chance was less than 5% ($P<0.05$). Odds ratios (ORs) with 95% confidence intervals (CIs) were calculated for disease susceptibility associated with specific genotypes and alleles.

RESULTS

From the initial examination for genotype frequencies, the *VDR-Fok*I polymorphism was found to be in HWE for both control and patient groups. For genotype analysis, GD patients featured a greater proportion of the CC homozygous genotype (44.3%) and a lower proportion of the TT homozygous genotype (12.5%) than was the case for normal controls (CC: 23.3% and TT: 28.9%; $P=0.003$, power = 0.87; Table 1). The OR for the risk of the appearance of the CC genotype, compared with the CT and TT genotypes, for the GD patient group was 2.10 (95% CI: 1.06–4.18) and 4.39 (95% CI: 1.82–10.61), respectively.

With respect to allelic analysis, we noted significantly increased C-allele (65.9%) and decreased T-allele (34.1%) frequencies for GD patients as compared to corresponding values for normal controls (C: 47.2% and T: 52.8%; $P=0.002$; Table 1). The OR for the risk of the C allele for individuals from the GD patient group was 1.93, with a 95% CI of 1.27 to 2.95.

Further subgroup analysis was performed, and no significant association between the anti-thyroid Ab levels and genotypes for GD patients was detected (Table 2).

TABLE 1. Genotype and allelic variant frequencies of the *VDR* exon-2 start codon when comparing healthy control subjects and Graves' disease (GD) patients

	Graves' disease patients n = 88 (%)	Normal controls n = 90 (%)	*P*
Genotype			0.003[a]
CC (FF)[b]	39 (44.3)	21 (23.3)	
CT (Ff)	38 (43.2)	43 (47.8)	
TT (ff)	11 (12.5)	26 (28.9)	
Allelic variant			0.002
C (F)[c]	116 (65.9)	85 (47.2)	
T (f)	60 (34.1)	95 (52.8)	

[a]Sample power = 0.87.
[b]OR for CC (compared respectively with CT and TT): 2.10 (95% CI: 1.06-4.18) and 4.39 (95% CI: 1.82-10.61) for GD patients.
[c]OR for C: 1.93 (95% CI: 1.27-2.95) for GD patients.

TABLE 2. Relationship between anti-thyroid Ab levels and *VDR* exon-2 start codon genotypes for GD patients

		Genotype[a]			
Anti-thyroid Ab level	Case number	CC	CT	TT	*P*
TRAb					0.838
>30%	68	28 (41.2)	32 (47.1)	8 (11.7)	
<30%	20	9 (45.0)	8 (40.0)	3 (15.0)	
AMiA					0.913
(+)	72	29 (40.3)	32 (44.4)	11 (15.3)	
(−)	16	6 (37.5)	8 (50.0)	2 (12.5)	
ATA					0.936
(+)	31	13 (41.9)	11 (35.5)	7 (22.6)	
(−)	57	25 (43.9)	21 (36.8)	11 (19.3)	

[a]Data are presented as n (%).

DISCUSSION

The etiology of GD would appear to be complex and involving multiple genetic and environmental influences. The most well-known genes contributing to the susceptibility of GD are the human leukocyte antigen (HLA) class-II genes and the cytotoxic T-lymphocyte antigen-4 (*CTLA-4*) (18,19). In addition, certain other genes would also appear to contribute to the inheritance of GD (20). Herein, we report a novel finding that demonstrates an association between the *VDR* polymorphism and an individual's susceptibility to GD. Our data reveal increased frequencies of appearance of the CC homozygous genotype and the C allelic variant of the *VDR-Fok*I for GD patients as compared to normal controls. Therefore, we suggest that the *VDR* could be able to be used as an appropriate candidate "susceptibility gene" to predict the development of GD.

VDR is a member of the nuclear receptor superfamily and is capable of modulating the transcription of target genes in response to $1,25(OH)_2D_3$ (21). The presence of the *VDR-Fok*I polymorphism may, putatively, result in the appearance of an alternative to the normal translation initiation site, leading to the production of a VDR variant featuring different length and biological activity to the normal site (22). In actuality, a shorter-length VDR variant, which is encoded by the *VDR-Fok*I CC (FF) variant, has been demonstrated to be transcriptionally more active and to feature a greater avidity to interact with a transcription factor, TFIIB, than is the case for a longer one, ff variant (23,24). Because such alteration in *VDR* expression may influence the relative potency of $1,25(OH)_2D_3$ upon the process of immunomodulation, it would be expected that the *VDR-Fok*I polymorphism could thus interfere with the development of GD. In this study, we attempted to evaluate the relationship between anti-thyroid Ab levels and the genotypes in this polymorphic site for GD patients, but failed to observe any association. Further study is necessary to elucidate the effect of *VDR-Fok*I polymorphism on immunological reactions.

In what would appear to be the earliest published case–control study relating to the association of the *VDR-Fok*I polymorphism and GD susceptibility, Ban et al. (14) observed the overexpression of the FF homozygote (48% vs. 33%; $P = 0.0118$) and the F allele (69% vs. 61%; $P = 0.0472$) for 131 female, Japanese GD patients when compared with 150 controls (14), the preliminary results of our study for both genotype ($P = 0.003$) and allelic ($P = 0.002$) analyses being quite compatible with these author's observations. Further analysis, however, reveals that the distribution patterns of genotype and allelic frequencies are similar for GD-patient groups when comparing the data from Ban et al. (14) with those deriving from our work, but are significantly different for control groups. With regard to various studies relating to the same polymorphism for European population, by contrast, the resultant data would appear to be somewhat conflicting, with either positive or negative association between the *VDR-Fok*I polymorphism and GD susceptibility among a German population and Polish and UK populations, respectively (15,16). The distribution patterns of genotype and allelic frequencies presented in these last-mentioned studies were also inconsistent for control groups of individuals from different ethnic origins, such a result, no doubt, reflecting the different genetic pools represented in different racial populations. To the best of our knowledge, the reasons for such an apparent discrepancy in the results generated from studies for various different ethnic and geographically located populations are unknown, but may be related to the interpopulation heterogeneity of genetic and environmental determinants of certain disease. Further, however, the possibility of the influence of certain other factors, such as population stratification, variation in study design, confounding sampling bias, misclassification of phenotypes and, almost certainly, statistical artifacts contributing to such discrepancy in results should not be excluded (25–27).

Most patients suffering from GD are diagnosed subsequent to their developing GD-related symptoms, due to an apparent lack of predictive markers for this disease. Therefore, the existence of a reliable marker for GD could lead to earlier diagnosis and treatment for this malady, and could exert a significant impact upon improved patient care and overall patient outcome. Our data indicate that the *VDR* exon 2 start codon T/C polymorphism is a candidate genetic marker to screen for an individual's susceptibility to GD. Individuals featuring the CC genotype at this site might be expected to experience quite a substantial risk of developing GD, although because the overall effect of $1,25(OH)_2D_3$–VDR interaction upon the development of GD would still appear to be unclear, further investigation in this realm is clearly necessary in order to elucidate the relative contribution of the *VDR-Fok*I polymorphism to the pathogenesis of GD.

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