Original Article

The influence of BsmI and TaqI vitamin D receptor gene polymorphisms on the intensity of hyperparathyroidism in Iranian hemodialysis patients

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Abstract

Background: The influence of vitamin D receptor (VDR) gene polymorphisms on the regulation of the parathyroid hormone is important in end-stage renal disease (ESRD) patients. We analyzed rs1544410 (BsmI) and rs731236 (TaqI) polymorphisms of VDR gene in hemodialysis patients to determine their relationship with serum intact parathyroid hormone (iPTH).

Materials and Methods: Ninety hemodialysis patients were included in this study. Patients were classified into four groups according to their serum iPTH level. Polymorphisms of VDR gene were surveyed using polymerase chain reaction-restriction fragment length polymorphism method with BsmI and TaqI enzymes in all the patients.

Results: Patients age ranged between 30 and 60 years (mean ± SD: 36.0 ± 11.4) and period undergoing hemodialysis 80 ± 71 months. Patients were divided into four groups based on the serum concentration of iPTH. The distribution of VDR gene allelic variation for BsmI and TaqI polymorphisms was different between the four groups of uremic patients. Analysis of data revealed a significant correlation between the TaqI variants and serum iPTH level. There was also a correlation between the BsmI variants and serum iPTH level in that patients with the BB genotype were more likely to have a higher serum iPTH level. However, the latter was not statistically significant.

Conclusions: Genotype of the TaqI and BsmI VDR gene polymorphisms is reported in Iranian patients with ESRD. Those with tt or BB genotypes may develop more severe secondary hyperparathyroidism.

Key Words: End-stage renal disease, hyperparathyroidism, polymorphism, vitamin D receptor

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INTRODUCTION

Most patients with chronic renal failure have secondary hyperparathyroidism (sHPT).[1] Although, with recent advances in the management of renal osteodystrophy, the number of patients with end-stage renal failure who develop severe secondary hyperparathyroidism is decreasing, some patients still manifest severe secondary hyperparathyroidism.[2] Stimulation of parathyroid function is caused by insufficient

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production of calcitriol by the kidney, calcium deficiency, and phosphate excess.[1] The incidence of secondary hyperparathyroidism among hemodialysed patients varies despite similar therapeutic management. This may be caused by genetic heterogeneity.[2] It has been suggested that vitamin D receptor (VDR) gene can influence the secretion of PTH. The effect of VDR polymorphisms in patients with chronic renal failure has been studied due to the critical role of vitamin D in these patients.[3] It has been suggested that genetic predispositions influence the degree of parathyroid hyperplasia in uremic patients. Allelic polymorphisms of the VDR gene in intron 8 (B/b alleles) and exon 9 (T/t alleles) have been associated with parathyroid gland function in the general population. Several studies have been conducted, looking for a possible relationship between VDR polymorphisms and parathyroid function in patients with primary and secondary hyperparathyroidism.[1] The calcitriol-VDR complex regulates parathyroid cell proliferation and parathyroid hormone (PTH) synthesis.[4,5] As a result, the interaction of calcitriol with its nuclear receptor inhibits PTH synthesis and parathyroid gland cell proliferation.[3] Vitamin D and its receptor also play a role in determining the set-point of calcium-regulated PTH secretion.[6] Therefore, mutations that inactivate the function of the VDR could lead to increased proliferation of the parathyroid cells.[7] and VDR genotypes may have influence on the degree of secondary parathyroid hyperplasia.[1] Hence, to assess the potential role of the VDR in intensity of secondary hyperparathyroidism, we evaluated 90 Iranian hemodialysis patients for the presence of inactivating mutations in the VDR gene.

MATERIALS AND METHODS

Patients

In this cross-sectional study 90 Iranian hemodialysis patients (56 men and 34 women; age between 30 and 60 years, mean ± SD: 36.0 ± 11.4) were selected from two dialysis units in Isfahan city. In this population, 43 patients had non-insulin-dependent diabetes mellitus and 17 patients had received oral calcitriol pulse therapy. Patients were undergoing hemodialysis for periods ranging from 2 to 280 months (80 ± 71). Patients received calcium carbonate orally, to maintain the serum phosphorus concentration at less than 6 mg/dL, and calcitriol or alfa-calcidol unless their serum calcium levels exceeded 11 mg/dL. Patients with liver disease were excluded from the study based on their serum Gamma Glutamy transferase (GGT) activity. None of the female patients were pregnant.

Patient blood sampling

Ten milliliters of venous blood was taken from each patient fasting overnight: 2 mL blood was collected into ethylenediaminetetraacetic acid (EDTA) evacuated tubes for DNA extraction and 8 mL blood was collected into a plain tube and sera were separated immediately for the analysis of intact parathyroid hormone (iPTH), 25(OH) Vit D, calcium, phosphate, total alkaline phosphatase, and albumin. All samples were stored at –20°C in aliquots and were analyzed within 2 months of collection.

Assay methods

Serum concentration of iPTH was measured using radioimmunoassay kit (intact PTH; Allegro; Japan Mediphysics Co, Tokyo, Japan). Serum 25 (OH) Vit D was assayed using a commercial competitive protein-binding assay employing an automated chemiluminescence method. Based on serum concentration of iPTH, the patients were divided into four groups representing relatively low (iPTH < 150 pg/mL) (group I), target (150 ≥ iPTH < 300 pg/mL) (group II), mild to moderate (300 ≥ iPTH < 600 pg/mL) (group III), and moderate to severe (iPTH ≥ 600 pg/mL) (group IV) elevations in PTH.[8]

DNA analysis

DNA was extracted from 0.1 mL of whole blood using a commercial kit (CinnaPure DNA kit, Cinnagen, Iran) according to the manufacturer's recommendations. Extracted DNA was stored at –20°C for further analysis.

VDR genotyping

Extracted DNA underwent polymerase chain reaction (PCR) for DNA amplifying before determination of VDR gene polymorphisms. Then, restriction fragment length polymorphism (RFLP) for determination of VDR gene polymorphisms within intron 8 and exon 9 was performed using BsmI and TaqI restriction enzymes, respectively. All PCR primers were designed based on sequences published previously by Afshari *et al*.[9] These primers and their product sizes are summarized in table 1 and the PCR protocols are described in table 2.

Restriction endonucleases for RFLP

BsmI (rs1544410) and TaqI (rs731236) enzymes were used in the Fermentas FastDigest kits (Thermo Fisher Scientific, Pittsburgh, PA, USA). The RFLP conditions and product sizes of RFLP are given in

Table 1: The primer sequences used for VDR gene polymorphism analyses

Primer name	Sequence (5'-3')	Length (base pair)	Amplified fragment (base pair)
Bsml forward	AACTTGCATGAGGAGGAGCATGTC	24	801
Bsml reverse	GGAGAGGAGCCTGTGTCCCATTTG	24	
TaqI forward	GGGACGCTGAGGGATGGACAGAGC	24	716
TaqI reverse	GGAAAGGGGTTAGGTTGGACAGGA	24	

VDR: Vitamin D receptor

table 3. Electrophoresis on the 3% agarose gel was performed in Tris-borate EDTA (0.5X TBE) buffer with a molecular weight marker of 50 base pairs (bp) (GeneRuler, Fermentas, Thermo Fisher Scientific) for separation of the digestion products. After electrophoresis digested products were stained using ethidium bromide [Figures 1 and 2]. The sites of bands represent the type of genotype, which the person had.

Statistical analysis

SPSS Software Version 19.0 (SPSS Inc.) and statistical package STATA 6.0 (Stata Corporation, College Station, TX, USA, 2001) were used for statistical analysis. Frequency and percentage of each of the genotype and allele were recorded. Hardy-Weinberg equilibriums were tested for comparing the observed and expected genotype and allele frequencies. For investigating the relationship between each allele with biochemical factors, statistical *t* test was used.

The significance of genotype frequency differences between any of the four groups was determined by the Chi-squared test. A probability value less than 0.05 was considered to be significant. For the comparison of serum levels of vit D, Alb, P, Ca in the four studied groups, we used one-way analysis of variance and for comparison of alkaline phosphatase (ALP) and iPTH we used Kruskal-Wallis test. Results are expressed as mean ± standard deviation (SD).

Table 2: PCR protocols used for VDR polymorphism analyses

PCR protocol	Polymorphism
Pre-denaturation: 94°C for 5 min	BsmI
30 cycles at:	(rs1544410, A>G)
Denaturation: 94°C for 30 s	
Annealing: 53.7°C for 30 s	
Extension: 72°C for 1 min	
Final extension: 72°C for 5 min	
Pre-denaturation: 94°C for 5 min	TaqI
30 cycles at:	(rs731236, C>T)
Denaturation: 94°C for 30 s	
Annealing: 60°C for 30 s	
Extension: 72°C for 1 min	
Final extension: 72°C for 5 min	

VDR: Vitamin D receptor, PCR: Polymerase chain reaction

Table 3: Incubation conditions and the expected product sizes for VDR polymorphisms in RFLP

Time of incubation (min)	Product size (bp)	Temperature (°C)	Enzyme
60	BB (801bp), Bb (801,480,321), Bb (480,321)	37	BsmI
5	TT (202, 514), Tt (514,237,169), tt (237, 169)	65	TaqI

RFLP: Restriction fragment length polymorphism, VDR: Vitamin D receptor

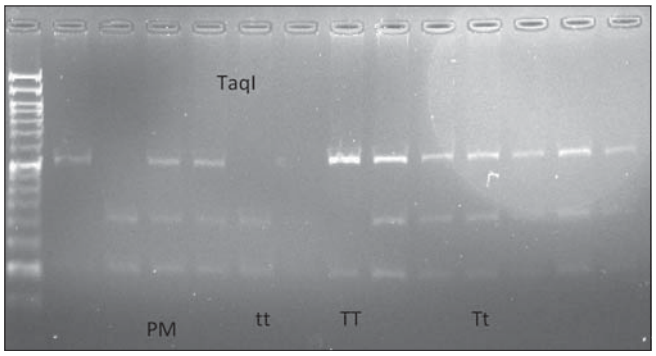

Figure 1: Polymerase chain reaction-restriction fragment length polymorphism for SNP rs1544410 with the restriction enzyme BsmI. Gel electrophoresis of agarose in 3% stained with ethidium bromide. PM, molecular weight marker of 50 bp

RESULTS

In this study we examined the alleles and genotype frequencies of VDR at positions TaqI and BsmI in hemodialysis patients. All frequencies, in four groups of patients were in Hardy-Weinberg equilibrium ($P < 0.001$). The distribution of the allelic variation for BsmI and TaqI polymorphisms was significantly different between the four groups of uremic patients ($P = 0.03, 0.04$) [Table 4]. According to data presented in table 4, the frequency of BB genotype is higher in groups III (300 ≥ iPTH < 600 pg/mL) and IV (iPTH ≥ 600 pg/mL) and that of bb genotype is higher in group I (iPTH < 150 pg/mL) and group IV (iPTH ≥ 600 pg/mL) of patients. The most frequent tt genotype for TaqI is in the group IV of patients (iPTH ≥ 600 pg/mL) [Table 4]. Comparison of the serum levels of 25(OH) vit D, P, Ca, and Alb between the four groups of patients revealed no significant difference by one-way analysis of variance ($P \geq 0.05$). But there was statistically significant difference for iPTH and ALP between the four groups by Kruskal-Wallis test ($P < 0.0001$) and ALP level paralleled that

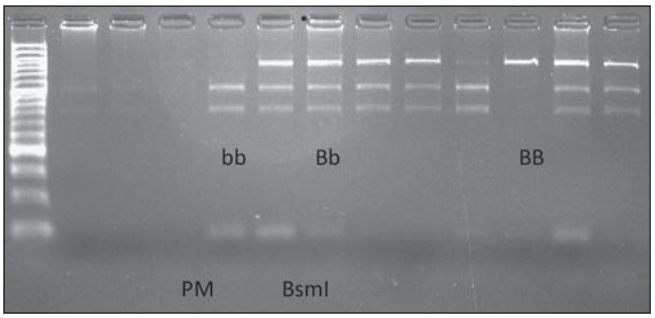

Figure 2: Polymerase chain reaction-restriction fragment length polymorphism for SNP rs731236 with the restriction enzyme TaqI. Gel electrophoresis of agarose in 3% stained with ethidium bromide. PM, molecular weight marker of 50 bp

of iPTH [Table 5]. Also, the calcium level seen in the BB genotype is less than those for Bb and bb genotypes ($P = 0.002$) [Table 6]. In comparison, the calcium levels in the tt genotype were significantly lower than those seen in Tt and TT groups ($P = 0.007$) [Table 7]. There was no statistically significant association between B and b alleles with biochemical factors ($P > 0.05$). However, for T and t alleles there was a significant correlation with serum calcium ($P < 0.05$). As shown in table 4, the frequency of B allele, in group III (300 ≥ iPTH < 600 pg/mL) and b allele in group I (iPTH ≤ 150 pg/mL) were more frequent in comparison with the other groups. For TaqI, T and t alleles in the first group (iPTH ≤ 150 pg/mL) and fourth group (iPTH ≥ 600 pg/mL), respectively, are higher.

The frequency of B allele, in group III (300 ≥ iPTH < 600 pg/mL), was more frequent (96%), and b allele in group I (iPTH ≤ 150 pg/mL) was more frequent (35%) in comparison with the other groups. For TaqI, T and t alleles in the first group (iPTH ≤ 150 pg/mL) and fourth group (iPTH ≥ 600 pg/mL), respectively, are higher.

Table 4: Frequencies of VDR polymorphisms genotype in the groups of the study

Polymorphism	Group (%)					Sig
	I	II	III	IV	Total	
BsmI						
BB	2 (2.2)	5 (5.6)	7 (7.8)	6 (6.7)	20 (22.2)	0.03
Bb	11 (12.2)	15 (16.7)	17 (18.9)	54 (60)	54 (60.0)	
bb	7 (7.8)	1 (1.1)	1 (1.1)	7 (7.8)	16 (17.8)	
B	13 (65)	20 (95.2)	24 (96)	17 (70.8)	74 (82.2)	0.04
b	7 (35)	1 (4.8)	1 (4)	7 (29.2)	16 (17.8)	
TaqI						
TT	14 (15.6)	6 (6.7)	7 (7.8)	9 (10.0)	36 (40.0)	
Tt	6 (6.7)	13 (14.4)	14 (15.6)	10 (11.1)	43 (47.8)	
tt	0 (0)	2 (2.2)	4 (4.4)	5 (5.6)	11 (12.2)	
T	20 (100)	19 (90.5)	21 (84)	19 (79.2)	79 (87.8)	
t	0 (0.0)	2 (9.5)	4 (16)	5 (20.8)	11 (12.2)	

VDR: Vitamin D receptor

DISCUSSION

Vitamin D deficiency has been shown to play a role in several clinical conditions. Because vitamin D exerts its effects through the VDR, nucleotide changes in the VDR gene may affect transcript levels, transcript stability, or the functional integrity of the VDR protein in such a way that downstream vitamin D pathways are adversely affected. Most of these nucleotide changes in the VDR gene occur as SNPs, which have been associated with diseases such as osteoporosis, cancer, diabetes, and so on. Genetic predisposition of certain ethnic patients to severe hyperparathyroidism has been linked to polymorphisms in VDR gene. This in turn may be dependent on VDR genotypes an individual possesses, which may influence VDR binding with vitamin D.[10] The influence of VDR gene polymorphisms on the regulation of the parathyroid hormone is important in end-stage renal disease (ESRD) patients. Since the discovery of the VDR effect on parathyroid cells, a large number of studies in this field have been conducted. In 1995, Carling *et al*.[11] demonstrated a relationship between BsmI polymorphism and primary hyperparathyroidism. Tsukamoto *et al*.[12] reported a higher incidence of the b allele on hemodialysis patients with secondary hyperparathyroidism. A study on Japanese patients undergoing hemodialysis indicated a protective effect of the B allele.[13] The main objective of this study was to investigate the association between VDR gene SNPs with the intensity of hyperparathyroidism in hemodialysis patients. Despite the growing body of evidence investigating the associations between VDR polymorphisms and hyperparathyroidism, the results are contradictory. This study showed a possibility of genetic susceptibility of the Iranian population to parathyroid hyperplasia. Our findings revealed that the tt variant of TaqI is linked to high serum iPTH levels. The results are partly in agreement with the findings of a study on Turkish patients with ESRD, showing that the TT variation

Table 5: Mean levels of serum biochemical factors in the four groups of hemodialysis patients

	Mean±S.E				*P* value
	Group I iPTH: <150 (*n*=20)	Group II iPTH: 150≥ <300 (*n*=21)	Group III iPTH: 300≥ <600 (*n*=25)	Group IV iPTH: ≥600 (*n*=24)	
ALP	285.85±25.71	386.19±55.25	448.64±77.32	1169.63±193.9	<0.0001
ALb	4.18±0.09	4.01±0.11	4.2±0.07	4.1±0.06	0.436
Ca	8.66±0.27	8.48±0.22	8.05±0.24	7.78±0.26	0.053
P	4.63±0.29	4.74±0.28	5.11±0.28	4.97±0.25	0.602
25 (OH) Vit D	97±19.67	76.76±14.66	70.24±12.13	58.54±7.22	0.261
iPTH	87.7±9.44	232.85±9.34	461.48±17.94	1141.53±99.86	<0.0001

ALP: Alkaline phosphatase, Alb: Albomin, Ca: Calcium, P: Phosphate, 25(OH) Vit D: 25 Hidroxy vitamin D, iPTH: intact parathyroid hormone

Table 6: The relationship of the VDR rs1544410 (BsmI) gene polymorphism with the biochemical parameters

	BB (*n*=20)	Bb (*n*=54)	Bb (*n*=16)	*P* value
ALP	416.5 (323.7, 599.7)	306.5 (230, 551)	511.5 (273, 953.2)	0.073
Alb	4.23±0.08	4.11±0.06	4.03±0.07	0.305
Ca	7.36±0.29	8.47±0.15	8.28±0.27	0.002
P	4.92±0.26	4.94±0.18	4.59±0.33	0.627
25 (OH) Vit D	68.5 (49, 108.2)	50.5 (38, 68.7)	54.5 (40.2, 95)	0.160
iPTH	546.07±99.5	445.77±54.0	661.55±180.7	0.267

Values expressed as median (25th and 75th percentile) or average±standard error. *P* values were obtained using Kruskal-Wallis test or one-way analysis of variance, according to the distribution of the variables, VDR: Vitamin D receptor, ALP: Alkaline phosphatase, Alb: Albomin, Ca: Calcium, P: Phosphate, 25(OH) Vit D: 25 Hidroxy vitamin D, iPTH: intact parathyroid hormone

Table 7: The relationship of the VDR rs731236 (TaqI) gene polymorphism with the biochemical parameters

	TT (*n*=36)	Tt (*n*=43)	Tt (*n*=11)	*P* value
ALP	331 (236.2, 550.5)	372 (253, 629)	524 (257, 600)	0.334
Alb	4.14±0.07	4.09±0.07	4.19±0.07	0.711
Ca	8.3±0.21	8.41±0.17	7.15±0.30	0.007
P	4.92±0.23	4.71±0.18	5.35±0.43	0.346
25 (OH) Vit D	61.5 (45.5, 89.7)	50 (37, 75)	52 (32, 81)	0.385
iPTH	491.16±96.5	471.96±57.3	691.02±150.3	0.395

Values expressed as median (25th and 75th percentile) or average±standard error. *P* values obtained using Kruskal–Wallis test or one-way analysis of variance, according to the distribution of the variables, VDR: Vitamin D receptor, ALP: Alkaline phosphatase, ALP: Alkaline phosphatase, Alb: Albomin, Ca: Calcium, P: Phosphate, 25(OH) Vit D: 25 Hidroxy vitamin D, iPTH: intact parathyroid hormone

of the TaqI VDR gene influences the development of hyperparathyroidism.[14] Also in our study the frequency of BB genotype is higher in groups III (300 ≥ iPTH < 600 pg/mL) and IV (iPTH ≥ 600 pg/mL) thus BB genotype may develop more severe secondary hyperparathyroidism but the relationship between BsmI variants and serum iPTH level was not statistically significant. Tagliabue *et al*.,[15] in their study concluded that patients with the B allele and BB genotype had a significantly lower serum PTH and alkaline phosphatase levels than patients with the b allele and bb genotype but the difference did not reach statistical significance. In our study, the calcium level seen in the BB genotype is less than those for Bb and bb genotypes (P = 0.002). These findings contrast with results of a study in north India that showed the serum calcium levels were significantly higher in BsmI "BB" genotype.[16] Our study revealed that for Ca, 60% of patients and for ALP, 63.3% of patients were out of normal range. The mean serum level of ALP in the fourth group (iPTH > 600 pg/mL) was higher in all groups (P < 0.0001).

Some studies suggest that the b allele of BsmI and the T allele of TaqI are more common variants in patients with primary hyperparathyroidism.[11,17,18]

Further studies including genetic association surveys are needed to describe the effects of VDR polymorphisms on disease development, in larger groups of population among different ethnicities. Researches have reported that certain population cohorts are more vulnerable to phenotypes related to severe hyperparathyroidism than other groups of various ethnicities. Therefore, large groups of population, need to be investigated for association of disease phenotype and SNPs of the VDR gene in different societies. This could explain the inconsistency of results among various related studies.

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