Relationship Between Vitamin D Receptor Gene FokI and ApaI Polymorphisms and Serum Levels of Fetuin-A, Vitamin D, and Parathyroid Hormone in Patients on Hemodialysis

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Introduction. Low fetuin-A and 1,25-hydroxyvitamin D3 (vitamin D) levels accompanied with high intact parathyroid hormone (PTH) contents are associated with cardiovascular disease in dialysis patients. The aim of present study was to evaluate the relationship between vitamin D receptor (*VDR*) gene FokI and ApaI polymorphisms with serum levels of fetuin-A, vitamin D, and intact PTH in hemodialysis patients.

Materials and Methods. Serum was obtained from 46 stable chronic hemodialysis patients and 43 healthy controls. Serum levels of intact PTH, fetuin-A, vitamin D, calcium, and phosphorus were measured. Genotyping of the *VDR* gene was performed using standard methods.

Results. Serum fetuin-A and vitamin D levels were significantly lower, whereas serum levels of PTH, calcium, and Phosphorus were higher in the hemodialysis patients than in the healthy controls. The FokI genotypes were more frequent in the hemodialysis patients than the control group ($P = .004$). With respect to FokI genotypes, intact PTH level was higher among the hemodialysis patients compared to the controls ($P = .02$). In contrast, vitamin D level was lower in the hemodialysis patients with ApaI genotypes compared to the control group ($P = .04$).

Conclusions. Our study shows that increased serum level of PTH and decreased fetuin-A and vitamin D levels may increase susceptibility of atherosclerosis in patients with hemodialysis through *VDR* gene FokI and ApaI polymorphisms.

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INTRODUCTION

Cardiovascular events are the main causes of morbidity and mortality in hemodialysis patients. Approximately 50% of mortality among the patients with end-stage renal disease (ESRD) originates from cardiovascular events.[1,2] Patients with ESRD are characterized with progressive atherosclerosis and particularly marked vascular calcification.[3] Both the formation of calcified atherosclerotic plaques and diffuse calcification of the media of central arteries are frequent findings in these patients.[4,5] Cardiovascular homodynamic consequences of this process are decrease in arterial elasticity, coronary artery perfusion, left ventricular hypertrophy, and myocardial ischemia and an increase in pulse wave velocity.[6-8]

There is growing evidence that shows physiologic inhibitors of vascular calcification play an important role in the process.[9,10] Fetuin-A, an acute-phase glycoprotein also referred to as α_2-*Herman Schemid glycoprotein*, is a circulating protein synthesized and secreted by the adult liver tissue that inhibits ectopic calcium-phosphate precipitation and vascular calcification.[11,12] Fetuin-A accounts for approximately 50% of the capacity of serum to inhibit the formation of spontaneous vascular calcified plaque from solutions supersaturated in calcium and phosphate.[13] Schafer and colleagues[14] reported that the absence of fetuin-A in fetuin-A-knockout mice resulted in immense extra osseous calcification. These findings suggest that the size of calcified atherosclerotic plaques may be changed based on the fetuin-A concentration and the regulators of calcium and phosphate homeostasis, including hormonal regulation, such as parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D3 (vitamin D) or calcitriol therapy.

In ESRD, high circulating levels of PTH lead to cardiovascular complications that increase morbidity and mortality.[15] Vitamin D deficiency is the most common cause of secondary hyperparathyroidism in CKD patients, because the kidney is no longer able to convert precursor of vitamin D to its active form.[16-18] In the investigation for vascular calcification risk factors, genetic association studies revealed that allelic polymorphisms of the gene coding for the vitamin D receptor (VDR) influence morbidity and mortality risk in hemodialysis patients. One polymorphism in particular, the BsmI polymorphism, was associated with survival in a study of 143 hemodialysis patients.[19] Furthermore, enough evidence exists to hypothesize that lower levels of fetuin-A and vitamin D or variations in VDR function induced by polymorphisms at the 3′ and 5′ regions of the *VDR* gene may alter mortality rate of these patients. The aim of present study was to evaluate the relationship between *VDR* gene FokI and ApaI polymorphisms with serum levels of fetuin-A, vitamin D, and intact PTH, as the main factors involved in vascular calcification in hemodialysis patients.

MATERIALS AND METHODS

Study Population

The study was performed in the Department of Biochemistry of Tabriz University of Medical Sciences. The ethics committee of the university approved the study protocol. Informed consent was obtained from all of the participants. The study groups comprised 43 healthy controls (20 men and 23 women) and 46 patients on hemodialysis (28 men and 18 women). Hemodialysis patients were excluded from the study if they had a history of hormone therapy with PTH, liver disease, and cardiovascular disease. The causes of ESRD were diabetic nephropathy in 18 patients (39.1%), hypertension in 2 (4.3%), polycystic kidney disease in 1 (2.2%), glomerulonephritis in 4 (8.7%), and other or unknown causes in 21 (45.7%). All of the patients were stable and were under regular hemodialysis for at least 6 months (range, 6 to 84 months), for 4 hours, 3 times per week.

Laboratory Methods

All blood samples were obtained from a peripheral vein after 12 hours of overnight fasting, just prior to the beginning of hemodialysis in the hemodialysis group. Subsequent plasma and sera were separated within 30 minutes and the samples were kept frozen at -70°C until assays were carried out. Total serum calcium and phosphorus concentrations were measured using commercial kits (Pars Azmoon Co, Tehran, Iran). Total plasma protein, albumin, and alkaline phosphatase levels were measured by the enzymatic colorimetric method with an automated chemical analyzer (Abbott Analyzer, Abbott Laboratories, Chicago, IL, USA). Serum concentrations of fetuin-A were measured using a human fetuin-A enzyme-linked immunosorbent assay (ELISA) kit in an ELISA plate reader (STATFAX2100, Multi-detection Multi Plate Reader, USA). Fetuin-A concentration was determined by interpolation with a standard curve. The analytical limit detection of the assay was 0.35 ng/mL, with an interassay coefficient of variation of 6.5% and an intraassay coefficient of variation of 5.1% (BioVendor Laboratory Medicine Inc, Brno, Czech Republic). Intact PTH was measured by ELISA (Immunodiagnostic Systems, Boldon, UK). Vitamin D was measured by ELISA (Immunodiagnostic Systems, Boldon, UK).

Vitamin D Receptor Genotyping

One-milliliter aliquots of peripheral blood samples were collected from the hemodilaysis and control groups and stored in the presence

of ethylenediaminetetraacetic acid. Genomic DNA was extracted from whole blood samples using the QIaAmp-DNA Blood Midi Kit (Qiagen, Hilden, Germany). Genotyping of the FokI and ApaI polymorphisms was performed using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. Polymerase chain reaction was performed for both of the FokI and ApaI polymorphisms. Initial denaturation at 95°C for 5 minutes, 35 cycles 94°C for 45 seconds, 60°C for 45 seconds, and 72°C for 45 seconds. The last cycle was followed by an extension step of 10 minutes at 72°C. Polymerase chain reaction products were checked by electrophoresis on 2% agarose gel. For the detection of the polymorphic FokI restriction enzyme site, 2 primers were used: downstream primer 5′-AGC TGG CCC TGG CAC TGA CTCTGCTCT-3′ and upstream primer 5′-ATG GAA ACA CCT TGCTTCTTCTCC-3′. For detection of the polymorphic ApaI restriction enzyme sites, the following primers were used: downstream primer 5′-CAG AGC ATG GAC AGG GAG CAA G-3′ and upstream primer 5′-GCA ACT CCT CATGGC TGA GGT CTC A-3′.

The PCR products were digested with the respective restriction enzymes according to the manufacturer's instructions as follows: at 37°C for 5 minutes with FokI and at 37°C for 20 minutes with ApaI (Fermentas M Medical Srl, MD, USA). The RFLP products underwent electrophoresis on 3% agarose gel and were stained with ethidium bromide and visualized under shortwave ultraviolet light, and genotype was determined according to the digestion pattern. Also FokI and ApaI polymorphisms were confirmed by PCR repetition and PCR product sequencing (genfanavaran). For FokI polymorphism, the presence of the restriction site, which generates 2 fragments of 196 bp and 69 bp, identified the f allele, while its absence, resulting in a single undigested 265-bp product, identified the F allele. Determination of *VDR* genotyping for ApaI polymorphism was made based on the ApaI cleavage pattern, and A allele indicated the absence of the restriction site, which produced 740-bp fragment, while A allele indicated the presence of the restriction site, which produced 530-bp and 210-bp fragments.

Statistical Analyses

All continuous variables were expressed as mean ± standard deviation. Numbers and their percentages were shown for categorical variables. The data were analyzed using the Student *t* test, chi-square test, Mann-Whitney U test, and the 1-way analysis of variance. The Spearman coefficient was calculated to determine the correlation between biochemical parameters. All statistical analyses were performed using the SPSS software (Statistical Package for the Social Sciences, version 13.0, SPSS Inc, Chicago, Ill, USA). *P* values less than .05 were considered significant.

RESULTS

Table 1 lists demographic and biochemical data of the study groups. There was no significant difference in the mean age and sex distribution between the two study groups. Serum fetuin-A and vitamin D levels were significantly lower in the hemodialysis patients than in the healthy control group (128.1 ± 88.8 ng/mL versus 184.7 ± 93.4 ng/mL, $P = .004$; 22.7 ± 7.0 ng/mL versus 36.2 ± 15.2 ng/mL, $P = .002$; respectively); however, significant differences in vitamin D was fund only in the hemodialysis group when analysis was done by sex. Table 2 shows that serum levels of fetuin-A did not differ between the men and the women in the hemodialysis patients and in the control group. A significant difference in serum intact PTH, calcium, and phosphorus concentrations was also found between the hemodialysis and control groups ($P < .001$ for all). In the subgroup analysis, these differences were found not only between the men and the women in both hemodialysis and control groups ($P = .04$ and $P = .02$, respectively), but also between the men and between the women in both study groups. There was no significant correlation between serum fetuin-A, vitamin D, and intact PTH levels in neither of the hemodialysis or control groups.

No significant difference was seen in the genotype frequencies between the two study groups in ApaI polymorphism ($P = .20$), but a significant difference was found between the hemodialysis and control groups in FokI polymorphism frequencies ($P = .004$). As shown in Tables 3 and 4, serum fetuin-A, vitamin D, and intact PTH levels were categorized by FokI and ApaI polymorphisms in the two study groups. Except the PTH level in FokI polymorphism in hemodialysis patients ($P = .02$) and vitamin D levels in ApaI polymorphism in the

Table 1. Demographic and Clinical Data for the Hemodialysis and Control Groups

Characteristic	Hemodialysis Group (n = 46)	Control Group (n = 43)	*P*
Mean age, y	60.3 ± 14.5	54.6 ± 17.8	.15
Sex			
Male	28	20	
Female	18	23	.10
Underlying diagnoses			
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)	…	…
Mean serum calcium, mg/dL	8.5 ± 0.43	9.5 ± 0.55	< .001
Mean serum phosphorus, mg/dL	6.05 ± 0.9	3.9 ± 0.6	< .001
Mean serum intact parathyroid hormone, pg/dL	367.3 ± 133.4	26.7 ± 15.6	< .001
Mean serum vitamin D, ng/mL	22.7 ± 7.0	36.2 ± 15.2	.002
Mean serum total protein, g/dL	6.0 ± 1.2	6.7 ± 1.6	< .001
Mean serum alkaline phosphatase, IU/L	411.2 ± 310.1	183.6 ± 59.4	.02
Mean serum albumin, g/dL	3.6 ± 0.7	4 ± 0.5	.08
Mean serum fetuin-A, ng/mL	128.1 ± 88.8	184.7 ± 93.4	.004

Table 2. Serum Levels of Vitamin D, Fetuin-A, and Intact Parathyroid Hormone (PTH) in Men and Women of Hemodialysis and Control Groups

Parameter	Men	Women	*P*
Mean serum vitamin D, ng/mL			
Hemodialysis group	24.5 ± 7.6	19.9 ± 4.8	.02
Control group	32.6 ± 13.9	39.3 ± 15.9	.08
P for study groups	.005	< .001	…
Mean serum fetuin-A, ng/mL			
Hemodialysis group	130 ± 89.4	125 ± 90.3	.90
Control group	197.9 ± 86.4	173.1 ± 99.5	.30
P for study groups	.01	.10	…
Mean serum intact PTH, pg/dL			
Hemodialysis group	336.8 ± 139	414.7 ± 111.8	.04
Control group	23.5 ± 17.8	29.6 ± 13.1	.02
P for study groups	.001	.001	…

Table 3. Serum Fetuin-A, Vitamin D, and Intact Parathyroid Hormone (PTH) Levels in FokI Polymorphisms in Hemodialysis and Control Groups

	FokI in Hemodialysis Group				FokI in Control Group			
Parameter	FF	Ff	ff	*P*	FF	Ff	ff	*P*
Participants (%)	15 (32.6)	18 (39.1)	13 (28.3)		26 (60.5)	15 (34.9)	2 (4.7)	
Mean serum vitamin D, ng/mL	20.6 ± 5.3	24.1 ± 8.7	23.2 ± 5.6	.60	39.9 ± 18	30.6 ± 7.2	31.5 ± 3.5	.40
Mean serum fetuin-A, ng/mL	133 ± 104.3	113.5 ± 82.3	142.5 ± 81.8	.70	187.6 ± 99.5	184.4 ± 91	148.5 ± 5	.80
Mean serum intact PTH, pg/dL	447.3 ± 126	319.3 ± 125.8	341.5 ± 116.5	.02	25.9 ± 13.2	28.1 ± 20	28 ± 14.5	.90

Table 4. Serum Fetuin-A, Vitamin D, and Intact Parathyroid Hormone (PTH) Levels in ApaI Polymorphisms in Hemodialysis and Control Groups

	ApaI in Hemodialysis Group				ApaI in Control Group			
Parameter	AA	Aa	aa	*P*	AA	Aa	aa	*P*
Participants (%)	10 (21.7)	23 (50)	13 (28.3)		16 (37.2)	16 (37.2)	11 (25.6)	
Mean serum vitamin D, ng/mL	24.1 ± 10.5	21.4 ± 5	24.1 ± 6.7	.60	41.5 ± 17.5	36.7 ± 15.2	27.9 ± 6.8	.04
Mean serum fetuin-A, ng/mL	100.2 ± 74	129.4 ± 84.2	147.1 ± 107	.50	168.6 ± 102.8	205 ± 103.4	178.5 ± 60.2	.60
Mean serum intact PTH, pg/dL	316.8 ± 163	382.1 ± 108	379.8 ± 150.2	.50	25.2 ± 12.3	33.3 ± 20.4	19.4 ± 6	.09

Table 5. Relationship Between Vitamin D Receptor Gene Polymorphisms and Serum Levels of Fetuin-A, Vitamin D, and Intact Parathyroid Hormone (PTH) in Hemodialysis and Control Groups*

	ApaI				FokI			
Parameter	**All**	**AA**	**Aa**	**aa**	**All**	**FF**	**Ff**	**ff**
Hemodialysis Group								
Participants	46	10	23	13	46	15	18	13
Vitamin D and fetuin-A	0.30 (.09)	-0.01 (> .99)	0.20 (.30)	0.40 (.20)	0.30 (.09)	0.10 (.70)	0.20 (.50)	0.50 (.10)
Vitamin D and PTH	-0.10 (.30)	0.20 (.60)	0.02 (.90)	0.40 (.02)	-0.10 (.30)	0.20 (.50)	-0.40 (.09)	0.20 (.50)
Fetuin-A and PTH	0.08 (.60)	0.30 (.40)	-0.09 (.70)	-0.07 (.80)	0.08 (.60)	0.20 (.40)	0.10 (.70)	-0.20 (.60)
Control Group								
Participants	43	16	16	11	43	26	15	2
Vitamin D and fetuin-A	-0.20 (.20)	0.07 (.80)	-0.30 (.20)	-0.20 (.50)	-0.20 (.20)	1.00 (.001)	-0.10 (.70)	…
Vitamin D and PTH	0.10 (.30)	0.20 (.50)	0.06 (.80)	-0.30 (.40)	0.10 (.30)	0.10 (.50)	0.20 (.40)	…
Fetuin-A and PTH	0.10 (.30)	0.70 (.10)	0.20 (.50)	-0.10 (.80)	0.10 (.30)	0.10 (.50)	0.30 (.30)	…

*Values are Spearman coefficients (*P* values).

control group (P = .04), there was no significant difference in serum fetuin-A, vitamin D, and PTH levels between the three genotypes in both study groups. Table 5 summarizes the relationship between *VDR* polymorphisms and serum levels of fetuin-A, vitamin D, and intact PTH in the hemodialysis and control groups; in patients with aa genotype, there was a significant positive correlation between vitamin D and PTH levels (P = .02, r = 0.4), but in control group, a positive correlation was observed between vitamin D and fetuin-A (P = .001, r = 1) only in FF genotype.

DISCUSSION

In this study, we compared the main factors involved in vascular calcification including serum levels of fetuin-A protein, vitamin D, and intact PTH between hemodialysis patients and healthy individuals. Also, we studied the association of these parameters with the two *VDR* gene FokI and ApaI polymorphisms. Vascular calcification is a common problem in patients with ESRD; the presence and extent of it is an alert sign for cardiovascular and all-cause mortality in these patients.[20,21] In Our study, serum fetuin-A and vitamin D levels in hemodialysis patients were significantly lower, whereas intact PTH and phosphorus levels were higher than those in the control group. Caplin and collegaues[22] have shown that serum level of fetuin-A is decreased in patients with ESRD. On the other hand, further studies have demonstrated the lower serum fetuin-A concentration is independently associated with increased coronary arterial and valvular calcification scores.[23,24] With deterioration of kidney function, a higher level of PTH is secreted to overcome the PTH skeletal resistance and also to maintain normal bone turnover. The clearance rate of phosphorus is also decreased, so this element is retained in hemodialysis patients.

It has been shown that fetuin-A can only inhibits the de novo formation of calcium phosphate and does not dissolve preformed mineral; thus, decreased fetuin-A retention in hemodialysis patients leads to increased secretion of PTH, and in turn, it can enhance susceptibility of vascular calcification and cardiovascular morbidity in hemodialysis patients.[25,26] We compared changes in serum fetuin-A, vitamin D, and intact PTH levels with each other, but we found no significant relationship between these parameters in hemodialysis patients and neither in the healthy controls.

Some studies have shown that fetuin-A level is comparable between male and female hemodialysis patients.[12] Our study also confirms this finding. Increased intact PTH and decreased vitamin D levels in both male and female hemodialysis patients in comparison to healthy control group indicate that deficiency of vitamin D in hemodialysis patients independent of sex influence may be considered as one of the main factors that works by increasing the production and release of PTH from the parathyroid glands.

To our knowledge, this study is the first to search evidence for a putative causal nature of the association between serum fetuin-A level and *VDR* gene FokI and ApaI polymorphisms. The FokI restriction site is located in a coding exon at the start region of the gene and alters the length and structure of the VDR; Unlike FokI polymorphism, the ApaI polymorphism site located

in a regulatory region at the 3′ end of this gene.[27,28] Our results showed that ApaI polymorphism was not distributed differently among hemodialysis patients and normal controls but FokI polymorphism was distributed differently between two study groups. In line with our findings, Amato and coworkers showed in 88 Italian hemodialysis patients that there was an increased frequency of the FF genotype in the hemodialysis patients as compared to a controlgroup.[29]

When individually assessed serum fetuin-A, vitamin D, and intact PTH, we found that: (1) none of polymorphisms were related to serum fetuin-A level in the two groups of the study; (2) vitamin D level was associated with the ApaI polymorphism in only the control group; and (3) intact PTH level was associated with in FokI polymorphism in the group of hemodialysis patients. Previous studies have suggested various different results about the influence of *VDR* gene FokI and ApaI polymorphisms on vitamin D and intact PTH concentrations in hemodialysis patients. Yokoyama and colleagues[30] reported the aa genotype of the ApaI polymorphism has been linked to higher intact PTH levels in predialysis Japanese patients with ESRD. Vigo and colleagues[31] reported serum PTH level in the FF group was significantly higher in patients with chronic kidney failure. The discrepancies between this and other studies could be a result of the influence of geographical location and ethnic differences related to distribution of the *VDR* gene polymorphisms in these populations.

Although it is known that the FokI polymorphism leads to change in the length and the structure of the *VDR*, but it is not entirely clear whether this polymorphism has any effect on the function of the VDR. Gross and colleagues[32] made clear that the two different receptors showed the similar affinity for vitamin D and the similar transactivation activity, whereas in other investigations this polymorphism was associated with changed vitamin D-dependent gene transcription.[33,34] However, a recent study has related FokI polymorphism with cardiovascular risk factors in the healthy people.[35] The present results and Vigo Gago and colleagues' findings[31] propose that FokI polymorphism might change the function of the VDR in patients with chronic kidney disease via effects on development or progression of cardiovascular complications.

CONCLUSIONS

Our findings expand previous observations about existence of a relationship between serum fetuin-A, vitamin D, and intact PTH levels and cardiovascular calcification, and subsequently mortality in hemodialysis patients. In addition, our results suggested that *VDR* gene FokI and ApaI polymorphisms could affect the management of cardiovascular events in hemodialysis patients. Further studies with larger samples of patients and study of other polymorphisms on *VDR* gene will improve our understanding of the contribution of this gene to vascular calcification and offer preventive and therapeutic interventions in hemodialysis patients.

CONFLICT OF INTEREST

None declared.

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