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Original Article

Fetuin-A and vitamin D receptor gene polymorphisms in hemodialysis patients

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Abstract

Introduction: Vascular calcification is a common complication in the chronic kidney disease (CKD) patients and the leading cause of morbidity and mortality in this patient. The aim of the present study was to evaluate a possible correlation between vitamin D receptor (VDR) gene FokI and ApaI polymorphisms with the expression of calcification biomarkers such as Fetuin-A and intact parathyroid hormone (iPTH) in hemodialysis (HD) patients.

Methods: In this cross-sectional study, serums were obtained from 46 stable chronic HD patients. The serum levels of iPTH, Fetuin-A, vitamin D, calcium, phosphorus, and VDR genotyping were determined by standard methods.

Results: Serum levels of Fetuin-A, calcium, and phosphorus did not differ between males and females, but significant differences in iPTH and vitamin D levels was found in the study patients [(336.8 ± 139.0 pg/dl) vs. (414.7 ± 111.8 pg/dl), P = 0.040 and (24.5 ± 7.6 ng/ml) vs. (19.9 ± 4.8 ng/ml), P = 0.020 respectively]. A significant correlations were found between serum phosphorus and levels of serum calcium (r = –0.4; P = 0.002), vitamin D (r = –0.5; P = 0.001) and iPTH (r = 0.4; P = 0.001). iPTH level in FokI polymorphism, were different between genotype groups in study patient (P = 0.020). There was a significant positive correlation between vitamin D and iPTH levels in patients with aa genotype (P = 0.020, r = 0.4).

Conclusion: These findings suggest that FokI (rs2228570) polymorphism in exon-2 of the VDR gene may play a role in iPTH levels. Fetuin-A deficiency or high level of iPTH and its association with VDR gene polymorphisms may be useful to identify the high-risk group susceptible to renal failure and atherosclerosis. Although VDR gene FokI and ApaI polymorphisms could affect the levels of Fetuin-A and vitamin D, their direct role on atherosclerosis needs further studies in the future.

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Introduction

Vascular calcification is a common complication in the chronic kidney disease (CKD) patients and the leading cause of morbidity and mortality in this patient.[1] Approximately 50% of deaths among the patients with end stage renal disease (ESRD) originate from cardiovascular events and approximately 30 times higher than the general population.[2] High calcium intake, age, dialysis vintage and high levels of phosphorus, C-reactive protein, and osteoprotegerin and low levels of Fetuin-A, osteoprotegerin and matrix-GLA protein are major risk factors for cardiovascular disease (CVD) in this patient.[3,4] It also has several biologic

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functions including vascular calcification and bone metabolism regulation, inhibition of insulin receptor tyrosine kinase, protease activity control, keratinocytes migration, and breast tumor cell proliferative signaling. Fetuin-A (α-2 Heremans-Schmid glycoprotein), a negative acute phase reactant protein and a prognostic factor for dialysis patients, is a circulating calcium-regulatory glycoprotein that synthesized by liver and inhibit vascular calcification by forming soluble mineral complexes that mainly consists of calcium, phosphate, and Fetuin-A.[5]

Previous studies showed that low serum levels of Fetuin-A are associated with increased CVD and as a risk factor for CVD in hemodialysis (HD) patient[6,7] and influences on mortality risk in this patients.[8] Furthermore, disturbances of calcium-phosphorus and vitamin D metabolism associated with CKD play a key role in the development of secondary hyperparathyroidism (SHPT), osteodystrophy, vascular calcification, and are associated with increased risk for CVD and mortality in HD patients.[9,10]

Vitamin D regulates calcium level, bone homeostasis and regulation of parathyroid hormone (PTH) secretion.[11-13] Decreased serum levels of 1,25-dihydroxy vitamin D ($1,25(OH)_2$vitD) cause an increase in PTH secretion and the development of SHPT. Increased serum levels of PTH and hyperphosphatemia are risk factors for CVD and increased mortality in HD patients.[14-16] Moreover, genetic-association studies have demonstrated that variations in vitamin D receptor (VDR) function induced by polymorphisms at the 3' and 5' regions of the VDR gene may effect on mortality risk in this patients.[17]

Previous study show that VDR gene can effect on mineral metabolism and bone mineral density.[18] If VDR gene polymorphisms influence the level of calcification factor, these polymorphisms may have roles in the pathogenesis of CVD, which effect on mortality. FokI polymorphism, as a start codon polymorphism, located in the 5' translated region and ApaI located in intron-8 at the 3' untranslated region of the VDR locus.[19]

Deficiency of 1, $25(OH)_2$vit D, low serum levels of Fetuin-A and SHPT may be influenced by FokI and ApaI VDR gene polymorphisms. The aim of the present study was to evaluate a possible correlation between VDR gene FokI and ApaI polymorphisms with the expression of calcification biomarkers such as Fetuin-A and intact parathyroid hormone (iPTH) in HD patients.

Methods

This cross-sectional study was performed in the Department of Biochemistry of the Tabriz University of Medical Sciences (TUMS) (Tabriz, Iran). Approval for the study was obtained from the TUMS Ethics Committee. Informed consent was obtained from all of the patients. A total of 46 HD patients (28 men and 18 women) were included in the study. None of the patients had a vitamin D therapy, hormone therapy with PTH and history of CVD. Because Fetuin-A is produced in the liver, patients with liver failure and liver cirrhosis excluded from the study. All of the patients were stable and were under regular HD for at least 6 months (6–84 months) 3 × 4 h/week by Fresenius-2008B hemodialyzer.

Pre-dialysis blood sampling from a peripheral vein was performed after 12 h of overnight fasting. Subsequent serums were separated within 30 min and samples were kept frozen at −70 °C until analysis was done. Calcium and phosphorus were determined by using standard laboratory techniques with commercial kits (Pars Azmoon Co.). Total plasma protein, albumin, and alkaline phosphatase were assayed by enzymatic colorimetric method with an automated chemical analyzer (Abbott Analyzer, Abbott Laboratories, Abbott Park, North Chicago, IL, USA). Serum iPTH was determined by enzyme-linked immunosorbent assay (ELISA) using the Immunodiagnostic system (Bolden, UK). Serum Fetuin-A was measured in diluted to a ratio of 1:10000 serum solutions using a Human Fetuin-A ELISA kit (BioVendor Laboratory Medicine, Brno,

Czech Republic) in an ELISA plate reader (STATFAX2100, Multi-detection Multi Plate Reader, USA). Fetuin-A concentration was determined by interpolation with a standard curve. Intra-assay and inter-assay variability were 5.1% and 6.5%, respectively. The detection limit of this Fetuin-A ELISA assay was 0.35 ng/ml. Vitamin D was measured by ELISA using the Immunodiagnostic Systems Ltd. (10 Didcot Way, Boldon Business Park, Tyne & Wear, NE35 9PD, UK).

Genomic DNA was extracted from whole blood samples using the QIaAmp DNA Blood Midi Kit (Qiagen, Hilden, Germany). Genotyping of the FokI (rs2228570) and ApaI (rs7975232) polymorphisms was performed using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. The FokI polymorphism was detected using forward primer 5'-AGC TGG CCC TGG CAC TGA CTC TGC TCT-3' and reverse primer 5'-ATG GAA ACA CCT TGC TTC TTC TCC-3' which amplify 265 base pair (bp) fragment containing FokI site. For the detection of the polymorphic ApaI restriction enzyme sites, one primer in intron-8 5'-CAG AGC ATG GAC AGG GAG CAA G-3' and the other in exon-9 5'-GCA ACT CCT CAT GGC TGA GGT CTC A-3' were used that yielding a 740 bp fragment containing ApaI site.[19]

PCR was performed for both of the FokI and ApaI polymorphisms at the following condition: after denaturation at 95 °C for 5 min, 35 cycles were performed with denaturation for 45 s at 94 °C, hybridization for 45 s at 60 °C, and elongation for 45 s at 72 °C; the last cycle was followed by an extension step of 10 min at 72 °C. PCR products were checked by electrophoresis on 2% agarose gel. The PCR products were digested with the respective restriction enzymes according to the manufacturer's instructions as follows: at 37 °C for 5 min with FokI and at 37 °C for 20 min with ApaI (Fermentas). Digested products separated in 3% agarose gels and visualized by ethidium bromide staining and genotype were determined according to the digestion pattern. Furthermore, FokI and ApaI polymorphisms were confirmed by PCR repetition and PCR product commercial sequencing (genfanavaran). Digested products for FokI polymorphism contain 196 bp and 69 bp and for ApaI polymorphism was 530 bp and 210 bp fragments that indicated f and a alleles respectively and undigested alleles as F and A (Figures 1 and 2).

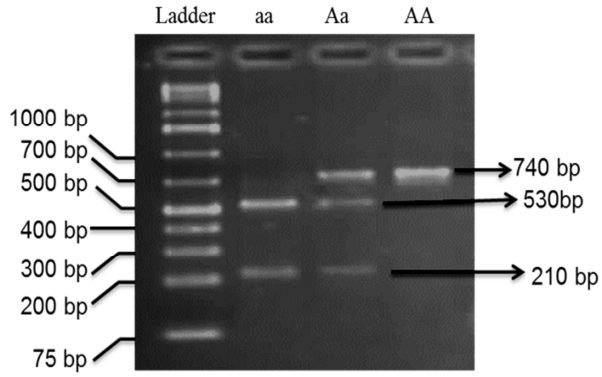

Figure 1. ApaI enzymatic digestion products as determined by agarose gel electrophoresis

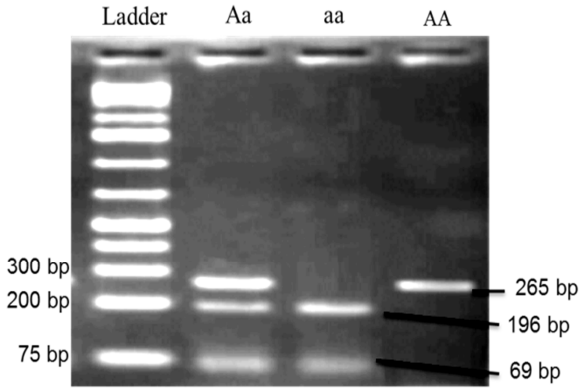

Figure 2. FokI enzymatic digestion products as determined by agarose gel electrophoresis

The SPSS for Windows (version 18, SPSS Inc., Chicago, IL, USA) was used for analysis of data. All variables were expressed as mean ± standard deviation. Numbers and their percent were showed when appropriate. The data were statistically analyzed using Student's t-test and one-way ANOVA. Differences among two groups were analyzed using Student's t-test, chi-square test, and Mann–Whitney U-test according to the characteristics of the variables and also correlations with the parameters were based on Spearman coefficient. $P < 0.05$ was considered as significant.

Results

Table 1 lists demographic and biochemical data of the study group. The underlying kidney diseases were: diabetic nephropathy 18 (39.1%), hypertension 2 (4.3%), polycystic kidney disease 1 (2.2%), glomerulonephritis in 4 (8.7%), and unknown causes 21 (45.7%). The mean serum Fetuin-A concentration was 128.1 ± 88.8 ng/ml. Serum levels of Fetuin-A, calcium, and phosphorus did not differ between males and females but significant differences in iPTH and vitamin D levels was found in the study patients [(336.8 ± 139.0) vs. (414.7 ± 111.8) pg/dl, P = 0.040 and (24.5 ± 7.6) vs. (19.9 ± 4.8) ng/ml, P = 0.020, respectively).

The correlations of vitamin D, Fetuin-A, calcium, phosphorus, and iPTH in the study patients have been shown in table 2. No significant correlation was found between vitamin D, Fetuin-A, calcium and iPTH levels in the study patients. On the other hand, significant correlations were found between serum phosphorus and levels of serum calcium (r = –0.4; P = 0.002), vitamin D (r = –0.5; P = 0.001) and iPTH (r = 0.4; P = 0.001).

As it has been shown in table 3, serum vitamin D, Fetuin-A, calcium, phosphorus, and iPTH levels classified by FokI and ApaI polymorphisms in HD group. Except iPTH level in FokI polymorphism (P = 0.020), serum levels of calcium, phosphorus, Fetuin-A, and vitamin D were not different between genotype groups in study patient.

Table 1. Baseline characteristics of study patients

Variable	HD group (n = 46)
Age (years) (mean ± SD)	60.30 ± 14.50
Sex (male/female)	28/18
Underlying diagnoses	n (%)
Diabetic nephropathy	18 (39.1)
Chronic glomerulonephritis	4 (8.7)
Hypertension	2 (4.3)
Polycystic kidney disease	1 (2.2)
Unknown etiology	21 (45.7)
Calcium (mg/dl) (mean ± SD)	8.50 ± 0.43
Phosphorus (mg/dl) (mean ± SD)	6.05 ± 0.90
iPTH (pg/dl) (mean ± SD)	367.30 ± 133.40
Vitamin D (ng/ml)	22.70 ± 7.00
Total protein (g/dl)	6.00 ± 1.20
Alkaline (IU/l)	411.20 ± 310.10
Albumin (g/dl) (mean ± SD)	3.60 ± 0.70
Fetuin-A (ng/ml) (mean ± SD)	128.10 ± 88.80
Fe (mg/dl) (mean ± SD)	63.50 ± 4.60
TIBC (mean ± SD)	360.00 ± 188.10
Ferritin (mg/dl) (mean ± SD)	23.50 ± 11.30

SD: Standard deviation; iPTH: Intact parathyroid hormone; TIBC: Total iron-binding capacity; HD: Hemodialysis

Table 2. The correlation between intact parathyroid hormone, Fetuin-A, phosphorus, vitamin D, and calcium levels in study patients

Parameters	Vitamin D		PTH		Calcium		Phosphorus	
	r*	P	r*	P	r*	P	r*	P
Fetuin-A	0.20	0.090	0.07	0.600	0.20	0.080	−0.10	0.300
Vitamin D	-		−0.10	0.300	0.20	0.100	−0.50	0.001
iPTH	-		-		−0.20	0.100	0.40	0.001
Calcium	-		-				−0.40	0.002

*P< 0.05 was considered meaningful; Spearman coefficient was calculated to determine the correlation between iPTH, phosphorus, vitamin D, Fetuin-A, and calcium levels; r: Correlation coefficient; iPTH: Intact parathyroid hormone

Table 3. Serum intact parathyroid hormone, phosphorus, vitamin D, and calcium levels in FokI and ApaI polymorphisms in hemodialysis patients

Parameter	FokI			P	ApaI			P
	FF	Ff	ff		AA	Aa	Aa	
n (%)	15 (32.6)	18 (39.1)	13 (28.3)		10 (21.7)	23 (50.0)	13 (28.3)	
Fetuin-A (ng/ml) (mean ± SD)	133.0 ± 104.3	113.5 ± 82.3	142.5 ± 81.8	0.700	100.2 ± 74.0	129.4 ± 84.2	147.1 ± 107.0	0.500
Vitamin D (ng/ml) (mean ± SD)	20.6 ± 5.3	24.0 ± 8.7	23.0 ± 5.6	0.500	24.0 ± 10.5	24.1 ± 6.7	24.1 ± 6.7	0.100
Calcium (mg/dl) (mean ± SD)	8.5 ± 0.5	8.5 ± 0.5	8.5 ± 0.3	0.600	8.4 ± 3.9	8.6 ± 0.5	8.4 ± 0.3	0.500
iPTH (pg/dl) (mean ± SD)	447.3 ± 126.0	319.3 ± 125.8	341.5 ± 116.5	0.020	316.8 ± 163.0	382.0 ± 108.0	380.0 ± 150.0	0.200
Phosphorus (mg/dl) (mean ± SD)	6.5 ± 1.0	5.8 ± 0.10	5.8 ± 0.7	0.20	6.3 ± 1.2	6.0 ± 0.8	5.9 ± 0.9	0.70

Differences among groups were assessed by ANOVA test; SD: Standard deviation; iPTH: Intact parathyroid hormone

Furthermore, the overall prevalence of genotypes in this study for FokI polymorphism was 32.6% FF, 39.1% Ff, and 28.3% ff, and for ApaI polymorphisms was 21.7% AA, 50% Aa, and 28.3% aa. The results of the relationship between VDR polymorphisms and serum levels of Fetuin-A, vitamin D, and iPTH in study group showed that in patients with aa genotype, there was a significant positive correlation between vitamin D and iPTH levels (P = 0.020, r = 0.4).

Discussion

CVD in ESRD patient compared with a healthy subject occurs at an earlier age, is more prevalent and is more severe. In the present study, we examined variation in the VDR gene would play in susceptibility of CVD in ESRD.

Our findings expand previous studies about existence of a relationship between serum level of Fetuin-A and all-cause mortality, and also confirmed the association between the metabolism of calcium and phosphorus and cardiovascular calcification and subsequently mortality in HD patients.[8] Further studies have demonstrated the decreased circulating level of Fetuin-A is associated with increased coronary arterial and valvular calcification scores. It has been shown that Fetuin-A can inhibit vascular calcification by inhibiting hydroxyapatite formation, so decreased Fetuin-A in HD patients leads to susceptibility of vascular calcification and cardiovascular morbidity in HD patients.[6,7]

Further studies have demonstrated the lower serum Fetuin-A concentration is independently associated with increased cardiovascular mortality in this study population. Pecovnik et al. reported that in patients undergoing HD, lower Fetuin-A levels are associated with higher mortality[8] and is a useful predictor for CVD and early mortality in HD patients.[20] Fetuin-A, a liver-derived circulating glycoprotein, appears to have diverse biologic activity. In the serum, act as a buffer in super saturation state by binding calcium and phosphorus, and it has a role to prevent vascular calcification by protecting vascular smooth muscle cells from the effects of calcium overload and calcification in the general population as well as uremic patients.[4] So reduced Fetuin-A level leads to high level of iPTH secretion that it is one of the main risk factor for CVD. In contrast our result, Hermans et al. in a cross-sectional study reported that Fetuin-A levels were not different between dialysis patients and healthy subject.[21] This difference may be the result from differences in demographic and sample size between our study and mentioned study.

In sex analysis, decreased vitamin D levels, and increased iPTH concentration in females in compare to male group indicate that a deficiency of vitamin D independent of sex influence may consider as one of the main factors that works by increasing the production and release of PTH from the parathyroid glands.

Correlation between serum phosphorus and certain factors of bone metabolism were apparent. With the deterioration of kidney function, the clearance rate of phosphorus is decreased, and a higher level of PTH is secreted to overcome to regulation maintain normal bone turnover. Furthermore, vitamin D level reduced because of kidney failure and subsequent absorption of calcium decreases. So a significant positive correlation between phosphorus and iPTH levels and a negative correlation between serum phosphors and vitamin D levels, and between serum phosphors and the level of calcium expected. In support of our results about phosphors and iPTH correlation, Li et al. show that higher levels of iPTH are incremental correlates of hyperphosphataemia in HD patient.[14] With this description, combination of hyperphosphatemia, high level of calcium and SHPT can lead to cardiovascular complications.

Our finding shows that there is no significant correlation between serum Fetuin-A, vitamin D, iPTH, phosphorus and calcium, and VDR FokI polymorphism as well as VDR ApaI polymorphism in this patient, except association between iPTH levels with VDR FokI polymorphism. Previous studies have reported various different results about the influence of VDR gene FokI and ApaI

polymorphisms on iPTH and Fetuin-A levels in HD patients. This is the first study to investigate the relationship between VDR gene FokI polymorphisms and Fetuin-A levels. The only study in this area that consistent with our result, Storrs suggested that ApaI polymorphism in intron-8 of the VDR gene may play a role in Fetuin-A levels and AA genotype for ApaI associate with higher levels of Fetuin-A biomarker.[22]

In contrast our finding about iPTH level, Kohama et al. find that iPTH levels were different in genotype of ApaI polymorphism but calcium, phosphorus, and vitamin D were not different in FokI and ApaI polymorphisms genotype.[23] Yokoyama and Shigematsu[24] reported the aa genotype of the ApaI polymorphism has been linked to higher iPTH in predialysis Japanese patients with ESRD. In favor of our result, Vigo et al. reported serum iPTH level in the FF group was significantly higher in patients with chronic renal failure.[25]

Furthermore, Santoro et al. reported that patients bearing either the Ff heterozygous or FF homozygous genotype had significantly higher PTH values than those bearing the ff genotype.[26] The difference in our and other study results could be a result of the influence of geographical location and ethnic differences or study sample size related to distribution of the VDR gene polymorphisms in these populations.

Limitations

Small numbers of patients and not using imaging technique for assessment of the extent of arterial calcification in this population were our study limitations that require consideration. For this reasons, the relations between calcifications, mortality, and calcification factor levels could not be explored.

Conclusion

The findings of this study shows that a low Fetuin-A level in HD patients and increased serum level of iPTH is associated with susceptibility of atherosclerosis in this patients, and also VDR gene FokI polymorphism could affect the levels of iPTH; so Fetuin-A deficiency or high level of iPTH and its association with VDR gene polymorphisms may be useful to identify the high-risk group susceptible to renal failure and atherosclerosis.

Conflict of Interests

Authors have no conflict of interest.

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