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ORIGINAL ARTICLE

Association of vitamin D receptor gene fok1 polymorphism with bone health in Pakistani population

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ABSTRACT

Background: Osteoporosis is the bone disease characterized by demineralization of the bone. One of the most important genetic factors responsible for the osteoporosis is the vitamin D receptor (VDR) gene polymorphism. A translocation polymorphism which changes the codon from ATG to ACG has been associated with bone mineral density (BMD) variation and severity of the disease in patients of osteoporosis. The objective of the study was to find association of VDR *Fok1* polymorphism with bone mineral density

Methods: This case control study was design to find the association of the VDR *Fok1* polymorphism with bone health in Pakistani osteoporotic patients. The study was conducted at Islamabad Diagnostic center from 2014 to July 2016. Total of 156 participant (osteoporatic patients *n* = 78 and normal health controls *n* = 78 case control) were enrolled in the study. Polymerase chain Reaction restriction length polymorphism (PCR-RFLP) was used to genotype VDR *Fok1* polymorphism. Bidirectional sanger sequencing was used to verify the PCR-RFLP results with randomly picked *n* = 10 samples from both controls and patients. Commercial kits were used to estimate serum calcium and vitamin D level while dual-energy X-ray absorptiometry scan was used to measure the bone mineral density. The data were analyzed statistically using Statistical package for the social sciences (SPSS).

Result: F-allele increased the risk for decrease bone mineral density and osteopenia nearly threefold [Odds Ratio (OR): 2.8; 95% Confidence Interval (CI): 3.2-19.0; $p \leq 0.001$]. The genotype frequencies (Ff + ff *vs.* FF) showed an increase risk of disease (OR = 6.79; 95% CI = 3.41-22.6; $p = <0.001$). The OR cannot be calculated for recessive model since there was no ff genotype in the control group. Serum vitamin D and calcium was significantly lower in patients with mutant polymorph and their BMD was also significantly lower as compared to controls

Conclusion: VDR *Fok1* polymorphism is significantly associated with low mineral bone density, serum calcium and serum vitamin D level in Pakistani cohort.

Keywords: VDR, *Fok1*, BMD, osteoporosis.

Introduction

Vitamin D is an imperative factor that is either of dietary sources or synthesized in the skin during sunlight. Vitamin D is essential for many biological functions, but the most important among these are bone health. It acts by binding to the vitamin D receptor (VDR) for downstream process. By binding to VDR it mediates, it modulates the transcription of gene which is involved in calcium binding protein osteocalcin [1-3]. The VDR gene consists of 11 exons and 75 kb long which occupy the q arm of chromosome 12 [4-6]. VDR is the main role player of the bone health and mass. It harbors many polymorphisms which are associated with increased risk of bone diseases. These polymorphisms increase the risk of bone disease because they cannot bind and process vitamin D as required. These polymorphs are

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named after the restriction enzymes which are used in its identification by Polymerase Chain Reaction- Restriction Fragment Length Polymorphism (PCR-RFLP). The widely studied polymorphs are ApaI, BsmI, FokI, and TaqI [7-10]. Many studies have also been conducted to establish an association of polymorphism with bone mineral density in elderly patients [11,12]. The FokI polymorphism is a T/C transition polymorphism (ATG to ACG) at the first of two potential translation initiation sites in exon II [13] which has been identified using the FokI restriction endonuclease [14].

Although the potential risk of bone and other disease are associated with VDR polymorphism [15]. But these risks can be modulated by race, ethnicity, and diet habit [16]. The function of most of the polymorphism associated with risk has yet to be elucidated. Thus, the association of the polymorphism does not necessarily be the cause of disease for all the population [12]. Numerous studies have been published on the role of VDR and bone health but there is no study if the polymorphs in Pakistani population could affect the bone health; hence, the present study was designed to find the possible association and risk of bone diseases in Pakistani population with bone disease and to compare with control group. A case control study was carried out to study FokI polymorphism with osteoporosis osteopenia and bone mineral density in Pakistani population.

Methods

Patients' recruitment and data collection

Osteoporotic patients visited the private clinic of Islamabad diagnostic center (IDC) during July 2013 till July 2014 was enrolled in the study. This study was approved by Institutional ethics committee of International Islamic University, Islamabad, Pakistan, and IDC and was in concordance to Helsinki Declaration. The patients were diagnosed clinically and dual-energy X-ray absorptiometry (DEXA) scan was performed to measure bone mineral density and stage the disease. Age, gender, and ethnic matched controls were also included in the study. Blood were collected in two different tubes, i.e., Ethylenediaminetetraacetic acid (EDTA) tubes for DNA extraction and plain tubes for serum extraction.

The genomic DNA was extracted using commercial DNA extraction kit DNeasy Blood & Tissue Kit [17]. Serum was obtained by centrifugation of the clotted blood at 6,000 rpm for 10 minutes. The serum was separated in clean tubes. Both serum and DNA were stored at −20°C for further analysis.

Genotyping

Genotyping of VDR *Fok1* polymorphism was performed as describe previously [18]. Briefly, a PCR was performed with a total volume of 20 µl. The composition of the PCR reaction was as 2 µl of 10× Taq Buffer, 1 µl of 20 mM $MgCl_2$, 2 µl 10 mM dNTPs 1 µl of 10 pmol of each primer, 0.2 µl of 5 U/µl Taq polymerase and 12.8 µl PCR grade water for each reaction. PCR product 265 bp was obtained using Forward 5′- AGCTGGCCCTGGCACTGACTCTGCTCT -3′ and Reverse 5′- ATGGAAACACCTTGCTTCTTCTCCCTC -3′.

Initial denaturation of 94°C for 2 minutes 1×, followed by 94°C for 30 seconds 58°C for 45 seconds, and annealing at 72°C for 45 seconds 30×, and final extension at 72°C for 10 minutes were done. The PCR product obtained were subjected to digestion by *Fok1* endonuclease enzyme in 20 µl of reaction volume, i.e., 12.5 µl of PCR product, 1.5 µl of *Fok1* endonuclease enzyme, and 6 µl of 1X buffer. The reaction mixture was incubated at 37°C for 4 hours and then subjected to 2% agarose gel electrophoresis. The digested product split in to two distinct 296 bp and 69 bp fragments figure. Genotypes were assigned after visualizing in UV transilluminator on the basis of the size of fragments.

Estimation of serum vitamin D and calcium

The level of serum vitamin D was measured by commercially available diagnostic kit by Immundiagnostik AG, Germany. Principal of competitive Enzyme Linked Immunosorbant Assay (ELISA) is used which allows accurate quantitative determination of the serum vitamin. Vitamin D is considered as low when it is below 30 ng/ml and toxic when its level is >100 ng/ml.

Serum calcium was measured by using commercially available calcium assay on the ARCHITECT Systems ™ (Abbott, USA) according to the manufacturer instruction. The results were interpreted as kit instruction provided.

DEXA scan imaging

DEXA scan imaging was done to find the bone mineral density. Dual-energy X-ray absorptiometry (DEXA) scan (Hologic, Waltham, MA) was used measure Bone Mineral Density (BMD) at hip and lumbar spine region. The reference value of T and Z scores was entered in the DEXA machine with software for the Asian reference value. We considered osteopenia when the T score of total lumbar spine or total hip was between −1 and −2.5, and osteoporosis was considered when the *T* score was <−2.5 [19].

Results

Seventy eight diagnosed patients were enrolled in the study where the male and female numbers were 25 (32%) and 53(67%), respectively. The mean age for the male was 45.04 years and the females were around 52.45 years. Seventy eight healthy controls were also enrolled in the study in whom 37 (47.4%) were male and 41 (52.56%) were females.

Serum calcium and vitamin D level

Calcium is important for many body functions. The normal range of free calcium is 8.4-10.4 mg/dl. Serum calcium was measured for all the patients and control group. The hypocalcemia for the patients were $n = 29$

(17.17%) in which male were *n* = 8 (10.15%) and female were *n* = 21 (26.92%), while the remaining patients were normal for serum calcium level. No controls were found to be hypocalcemic.

Vitamin D is mainly involved in the bone health therefore serum vitamin D was measured for all the patients and control group. The hypovitaminosis D for the patients were *n* = 50 (61.10%) in which male were *n* = 31 (39.74%) and female were *n* = 19 (24.35%), while the remaining patients were normal for serum vitamin D level. The hypervitaminosis D for the patients were *n* = 3 (3.84%) in which male was *n* = 1 (1.74%) and female were *n* = 2 (2.56%), while the remaining patients were normal for serum vitamin D level.

Bone mineral density

The bone mineral densities were measured for all the patients and control using DEXA scan. The osteopenia for the patients were *n* = 41 (52.56%) in which male was *n* = 16 (20.51%) and female were *n* = 25 (32.05%). The osteoporosis for the patients were *n* = 19 (24.35%) in which male was *n* = 4 (5.21%) and female were *n* = 15 (19.23%), while the remaining patients were normal for bone mineral density. All the control individuals were normal for bone mineral density.

Goodness-of-fit test for Hardy-Weinberg equilibrium and comparison of Fok1 polymorphs between cases and controls

There was no deviation from the Hardy-Weinberg genetic equilibrium for the genotypic distribution of VDR Polymorph in group as shown in Table 1. In the analyzed VDR polymorph, Wild (FF) genotype was with highest frequency, the Ff heterozygous was found to be modest frequency, and the Mutant (ff) genotype was with lowest frequency. The wild genotype for the disease group were *n* = 45 (57.69%) and control were *n* = 72 (92.30%). The heterozygous genotype for disease group were *n* = 29 (37.69%) and control were *n* = 6 (7.69%). The homozygous mutant genotype disease group were *n* = 4 (5.12%), and no control was found for homozygous mutant as shown in table.

Association analysis

The F-allele which is minor allele and increase the risk of functionally deficient form of vitamin D receptor increased the risk for decrease bone mineral density and osteopenia nearly threefold [Odds Ratio (OR): 2.8; 95% Confidence Interval (CI): 3.2-19.0; $p \leq 0.001$]. The genotype frequencies (Ff+ff *vs.* FF) were also compared under dominant model and they even showed an increase risk of disease (OR=6.79; 95% CI=3.41-22.6; $p \leq 0.001$). The OR cannot be calculated for recessive model since there was no ff genotype in the control group. In case of recessive model (ff *vs.* FF + Ff), OR for ff is indefinitely large.

Discussion

Bone is the structural framework of the body and is metabolically active to support the weight of the body. Osteocytes, osteoclasts, and osteoblast are continuously active to maintain the bone health to be strong enough

Table 1. *Genotypic, allelic distribution, and risk assessment.*

	Genotype distribution				
	FF	**Ff**	**ff**	**Total**	**HWE (p-value)**
Disease group	45 (57.69%) %)	29 (53.69%)	4 (5.12%)	78	0.80
Control	72(92.7%)	6 (7.69%)	0 (0%)	78	0.72
Total	117	35	4	156	0.48
	Allelic distribution				
	F	**f**		**Total**	
Disease group	119 (76.28%)	37 (23.71%)		156	
Control	150 (96.15%)	6 (3.84%)		156	
Total	269	43		312	
	Genetic model and statistical data				
Genetic model	**Odds ratio (95% CI)**	**Chi-square**	**Degrees of freedom**	**P**	
Allele contrast (F vs. f)	2.8 (3.2-19.0)	25.92	1	<0.001	
Dominant (Ff+ff vs. FF)	6.79 (3.41-22.6)	24.92	1	<0.001	
Recessive (ffvs FF+Ff)	Infinite*	Infinity	1	0.12	

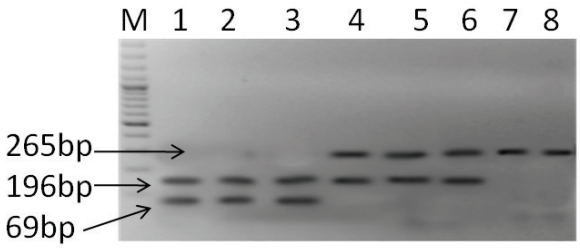

Figure 1. *Restriction Endonuclease digestion for FokI polymorphism. M is DNA ladder Lan 1, 2 and 3 indicate ff genotype consisting of 196 and 67 bp fragments, lane 4, 5 and 6 shows Ff genotype consisting of 265 and 196 bp fragments, while lane 7 and 8 indicated undigested product of 265 bp is the FF genotype.*

to support body. These cells perform their function in close coordination to calcium, magnesium, parathyroid hormones, and vitamin D [20]. Additional factor which affect the concentration and availability of these minerals and hormones is the genetic make up of the individuals along with exposure to the environment. Hence, differential genetic makeup (polymorphs) of the vitamin D receptor affect the availability these minerals and vitamins. These polymorphs behave differentially in different population and ethnicity. Therefore, the presence of bone disorders are different in different population [21,22]. Hence, the present study was designed to find the association of *Fok1* polymorphism with bone health in Pakistani population. A case control study was performed in which the wild genotype for the disease group were $n = 45$ (57.69%) and control were $n = 72$ (92.30%). The heterozygous genotype for disease group were $n = 29$ (37.69%) and control were $n = 6$ (7.69%). The homozygous mutant genotype disease group were $n = 4$ (5.12%) and no control was found for homozygous mutant. The association studies found that the Ff, ff genotype, and frequency of the f allele is significantly associated with the low bone mineral density and the risk of osteoporosis.

The f allele which is minor allele and increase the risk of functionally deficient form of vitamin D receptor increased the risk for decrease bone mineral density and osteopenia nearly three fold (OR: 2.8; 95% CI: 3.2-19.0; $p \leq 0.001$). The genotype frequencies (Ff+ff *vs.* FF) were also compared under dominant model and they even showed an increase risk of disease (OR = 6.79; 95% CI = 3.41-22.6; $p \leq 0.001$).

Mohammadi et al. [23] reported a significant association between the Fok1 and Bsm1polymorphism with osteoporosis in more than 50 reviewed studies [23]. The finding of these reported studies is consistent with our findings.

A study in Iranian population also showed similar results to our study [FF genotype $n = 45(57.69\%)$] the FF genotype was (56.5%) and the remaining were either Ff or ff genotypes [24]. Das et al. [25] reported avery similar results to our finding FF was 59%, Ff in 36%, and ff in 5% in Indian population, while in our study FF is 57.69%, Ff is 37.69%, and ff is 5.12% [25]. An increase heterozygous and homozygous for minor allele was reported in Italian population (FF = 33.2%, Ff = 32.8%, ff = 34%) [26]. A study in Spanish population also reported similar findings as to the Italian population FF = 40.4, Ff = 48.0, ff = 11.6% [27].

The association of lower BMD with ff genotypes is also frequently reported. Abrams et al. [28] and Hossein-Nezhad [24] reported that Ff and ff genotype increase the risk of risk of lower bone mineral density. The finding both way the genetic association of the genotype with lower BMD and frequency of the genotype in studied population is similar to our study.

Conclusion

Genetic testing of the VDR polymorphism should be considered while treating the patients for osteoporosis and osteopenia in Pakistani population.

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Declaration of conflicting interests
The authors of this article have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

Ethical approval
The study was performed in accordance with the Declaration of Helsinki and was approved by the Research Ethics Committee of Islamabad Diagnostic Centre Pakistan (IDC/REC/13-1).

Consent for publication
Written informed consent was obtained from all participants.

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