Association of Vitamin D3 Receptor Polymorphism with Bone Density

By

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Bone diseases
Gene polymorphism
Bone mineral density.

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بسم الله الرحمن الرحيم

Department of Bioinformatics and Biotechnology

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Dated: _ _

FINAL APPROVAL

It is certificate that we have read the thesis submitted by Mr. Najam Farooq and it is our judgment that this project is of sufficient standard to warrant its acceptance by the International Islamic University, Islamabad for the M.S Degree in Biotechnology.

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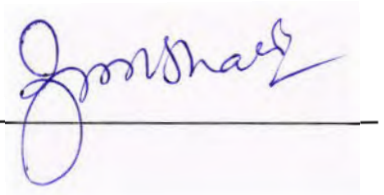

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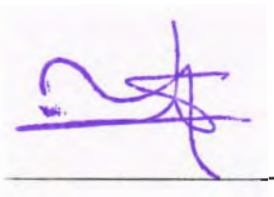

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a partial fulfillment of requirement for the award of the
Degree of MS. Biotechnology

DEDICATED

To

Hadi Raza, my lovely Child

DECLARATION

I hereby declare that the work present in the following thesis is my own effort, except where otherwise acknowledged and that the thesis is my own composition. No part of the thesis has been previously presented for any other degree.

Date: January 22, 2016

Najam Farooq

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List of Abbreviations

1, 25-$(OH)_2D_3$	1, 25-dihydroxyvitamin D3
25-(OH) D_3	25-hydroxyvitamin D3
BMD	bone mineral density
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleic triphosphate
DEXA	dual energy X-ray absorptiometry
HWE	Hardy-Weinberg equilibrium
LD	linkage disequilibrium
MAF	minor allele frequency
PCR	polymerase chain reaction
RFLP	restriction fragment length polymorphism
SNP	single nucleotide polymorphism
UTR	untranslated region
VDR	vitamin D receptor

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I bow my head in deep gratitude to **ALLAH, The Almighty**, Who is the intact source of knowledge and wisdom to me and all mankind. I owe my deep respect to **Prophet MUHAMMAD (ﷺ)**, the city of knowledge and mercy for the entire universe.

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Najam Farooq

Abstract

Osteoporosis is the bone disease characterized by demineralization of the bone. Among the key genetic factors responsible for the osteoporosis include the vitamin D receptor (VDR) gene polymorphism. A T/C polymorphism which is found at the VDR gene translation initiation site changes the codon from ATG to ACG, and makes FokI restriction endonuclease site, is being linked with BMD (bone mineral density) variation and the degree of severity of the disease in patients of osteoporosis. In case of "f" the translation starts from the first ATG and make the Fok1 restriction site, while the allele "F" imitation of translation starts from the second ATG and result no restriction site. In this study, we have found FokI polymorphism association in a group of patients with osteoporosis. The osteoporotic patients were n=19(24.35%) the patients with osteopenia were n=41(52.56%), and normal n=18 (23.79%) groups alone with normal control. The wild type allele for the disease group were n= 45(57.69%) and control were n=72 (92.30%). The heterozygous allele for the disease group were n= 29(53.6917) and control were n=6(7.69%). The homozygous mutant allele for the disease group were n= 4(5.12%) and no control was found for homozygous mutant. The association between markers of bone health via BMD (Dexa Scan) and Fok1 polymorphism of the Vitamin D Receptor gene in 78 patients and control was studied. Serum Calcium and Vitamin D was also measured. The defective polymorphism of Fok1 at the initiation site of VDR was significantly linked with Bone health (BMD, serum calcium, and vitamin D). Our results are suggestive of pathogenic association among the VDR Fok1 polymorphism and bone health.

Key words: BMD, VDR, Fok1

1. INTRODUCTION

The endocrine system of vitamin D is essential for the maintenance of homeostasis of bone and calcium. The active type of vitamin D is 1, 25-dihydroxyvitamin D (calcitriol), the circulating level of which is regulated strictly and performs via a specific receptor in the mediation of its shares of genomics on almost all aspects of calcium homeostasis. Additionally, vitamin D also affect the metabolic pathways, like as those involved in the cancer and immune response (Fig. 2.2, Haussler *et al.,* 1998). The dietary Vitamin D or the 7-dehydrocholesterol inactive and cannot perform its physiological function and must be converted to its active form to perform its physiological activity. In the skin hydroxylase enzyme act pro vitamin D3 to produced vitamin D3 in; Though, in humans, the sources of pro vitamin D and vitamin D other than kidney only can add significantly to blood or plasma levels of these precursor of vitamin D3 in certain pathological condition like; chronic renal failure, pregnancy and in other pathological disorders including granulomatous disorders, sarcoidosis, rheumatoid arthritis and tuberculosis. Vitamin D play its role after it is attached with its receptor and it acts as a transcription factor which is ligands activated. The transcription of gene is regulated by vitamin D receptor which initiate by binding ligand to vitamin D receptor (VDR), making heterodimer of the X receptor (RXR) of retinoid and response element attachment to the heterodimer and attachment of protein in pre-transcription initiation complex. Therefore, alteration or modification in the VDR gene can results the disruption of the gene activation pathway. This disruption in the pathway leads to pathological changes like altering calcium metabolism, immune function and cell proliferation. This can be explaining by protein sequence change for example, a VDR gene deleterious mutation causes erratic monogenic disorder known as 1, 25-dihydroxycholecalciferol resistant rickets (Sone *et al.,* 1990). Different variation like SNPs, CNV and other sequence variations (polymorphisms) are also found in the vitamin D receptor (VDR) gene. The significance and pathophysiology of these variations although has been analyzed but the effect on the expression of vitamin D receptor and its

role are not known. Studies are required to establish the association between these variation changes and the level of metabolite.

Changes in sequence with a frequency of 1% or greater in population is known as polymorphism. The polymorphism may be in the coding region or non-coding regions. These variations may pathogenic or nonpathogenic depending upon the amino acid change. Although changes in the introns (non-coding) gene sequence do not translate into protein. Sequences alteration in the controlling part like promoter or other regulatory elements would then affect expression level of the gene, and therefore the quantity of the protein. For example, the integrity of mRNA would get effect with the alteration in 3' (UTR) sequence leading to ambiguity to protein translation while the un-translated region 5'-promoter VDR gene may affect the patterns and mRNA expression levels.

The variations or changes in the coding regions or exonic DNA may take place which results changes in protein sequence and structure. Some variations in the exonic region do not alter the sequence of protein, which is known as synonymous polymorphisms. Mostly of these alteration delete or create cutting sites for restriction enzymes. These restriction enzymes digest PCR product creating segments of DNA with different lengths. These DNA fragments can be separated by electrophoresis. This tool is used for the identification of polymorphisms is termed as restriction fragment length polymorphism (RFLP). But now a day with the advancement of technology finding the allelic and genetic variation is now easier and quicker with the help of DNA sequencing, but PCR-RFLP is still the method of choice to screen large population.

The exploring of genetic variants and association with the risk of genetic disease may help in designing the preventive medicine. In general, to test the susceptibility of the polymorphism we used case control study and try to find out that whether the frequency of the polymorphism was high in cases as controls. The risk was calculated by using different statistical tool like Pearson correlation coefficient and odds ratio. If the risk is high, then it gives a good idea that the polymorphism is involved in the disease. Sometime the association of the disease is interrupted by the protective role of the polymorphism in the nearby gene. Thus knowing a polymorphism may sometime be not

helpful to reach a conclusion therefore a set of polymorphism should be studied to find out the possible association between these polymorphisms. Understanding their association at the functional and genetic level is important to establish haplotype.

A number of different restriction enzymes help to find RFLP site in gene of vitamin D receptor; some studies using PCR-RFLP has been describe. The examples include Tru9I (Ye *et al.,* 2000), TaqI (Morrison *et al.,* 1994), BsmI and EcoRV (Morrison *et al.,* 1992) and ApaI (Faraco *et al.,* 1989). All these RFLPs are between 9 and 8 exons and found in the area of unknown function. In the early nineteen another important polymorphism was studied by PCR-RFLP named as FokI polymorphism. (Gross *et al.,* 1996, Saijo *et al.,* 1991) The polymorphism is T>C change and is situated in exon 2. The nucleotide change is found in the initiation site therefore when base changes from cytosine to thiamine it will create another start site resulting truncated protein with different size. In case of transcription factor, it has been find out that smaller protein (424 aa) are more effective in transactivation than Most of the long form (427 aa) as a TF. The gene and its expression are certainly restricted to cell and tissue type, therefore the effect of polymorphism in different genes and cell type can produce different affects (Uitterlinden *et al.,* 2004a).

Novel polymorphisms can be searched out using direct sequencing approach, for example, Brown *et al.,* (2000) found two new polymorphisms a Cytosine > Thiamine change in the vicinity of 2^{nd} exon in 7^{th} exon, an insertion / deletion. Similarly, Arai *et al.,* (2001) find a new polymorphism in (Cdx2) gene using the Sanger sequencing in female population of Japan. This polymorphism is in the promoter region of VDR and a nucleotide change of G > A. The polymorphism is named after the name of TF known as CDX2 tract.

The ethnical finding is also important; the frequency and effect of polymorphism may be different in different population. According to the study there were several polymorphisms at the VDR gene's 3 prime un-translated region (Fang *et al.,* 2003). While Morrison *et al.,* (1994) reported 13 different polymorphisms and Durrin *et al.,* in (1999) reported 7 different polymorphisms. It is very interesting that there were just two

groups same in both articles. But the fact was that these studies were from different population.

The relationship of a polymorphism with disease not as a matter of course implies that the affiliation is pathogenic rather it figures the danger of the sickness. The association of genotypes of polymorphism with different other genotypes with in the same population is called haplotype association while the association of allele with the allele of the other polymorphism is called linkage disequilibrium (LD) (Wall and Pritchard, 2003). The association will be high if there is very low rate of recombination.

In practice, we test the occurrence of 1 allele to the occurrence of the allele in adjacent related gene to find out linkage disequilibrium. In linkage disequilibrium the alleles are represented with blocks. The blocks which are close to each other are called haplotype. The size of the blocks may be different ranging from 10-20kb and they are used in determining the cause of certain genetic disease. The advantage of the LD score calculations to find the allele haplotype association; By finding the haplotype association determining a single polymorphism is indicative of the other polymorphism of the same gene. Then, once we know the carries risk allele haplotype, different techniques can be used to determine that whether polymorphism is really responsible for the observed phenotype. Many studies were done to find out the LD of the VDR gene polymorphism. Although the available information is very low but there are certain polymorphisms with high degree of LD score. There are five different haplotype associations between the TaqI, BsmI, ApaI and EcoRV (Uitterlinden *et al.,* 2004). In addition, Uitterlinden *et al.,* (1996) showed strong linkage disequilibrium between BsmI genotypes and 3 'UTR (VNTR) genotypes. Thus, research on the diverse polymorphism of vitamin D receptor and haplotype analysis can provide us with better understanding of the disease.

In a late article, (Nejentsev *et al*., 2004) Block 94 kb inside of a 164 kb area of 12q12-q14 chromosome around the VDR quality. 245 polymorphisms were observed which appears, by all accounts, to be 3 pieces of LD. Polymorphism inside of every square has diminutive, if any, LD with polymorphisms in an alternate piece. They looked at the LD hinders from four unique populaces find noteworthy likeness to the LD designs

in all European populaces, yet not with the African populace. Along these lines, they reasoned that European populaces show relative point of preference to identify beginning relationship of the ailment, on the grounds that less label polymorphisms are expected to describe a typical variety. They likewise demonstrated that African populaces have more differences of haplotypes. Extra haplotype investigation can likewise help the fine mapping of a shrewd variety. In this way, hereditary examination in populaces of diverse beginning encourages the investigation of complex sicknesses.

Extremely late studies have started to reveal insight into the practical impacts of polymorphisms at the non-coding locales of the VDR quality. Tooth *et al.*, 2005 examined 15 haplotypes '1a/1e, 1b promoter locale and 3' UTR 5 and discovered extremely solid relationship with the danger of bone crack. Also, they performed a useful examination demonstrating that variations conveying the promoter first/1a with an expanded danger had lesser mRNA values. It was likewise demonstrated that, in an osteoblastic cell line, the vicinity of the 3 'UTR of danger haplotype brought about a 30% expansion of mRNA debasement. D'Alesio et al., 2005 examined two polymorphisms at the promoter district of the VDR quality (GA-1521C and 1012G). They found that an essential change in one of the option locales prompted a radical change in the development of protein-DNA complex with a littler stature of eleven years to grown-up size.

1.1 Objective of Study

This research involves several approaches including molecular genetics, genetic epidemiology, bio-statistics and bioinformatics. The objective of the current study includes:

i. Measurement of Serum vitamin D3 and total calcium of the patients diagnosed with vitamin D deficiency.
ii. Genetic polymorphic study of VDR gene and its association with bone density in Pakistani population.

2. REVIEW OF LITERATURE:

The dietary vitamin D or the 7-dehydrocholesterol inactive and cannot perform its physiological function and must be converted to its active form to perform its physiological activity. The vitamin D can act after binding to the receptor. There are many variations in the receptor which result the incapability of the receptor to accept the optimum vitamin D.

2.1 Structure of the VDR gene

According to Miyamoto *et al.,* (1997), it was possible in ten years to make the VDR genomic structure clear after cloning of human VDR cDNA in 1988 by (Baker *et al.,*.1988). Simultaneously, Croft *et al.,* (1998) demonstrated multiple tissue specific transcripts (exon 1d to 1f) which differ in the VDR gene at the 5. Although much of what has become known before the Human Genome Project was completed, the data Croft *et al.,*.1998) is not yet integrated into the gene databases Celera or NCBI. The size of the VDR gene and the exact location of 5'-terminal exons were still unknown (Fig. 2.1). At first the position of the VDR gene on the physical map of chromosome 12 was indicated mapping link (Labuda *et al.,*.1992) and later made better by fluorescence in situ hybridization (FISH) and mapping by radiation hybrid (Taymans *et al.*.1999).

However, in the above mention studies only the relative location of the VDR gene was find out and it is inconclusive to understand the role of polymorphism with the disease. Therefore, association studies are required to found the possible risk or cause of the genetic disease associated with the polymorphism. To know further details of the associated risk and disease in the VDR gene haplotype association studies are required. Therefore, the nearby genes and polymorphism of the VDR gene should be studied to explain the Haplotype.

2.2 VDR polymorphisms

VDR polymorphisms studies were initially focused on the 3 'end of the gene, from a combination of the VDR polymorphisms with BMD along with bone remodeling was reported by (Morrison *et al.,* 1992 & Morrison *et al.,* 1994). A partial correction of

the data on the association of BMD appeared a few years later in which the association was much diminished (Morrison *et al.,* 1997). A number of polymorphisms VDR are shown that were known before the start of the project. In the region of intron 8 and exon 9, Apa I26, EcoR V23, Bsm I23, I24 and I27 Taq Tru9 length polymorphism restriction fragment (RFLP) were discovered and used in association studies. For the 3' untranslated region (3`UTR) of the VDR gene sequenced two individuals who were homozygous for the most common haplotypes BSM-Apa-Taq: Bat-bat and bat bat and reported 13 distinct polymorphic sites, including a poly (A) -tract with a variable number of adenosine. (Morrison *et al.,*.1994; Durrin *et al.,* 1999) extended this method and sequenced the 3 'UTR in eight subjects and identified seven polymorphisms, four of which were mutual and 3 were uncommon in the 8 subjects analyzed. However, the number of individual selected for the study is very low in number, it is likely that most yet discovered and undiscovered polymorphisms exist in the 3 'UTR complete.

So far, only two polymorphisms have been reported in the exons encoding the VDR gene. One is the TaqI RFLP, which is located in exon 9, but this does not alter the VDR protein, amino acid sequence. Another is the Fok I RFLP Compared to the original sequence of the VDR cDNA, according to Saijo *et al.,* (1991) there are two sites of early potential translation initiation (ATG) and comparisons of subsequent sequences showed a T to C polymorphism occurs (ATG to ACG) to first possible starting site (Gross *et al.,* 1996). This polymorphism is also known as polymorphism of initiation codon (SCP), and is defined by the sequence using the Fok I restriction enzyme in a RFLP test (Arai *et al.,* 1997).

So, two protein variants may occur for the two start sites available: a long version of the VDR protein (T allele nucleotide allele detected as "f", also called the M1 form, namely methionine d first position) with a shortened protein by 3 amino acids (C allele nucleotide allele detected as "F", also referred to as M4 form, ie, methionine at the 4th position).

This is the only protein known polymorphism in the VDR gene so far (Brown *et al.*, 2000). Sequenced the coding region VDR in parathyroid tumors 59 to find mutations (Brown *et al.*, 2000). In addition to the previously reported polymorphisms Taq I and Fok I, they reported no polymorphism in the coding region and intron polymorphisms found two relatives of exon 2 and 8.

Another VDR polymorphism was found by sequence analysis of a target area in the VDR gene promoter region. After (Yamamoto *et al.*, 1999) found a binding site for CDX-2, a factor- specific intestinal transcription that in the region of the VDR promoter 1a (based on the genomic structure of the VDR Miyamoto *et al.*,.1997) & (Arai *et al.*, 1997) reported of a G to a sequence variation in this binding site between Japanese women (Arai *et al.*, 2001).

2.3 Metabolism of Vitamin D

Sunlight light exposure of the skin is required for the production of Vitamin D3 from 7-dehydrocholestrol or taken through dietary sources. 25-hydroxylation of vitamin D3 is the principal step in the production of active vitamin D (Jones *et al.*, 1999). Vitamin D3 25-hydroxylase is the enzyme which catalyzes the reaction and converts the inactive vitamin D3 to active vitamin. Hydroxylation of vitamin D3 25 (OH) D3 take place mostly in the while in small amount occurs in other tissues including the kidney, osteoblasts and endothelial cells (Axen *et al.*, 1995; Ichikawa *et al.*, 1995; Reiss *et al.*, 1997). There is controversy about the function of the enzyme that whether an enzyme can catalyze the 25- hydroxylation and the biochemical function of the enzyme is unclear. The major circulating form of vitamin D3 is 25 (OH) D3 and a very little amount of vitamin 25 (OH) D2 in humans (Jones *et al.*, 1999). When there is increase in the synthesis of vitamin D3 it increases the calcium deposition in the bone thereby decreasing the serum calcium level. Expression 1α-hydroxylase has not only been confirmed in the proximal convoluted tubules but also in the further distal regions of the nephron called the thick ascending loop of Henle, the distal convoluted tubule and the cortical collecting ducts (Zehnder *et al.*, 1999).

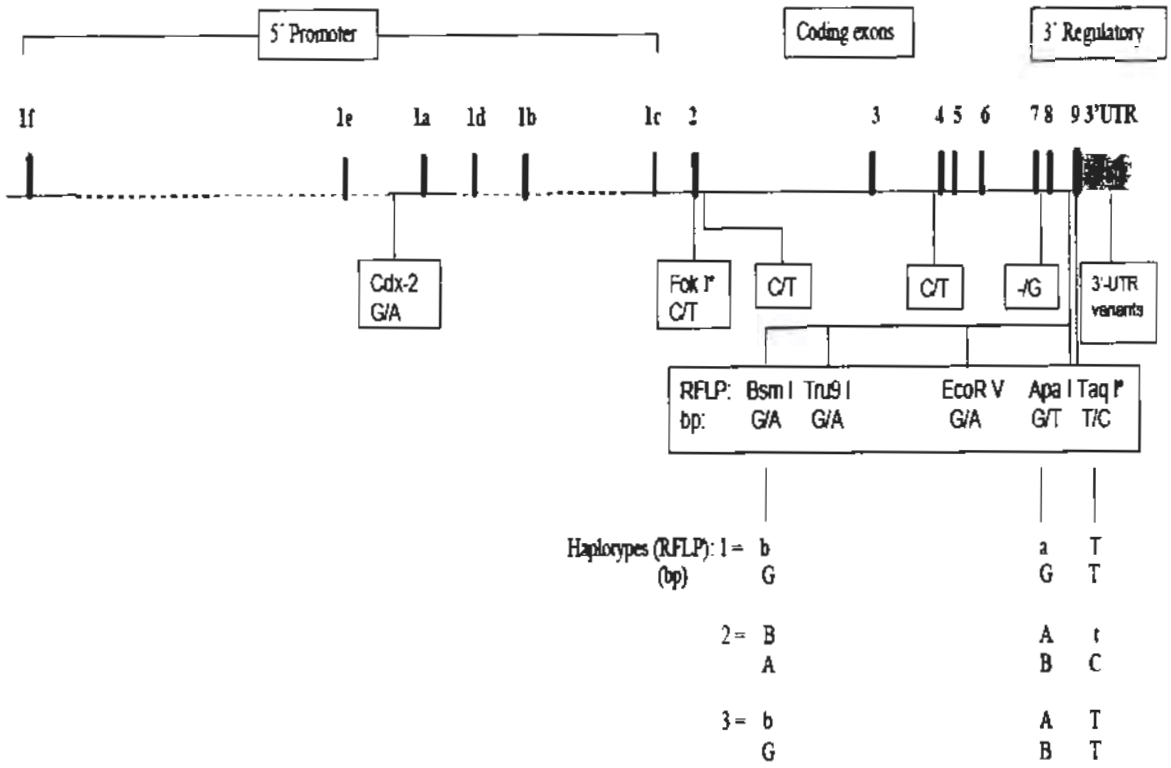

Figure 2.1: The genomic structure of the VDR gene adopted from Renate *et al.*, 2011

A number of publication showed extra renal expression of 1α-hydroxylase enzyme such as keratinocytes (Bikle *et al.*, 1986; Fu *et al.*, 1997), testes brain (Fu *et al.*, 1997; Zehnder *et al.*, 2001), the cultured bone cells (Howard *et al.*, 1981), macrophages (Overbergh *et al.*, 2000; Monkawa *et al.*, 2000), placenta (Delvin and Arabian,1987; Diaz *et al.*, 2000), cells of the prostate (Schwartz *et al.*, 1998), colon adenocarcinoma (Cross *et al.*, 1997; Tangpricha *et al.*, 2001), non-small lung carcinomas in small cells (Jones *et al.*, 1999b), and pancreatic islet cells (Zehnder *et al.*, 2001). The function of 1α-hydroxylase in the extra renal tissues has not been explained but assume to have involvement the local production of 1,25 (OH) 2D3 autocrine or paracrine for regulation. 1,25 (OH) 2D3 perform its function after binding with high affinity to the vitamin D receptor (VDR) (Haussler *et al.*, 1997; Jones *et al.*, 1999) in a many target tissues. After binding to VDR homodimers complex either form a heterodimer with the retinoid X

receptor (RXR), or other steroid receptors, after binding to response elements of vitamin D (VDRE) promotes activation target genes (Figure 2.1).

The gene products which can be up or down regulated at the transcriptional level can be seen in Figure 2.1. Mechanism of Action 1,25 (OH) 2D3 after binding of the complex to form VDR either homo or heterodimers with, e.g. retinoic X receptor. Binding VDRE fascinates coactivators and initiating transcription of target genes. Inactivation of 1,25 (OH) 2D3 24 begins hydoxylation by the enzyme 25-hydroxyvitamin D3 24-hydroxylase (24-hydroxylase). 24-hydroxylase is not only restricted to Vitamin D responsive traditional fabrics such as kidney and intestine, but rather is ubiquitously expressed (Jones *et al.*, 1999a). 1, 25 (OH) 2D3 up regulates RNA co-activator 1,25 (OH) 2D VDR RXR VDRE polymerase II 13 the gene for 24-hydroxylase and its promoter comprises two VDREs (Ohyama *et al.*, 1994; Jones *et al.*, 1999).

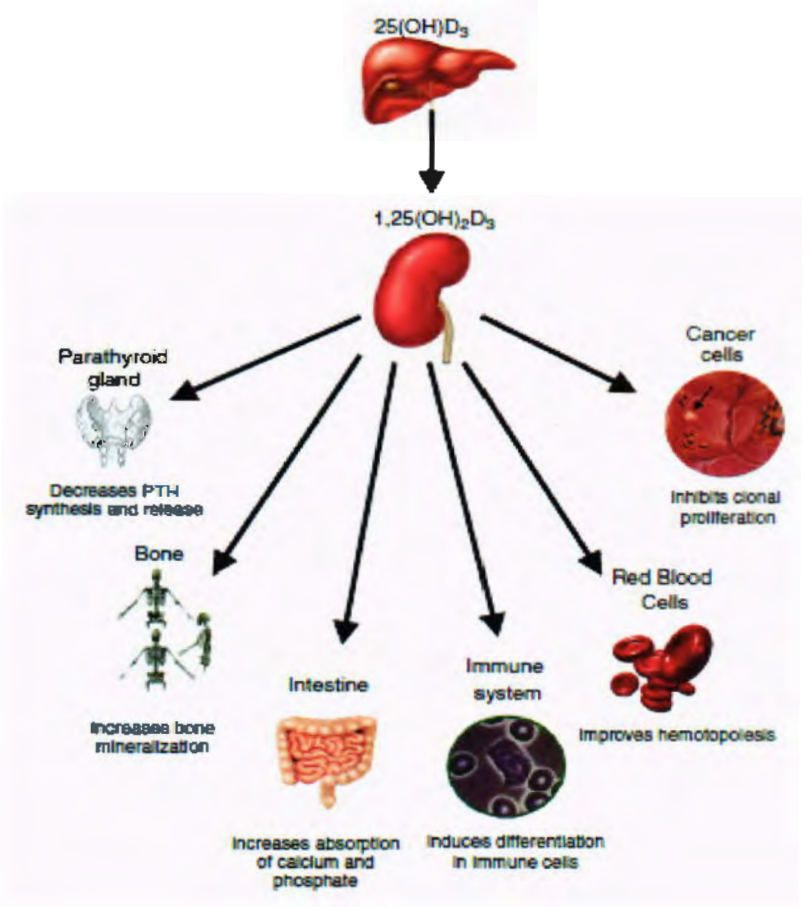

Figure 2.2: Vitamin D effect on target cells. (Adopted form Jose *et al.*, 2005)

2.4 VDR POLYMORPHISM AND ASSOCIATION WITH DISEASE

2.4.1 VDR polymorphism and renal patients

Vitamin D has a complex physiological role and act in many organs as bioactive molecule performing a vast variety of functions therefore the effect of VDR polymorphisms in patients of kidney disease have been elucidated. The patients of the kidney disease suffer from secondary hyperparathyroidism. The reason for this is that parathyroid cells and parathyroid hormone (PTH) synthesis is regulated by Calcitriol-VDR complex (Szabo *et al.*, 1989; Patel *et al.*, 1994). Thus, the complex of the calcitriol and VDR inhibits proliferation of the parathyroid gland cells and synthesis of PTH. To stop SHPT and reach to the cause of hypercalcemia the patients of SHPT uremia were given vitamin D metabolites. Some patients were found resistant to treatment with processing to calcitriol bolus of acting directly on the parathyroid gland, after determining the presence of VDR on parathyroid gland,

The SHPT affects the nodular growth and the VDR compactness in the nodules He found that nodular growth decreases in SHPT and the VDR density also decreases as compared to hyperplastic cell in the surrounding gland environment Fukuda *et al.*, (1993).

With the discovery; that VDR effect parathyroid cells has unlocked a new and exhilarating field of research. Carling *et al.*, 1995 studied the association of BsmI polymorphism with primary Hyperparathyroidism. These results compel scientist to explore the association of VDR polymorphism and secondary hyperparathyroidism in renal disease patients. Tsukamoto *et al.*, (1996) published a higher frequency of allele b in hemodialysis patients with secondary hyperparathyroidism. Then Fernandez *et al*, (1997) perform a large study to exclude the effect of time on hemodialysis as a vital risk factor for secondary hyperparathyroidism. In addition; they divided patient population into two groups based on PTH and found that there was no difference in the distribution of the BsmI polymorphism between high PTH group and the normal population. However, allele B was found in high frequency in patients than controls. Thus, even after being treated in the same conditions as in the other group, patients with B allele showed a relative hypoparathyroidism. It is crucial to mention that all the interfering factors were omitted from this group with patients any other factor affecting parathyroid function (diabetes, parathyroid hypofunction, hyperalbuminemia and hypercalcemia). Similar results were observed by other researchers (Tagliabue *et al.*, 1999, Nagaba *et al.*, 1998).

The major risk factors were excluded and the BB genotype were again tested and found that the PTH level is lower than the normal moreover it was also observed that the patients did also have increased level of calcitriol (Marco *et al.*, 1999). In order to outline the clinical inferences of these findings, the response of different genotype of patients with a single bolus of calcitriol was tested (Marco *et al.*, 2001). The BB genotype patient's further decrease of PTH levels even supplemented with phosphorous and calcium. It has been found out that patients with the BB genotype get the severe hyperparathyroidism and removal of parathyroid is some time required. However, the

patients with bb genotype required a longer dialysis time and therefore develop delayed (Borras *et al.*, 2003).

According to Marco *et al.*, (2001) bb genotype was overrepresented among hemodialysis survivors. It has been found out in the cox population that BB are at four-time higher risk of mortality as compared to bb genotype, but the mechanism involved in this benefit is unknown. However, it has been found out that the Patients with BB genotype represent with low level of PTH, which in itself is a risk factor for increased mortality factor. As VDR is present on each organ and system and their presence on the cardiac muscles and vascular tissues indicated a relationship to the polymorphism and VDR influence mortality. In addition, the association of genotypes with BsmI hemoglobin (Erturk et al., 2002) and bone mineral loss (Karkoszka *et al.*, 1998) could explain the increased survival of some patients.

The determination of higher risk of SHPT along with a specific BsmI genotype is difficult due to the diverse and occasionally contradictory results in the literature (McCarey *et al.*, 1998; Schmidt *et al.*, 1997). Secondly, because of the fact that the calculation of the risk tale place with numerous variables, and some of those variations in many studies (sample size, the design of the study, etc.). However, all of the published reports specify that there is some impact of BsmI genotype on the progress of SHPT. The BsmI genotype affiliation with PTH was also tested after renal transplantation. In this case, all the reports published so far established the association of genotype bb with increased PTH values (Messa *et al.*, 1998; Giannini *et al.*, 2002). However, the occurrence of the bb genotype seems to be related to a superior recovery of the bone mineral density three months after transplantation (Falkiewicz *et al.*, 2005; Torres *et al.*, 1996).

Although it is a fact that many polymorphisms effect the disease and risk but the other polymorphism were not assessed in the renal failure. There is only one published paper according to Vigo *et al.*, (2005) FokI FF genotype shows high level of PTH in renal disease patients. As VDR FF genotype directly affect the 1,25 (OH) 2D3, it would

be reasonable to expect the contrary result. However, the researchers state that, before the renal failure, patients with the FF phenotype had low level of plasma PTH. So after kidney failure the 1,25 (OH) 2D3 decreases with decreasing control on the secretion of PTH which results in the increase level of PTH in FF patients. In pre-dialysis patients, levels of 1,25 (OH) 2D3 are low. Thus, the researchers debate that the greater sensitivity of the FF genotype is on the suppression of PTH. Numakura *et al.*, (2005) studied the effect of Taq1 polymorphism and the risk of having post-transplant diabetes mellitus (DM). According to Yokoyama *et al.*, (1998) the Apa1 aa polymorphism is linked with PTH and osteocalcin in pre-dialysis patients and with a higher sensitivity for detecting the calcium changes and, consequently, to regulate the PTH secretion

2.4.2 VDR polymorphisms and cancer

1,25 (OH) 2D3 is so diversely involved in the biology of humans that certain cancer has been associated with the serum vitamin D level and vitamin D receptor polymorphism. These cancers include breast cancer, colon and prostate cancer. Like association of bone disease and renal disease the results are provocative and even inconsistent. These apparent denials can be explained by differences in the serum vitamin D level of the population ethnicity and sample size.

The first report of VDR polymorphism with cancer association was published by Ingles *et al* (1997) He studies the association of prostate cancer with the VDR (poly A) polymorphism in US population. Taylor *et al.*, (1996) studied the association of Taq1 polymorphism of VDR and a higher risk of prostate carcinoma. The genotype tt shows lower risk of prostate cancer and high level of. These results were confirmed by another study in European population (Cerro *et al.*, 1999). While there are certain reports which does not show any association between the taq1 or poly "A" polymorphism and prostate cancer. Kibel *et al*,. (1998) and Cheteri *et al.*, (2004) found no linkage between the two polyA or TaqI polymorphisms and death from prostate carcinoma in American patients. Gsur *et al.*, (2004) studied the association of TaqI polymorphism and higher risk of prostate carcinoma and found no association in Japan, a dearth of association between

TaqI and the risk of prostate cancer has been studies (Furuya *et al.*, 1999, Habuchi *et al.*, 2000).

The other VDR polymorphisms and association with prostate cancer are contradictory like the taq 1. Fok1 association was established in some studies e.g Xu *et al.*, (2003) find the association while Correa-Cerro *et al.*, (1999); Chokkalingam *et al.*, (2001), Cheteri *et al.*, (2004) did not found any association between the Fok1 polymorphism and prostate cancer. Similarly, the Bsm1 polymorphism and its association are also contradictory. An association was established between the Bsm1 polymorphism and prostate cancer in Japanese and US population (Habuchi *et al.*, 2000; Ingles *et al.*, 1998). While in North American population there was found no association between the risk of prostate cancer and BsmI. (Cheteri *et al.*, 2004) Similarly no association was found between the risk of prostate cancer and BsmI in Chinese population (Chokkalingam *et al.*, 2001).

The most common cancer in female is the breast and studies have been done extensively on the association of breast cancer and risk factors. Studies have been done on the association of breast cancer and VDR polymorphism; like the other studies different picture of the association studies come in to view. The Taq1 polymorphism was studied and the majority of reports show no association between the breast cancer and taq1 polymorphism (Dunning *et al.*, 1999; Newcomb *et al.*, 2002). On the other hand, Lundin *et al.*, (1999) showed a linkage between the TaqI polymorphism and risk of metastases. Similar finding was also reported by Curran *et al.*, (1999). The BsmI showed a contrary trend to the association detected in reports (Ruggiero *et al.*, 1998) and showed no association between the breast cancer and the polymorphism. The association of FokI with breast cancer is also contradictory. Some reports found an association (Ingles *et al.*, 1997) while the other did not found any association (Bretherton-Watt *et al.*, 2001).

The studies investigating the association between the Ploy A and cancer found surprising that poly A polymorphism is preventive to tumor (Ingles *et al.*, 1997; Curran *et al.*, 1999). The association's studies with these polymorphisms with other cancer are

limited. The association of colon cancer with Bsm1 polymorphism showed an association (Speer *et al.*, 2000) while the FokI results are conflicting showing no association (Ingles *et al.*, 2001). The FF genotype of Fok1 polymorphism was also found to be protective in association with malignant melanoma (Hutchinson *et al.*, 2000). Similarly, an association was also seen between the renal carcinoma and TaqI polymorphism. (Ikuyama *et al.*, 2002) Recently, Halsall *et al.*, (2005) described a new polymorphism in in the exon 1 of the *transcription start site (A*>G1012). The researchers also study that the allele was overrepresented in melanoma and it was linked to metastasis, especially when combined with the f allele of the polymorphism Fok1. They established that one A>G1012 polymorphism could have additive predictive power.

2.4.3 VDR polymorphism and nephrolithiasis

The most studied and first association is between the calcium and treatment of VDR polymorphism. Affective metabolism and bioactivity of the calcium is responsible for the efficient mineralization of bone. The defective calcium handling in the body can cause various metabolic changes like higher excretion or increase retention in the body leads to different pathologies. One altered calcium metabolism is Nephrolithiasis. Nephrolithiasis is a multifactorial disease resulting from the interaction among environmental effects, hormonal and genetic factors.

A predisposing factor to calcium oxalate kidney stone is increase urinary calcium and decrease citrate and magnesium level. The first risk study was done by Ruggiero *et al.*, (1999), who found association between the Bsm1 "bb" with the increase urinary calcium putting the high increase risk of kidney stone. This study was verified by Mossetti *et al.*, (2003) who studied the decrease citrate level is associated with bb genotype. The studies confirm that b allele is a risk factor for kidney stones. Like the other association studies there are some conflicting results as well. In the TaqI polymorphism similar conflicting results were seen. Nishijima *et al.*, (2002) showed an association of the T allele with a high excretion of calcium and, hance, to an increased formation of calculus. However, the outcomes of Mossetti *et al.*, (2003) demonstrate a link among the T allele and hipocytraturia, resulting in stone formation conditions. On

the other hand, there are also reports demonstrating the taq1 polymorphism and stone formation (Ozkaya *et al.*, 2003). Similar conflicting results have been found about Fok I and Apa I (Bid *et al.*, 2005).

2.4.4 Diabetes and VDR Polymorphism

Association of vitamin D with the most studied metabolic disease, Diabetes Mellitus has been established in both types. The type 1 diabetes is autoimmune disease cause by T cells (Bach *et al.*, 1994). In addition, the vitamin D and its metabolites suppresses the activation of T cells by binding to vitamin D receptor (Bhalla , 1984) and accordingly, the VDR gene polymorphisms may be linked with disease T lymphocytes self-immune mediated. On the other hand, it has been labelled that, in experimental animals, the vitamin D is required for the normal release of insulin and the optimum glucose metaolosim (Norman, 1994), both of which are a defect of type II diabetes mellitus.

In addition, the β-cells have VDR (Ishida and Norman, 1988) and the secretion of insulin is reduced by hypovitaminosis D and become normal with supplementation 1-25 (OH) vitamin D (Bourlon *et al.*, 1996). It is not unusual that the reults of different studies are different. In type 1 diabetes, BsmI polymorphism was related to the sensitivity of presenting the disease in South Indians, (McDermott *et al.*, 1997) Taiwan (Chang *et al.*, 2000), Croats (Skrabic *et al.*, 2003) and the Japanese. (Motohashi *et al.*, 2003) In Finnish (Turpeinen *et al.*, 2003) and Chile (Angel *et al.*, 2004) populations association can be made. On the same model ApaI, TaqI and FokI were found linked with type 2 diabetes in some reports (Chang *et al.*, 2000) and not related in some other (Turpeinen *et al.*, 2003). A study also reported the association of 98 different polymorphisms with type 2 diabetes mellitus Nejentsev *et al.*, (2004). They studied the susceptibility in 3000 families in UK. The outcomes of this study indicated that none of the sequences studied had a key effect in type 1 diabetes.

In type 2 diabetes link BsmI and the onset of the disease have been seen in the Hungarian (Speer *et al.*, 2001) and (Ortlepp *et al.*, 2001) Germans but not in French, (Ye

et al., 2001) Bangladesh (Hitman *et al.*, 1998) or Polish. (Malecki *et al.*, 2003). Similar results were found on other most common polymorphisms (Muray *et al.*, 2003).

2.4.5 VDR polymorphism and other diseases

The VDR polymorphisms association has also been studied in the other disease the list of associated are disease is very vast. One group studied the association of BsmI genotype and hypertension in healthy men (Muray *et al.*, 2003). On the other hand, in Korean population, no relationship was reported (Lee *et al.*, 2001). Ortlepp *et al.*, 2001 reported an increased susceptibility to calcific aortic valve stenosis in patients with allele B, but also an absence of relationship among the BsmI polymorphism and severity of coronary artery disease (Ortlepp *et al.*, 2003). But the same researchers described a rise in vulnerability to myocardial infarction (Ortlepp *et al.*, 2005) associated with the presence of the allele B. The results are in agreement with those of Kammerer *et al.*, (2004); reports a combination of BB genotype with a thickness of the intima-media higher in the carotid artery.

Another disease lupus erythematosus was associated the Bsm1 polymorphism of VDR (Ozaki *et al.*, 2000). Huang *et al.*, (2002) stated a positive relationship among the B allele of the BsmI polymorphism and the occurrence of systemic lupus erythematosus in Japanese and Chinese population. The same Chinese group later studied and stated proof of an absence of relationship among the FokI polymorphism and disease (Huang *et al.*, 2002). Chrone disease was associated with Taq1 Apa1 and Fok1 polymorphism and susceptibility to the disease (Simmons *et al.*, 2000). Similarly, BsmI and FokI was associated with primary biliary cirrhosis and autoimmune hepatitis (Vogel *et al.*, 2002). In multiple sclerosis, a greater incidence of the haplotype BA was found (Fukazawa *et al.*, 1999). According to Partridge *et al.*, (2004) and Tajouri *et al.*, (2005) and FokI polymorphisms is linked with amplified risk of multiple sclerosis.

The outcomes found in patients with Graves' disease differ depending on the ethnic origin. In Japanese, the Fok F allele polymorphism was overrepresented between patients with the disease (Ban *et al.*, 2000). In Caucasians, Collins *et al.*, (2004) have

found no linkage of any of the polymorphisms studied (10 polymorphisms, BsmI and ApaI including) with amplified susceptibility to suffer from Graves' disease. However, in the eastern Croatian presence of BB or AA genotypes was described to have a defensive effect (Stefanic *et al.*, 2005). In German and Polish, b allele of BsmI and F allele of FokI was associated with increased risk of Graves' disease (Ramos-Lopez *et al.*, 2005).

Bellamy *et al.*, (1999) reported the TaqI polymorphism association with TB in African patients. He found that the tt genotype had some defense against TB. The following year, Selvaraj *et al.*, (2000a) published the same genotype was associated with higher vulnerability to developing the disease, but only in women. To add more confusion, Wilkinson *et al.*, (2000) have shown that the effect of genotype was identified only with little serum levels of 25 (OH) D3. Since then, a number of papers have been published presenting different susceptibility results (Delgado *et al.*, 2002) or even the response to treatment (Roth *et al.*, 2004). However, a recent report by Lewis *et al.*, (2005) conducted a systematic review and meta-analysis and found that the results were not conclusive and the studies were underpowered.

Many articles have been published displaying a linkage between BsmI, ApaI and TaqI haplotypes and the risk of primary hyperparathyroidism (pHPT). Majority of them have been done on a Swedish population, showed a increased frequency of bat haplotype in patients with an adenoma (Carling *et al.*, 1997) and in postmenopausal women with pHPT (Carling *et al.*, 1998). However, no relationship was found in Spanish or the Canarian population (Sosa *et al.*, 2000). There is a current article by (Halsall *et al.*, 2005) in which they found that, in patients without a family history of psoriasis, the existence of the allele polymorphism A-1012G will confer safety against psoriasis. This safety may be further enhanced in combination with the F allele of the polymorphism Fok I.

2.4.6 Bone Biology and VDR polymorphisms

Morrison *et al.*, 1992 described for the first time an association among the polymorphism and BsmI osteocalcin levels. In this work, the incidence of the allele homozygous bb has been linked to increased bone mass density (BMD) in the normal

population and twin pairs. This paper, with a second published in Nature Journal in 1994 (Morrison *et al.*, 1994), in which the presence of BB genotype was associated with reduced BMD in postmenopausal women, the land base of dozens of articles published in the later years.

The studies were carried out in the normal population by Hustmyer *et al.*, 1994, among women premenopausal (52- 55), among women postmenopausal (Houston *et al.*, (1996), in older women Ferrari *et al.*, (1995), in healthy women Riggs *et al.*, (1995) and in women with osteoporosis Riggs *et al.*, (1995) or even comparing black and white women Fleet *et al.*, (1995) All these studies were conducted in the population of different origin as caucasic (Houston *et al.*, 1996), North America Riggs *et al.*, (1995), and South America (Lazaretti Castro *et al.*, 1997).

All of these studies have stated contradictory results, some of them confirming, others finding no relation or even reported opposite effect (Houston *et al.*, 1996). These differences could be explained by alterations in diet (Krall *et al.*, 1995), genotype distribution in different populations (Matsuyama *et al.*, 1995) or even the size of the sample used in all studies. Overall, this seems to be recognized is that the BsmI genotype effect on BMD is relatively low (2-3%) and extremely influenced by other non-genetic factors such as diet.

Two recent meta-analyses by Thakkinstian *et al.*, (2004) gathering all published data from 1994, showed an encouraging association between the allele b and bone mass density. In addition, it was shown that the haplotypes Bat and Bat were significantly linked with osteoporosis.

The association between the bone biology and the polymorphisms of VDR gene has been studied extensively. Almost all polymorphisms were studied with association with the bone health. For example, the Fok polymorphism was also linked with differences in bone mineral density (Arai *et al.*, 1997). On the other hand, opposite conclusions were drawn by Ferrari *et al.*, (1998); found no association between the

disease and vitamin D receptor polymorphism. Another polymorphism CDX-2 was also associated with BMD in Japanese population; the G allele is linked with low BMD of the lumbar spine.

In 1997, two nearly simultaneous documents stated for the first time the possible relationship between VDR polymorphisms and the risk of osteoarthritis. In one of them, Keen *et al.,* (1997) associated the TaqI polymorphism with increased risk of osteoarthritis. Uitterlinden *et al.,* (1997) studied haplotypes of the BsmI, ApaI and TaqI polymorphism and concluded that the bat haplotype was linked with a low prevalence of osteoarthritis of the knee. In Japanese women the association results of the haplotype were contrary to the previous haplotype association studies.

A study in Belgian population does not associated Bsm1 polymorphism with the osteoarthritis (Aerssens *et al.,* (1998). Similar results were also found between the Bsm1 polymorphism and the osteoarthritis.

2.4.7 Osteoporosis and VDR polymorphisms

Osteoporosis is a systemic skeletal disease with low bone mass and micro architectural weakening of bone tissue, with a consequent rise in bone fragility and vulnerability to fracture. It is a complex genetic disease, which involves the interaction between environmental and genetic factors. Because of the importance of the endocrine system of vitamin D for bone homeostasis itself, it quickly became a target for genetic association studies. Many genetic association studies have since shown an association among polymorphisms of the VDR gene (shown in Fig. 2.1) to a decrease in bone mineral density (BMD), and an increased risk of fracture, but it is also many negative studies.

BMD is one of the most important indicators of osteoporosis, especially in postmenopausal women. Low BMD in this population results from low peak BMD and or more rapid bone loss with aging, and is a significant cause of fracture risk. Morrison

et al., (1994) first demonstrated that the Bsm I RFLP in the last intron of VDR gene is linked to the serum osteocalcin, a biochemical marker of bone important turnover. They then found the Bsm I RFLP to be linked with differences in BMD in a double-and postmenopausal women. Although the first comment on the study of twins (but not in postmenopausal women) were withdrawn (Morrison *et al.,* 1997), in the years many articles were published the same RFLP analysis in relation to BMD. However, as controversial observations of the relationship between RFLP and commonly used BMD were reported. Three association studies (Cooper and Umbach, 1996, Gong *et al.,* 1999, Thakkinstian *et al.,* 2004) summarized between 3 'and Fok I VDR polymorphisms and BMD, but even contradictory conclusions were drawn and some questions remained of these analyzes.

The G sequence variation in the CDX-2 binding site just upstream of exon 1a was seen to be linked with BMD (Arai *et al.,* 2001). This site is proposed to play a significant role in the specific tract of the VDR gene transcription. The small intestine is the site where calcium absorption occurs mainly, the CDX-2 site is believed to influence the regulation of vitamin D, calcium absorption. The A-allele was shown that the higher affinity than the G allele for binding to CDX-2 transcription factor, and thus having a higher transcriptional activity (Arai *et al.,* 1997). In addition to expression of VDR in the intestine, the A-allele, can increase the VDR transcription downstream genes, particularly calcium transport proteins such as TRPV5, TRPV6, calbindin D9k, and calbindin D28K. This can improve the intestinal absorption of calcium and lead to increase in bone mineral density. Indeed, the rise in BMD was confirmed for Japanese postmenopausal women who wear the A-allele. However, because the population analyzed included only 55 postmenopausal women, the power of the study was low. In addition, the study did not analyze the relationship with fracture, the most clinically relevant criterion in osteoporosis. The association of this polymorphism with other osteoporosis evaluation criteria such as the parameters of fracture and bone geometry is therefore interesting to study in other preferably large populations, including those of origin different ethnic as Japanese.

The fracture is a clinical import endpoint to evaluate genetic studies of osteoporosis but also has some disadvantages. The etiology of the different types of breaking (e.g., vertebral fracture by fracture ratio of the hip fracture ratio of the wrist) is likely to be different.

3. MATERIAL AND METHODS

This study was conducted at the Islamabad Diagnostic Centre, Islamabad. The institutional ethical committee approved the study (attach ethical approval form). The study was according to the Helsinki declaration. The bone disease patients were enrolled in the study. The informed consent was obtained in written from all the study participants. The presences of bone disease were obtained by clinician.

3.1 BLOOD SAMPLING

The patients visiting the rheumatology clinics were enrolled in the study. Blood samples were collected randomly from patients visiting rheumatology clinic. Age and gender matched control samples from the healthy individual were also collected .10 cc blood were collected from each study individual. The 5-cc of blood samples were put in the in BD vacutainers (BD Franklin lakes NJ, USA) containing anti-coagulating agent ACD (Acid Citrate Dextrose) solution A for DNA extraction. Blood for serum was also collected in Gel tubes for serum Vitamin D3 and calcium level. After that the blood sample was stored at 4^0C.

3.2 DNA EXTRACTION

Genomic DNA was secluded from venous blood tests utilizing the standard phenol/chloroform strategy (Sambrook et al., 1989). Every blood test (5 ml) was exchanged to a 50 ml hawk tube. Lysis buffer was added to every specimen (three times the volume of blood) and put on ice for 30 minutes. The tubes were centrifuged at 1200 rpm and the supernatant was disposed of. A second wash with lyses buffer was performed to uproot remaining RBCs. The subsequent lymphocytes pellet was re-suspended in 4.75 ml STE support (pH 8.0). To this 250 ul of 10% SDS was included drop astute while vortexing. At long last 10 ul of 20 mg/ml proteinase K was included and tubes were set in water shower at 55 °C overnight. Following day 5 ml equilibrated phenol (pH 8.0) was added to every specimen, brooded on ice for 10 minutes and centrifuged at 3200 rpm for 30 minutes. The watery layer was exchanged with slice tips to a different marked tube and 5 ml of chilled chloroform: isoamyl liquor (24:1) was added to every example, hatched on ice for 10 minutes and centrifuged at 3200 rpm for

30 minutes. The subsequent watery layer was isolated and 10mg/ml RNase was added to every example and brooded at 37 C° for 2 hours. To this arrangement 250 ul 10% SDS was included alongside 10ul of 20 mg/ml proteinase K and hatched in water shower at 55°C for an hour. A Second phenol chloroform extraction was performed correspondingly. At last the DNA was hastened with 500µl 10M ammonium acetic acid derivation and approach volume of chilled isopropanol. The tubes were set at - 20°C overnight. On the next day the tubes were spun at 3200 rpm for an hour at 4°C and the supernatant was removed. DNA pellet was washed with 5 ml chilled 70% ethanol and centrifuged for 40 minutes at 3200 rpm. The ethanol was tossed and the DNA was air dried. The DNA pellet was then re-suspended in 200-300 µl of 10 mM Tris support (pH 8.0). The samples were then kept at 55 °C for a day to guarantee complete suspension of the DNA in cradle.

The optical densities of the specimens were taken by spectrophotometer (Nanodrop 2000c; Thermo Scientific, USA) at 260nm and 280nm. OD in a scope of 1.7-2.0 was considered as perfect proportion (Senguven *et al.*, 2014). Subsequent to taking Optical densities, tests were moved in 1.5ml marked Eppendorf tubes and were put away at 4oC.The specimens were put away at - 20oC in the wake of making 100ng dilutions of stock DNA. These dilutions were utilized for PCR amplification

3.3 PRIMER DESIGNING

Primers single stand stretch of nucleotides that bind very specifically to the complementary sequence of DNA to initiate strand synthesis. The sequence to be amplified should be known to design primers. The primer 3 online free software were used to designed primers for Fok1 mutation. Forward primer (5 -AGC TGG CCC TGG CAC TGA CTC TGC TCT- 3) and Reverse primer (5 - ATG GAAACA CCT TGC TTC TTC TCC CTC-3). The PCR products were digested using Fok1 restriction enzyme to cut the strand at specific position to give the information about the genetic status of the individual.

3.4 POLYMERASE CHAIN REACTION

PCR-RFLP was used to detect the genetic makeup of the *FokI* polymorphic sites of the gene encoding Vitamin D receptor. The DNA samples were first amplified using polymerase chain reaction. Polymerase chain reaction (PCR) is used to amplify and concentrate the region of DNA or gene of interest (Hazar *et al.*, 2012). To amplify the gene or sequence of DNA a template DNA, a pair of specific primers is required. The final volume of PCR was kept to be 25ul according to the detail given in table 3.1

The PCR cyclic program to amplify the *FokI* polymorphic sites of VDR gene was performed in three steps as mentioned in (Hazar *et al.*, 2012). 1 cycle at 95°C for 5 minutes to initially denature, then followed by 37cycles at 94°C for 1 min, 58°C for 30sec, 72°C for 1min and final extension at 72°C for 5 min. PCR products were separated by 2.5% agarose gel and visualized by ethidium bromide.

3.5 RESTRICTION DIGESTION (RFLP)

To analyze or screen for known mutation the most popular technique restriction fragment length polymorphism (RFLP) was used. RF. 20µl of reaction volume was used for the digestion reaction. The PCR product was 12.5 ul, enzyme FokI endonuclease was 1.5 µl of 1X enzyme buffer was 1 µl of and 5ul water were used. The digestion mixture was incubated at 37°C overnight at thermal cycler. The amplified 150 bp fragment was digested with FokI, which split into two fragments of 110 and 40 bp after digestion if FokI site is present. (Hazar *et al.*, 2012). After digestion ethidium pre-stained agarose gel was used to separate the bands. 10bp ladder as a marker was used to verify the band size. Electrophoresis was performed for 50 minutes at 130 volts for. The image was taken by using UV gel documentation system (Uvitec Cambridge, UK). The image was saved and the results were interpreted.

Table 3.1: Details of reagents used in Polymerase chain reaction (PCR)

Sr. No	Reagents	Stock conc.	Final Vol.
1	Taq buffer	10X	2µl
2	$MgCl_2$	25mM	2µl
3	dNTPs	50mM	1µl
4	Forward Primer	10uM	1µl
5	Reverse Primer	10uM	1µul
6	*Taq* DNA Polymerase	5U	0.25µl
7	DNA	100ng	1µl
8	dH_2O		11.75µl
		Total Volume	**20µl**

3.6 Biochemical Assays

3.6.1 Measurement Serum vitamin D3:

Serum vitamin D3 was analyzed on for all the serum samples. The assay is based on competitive ELISA technique with 25(OH)-vitamin D specific monoclonal antibody. As vitamin D is not free in the serum and it is bound with vitamin D binding protein. To analyze vitamin D accurately it must be separated from those binding proteins. The vitamin D releasing reagent is used for this purpose. Therefore, Standards, controls and patient samples which were to be analyzed were incubated with releasing reagent. A 25(OH)-vitamin D pre-coated micro plate was used to carry out the reaction by mixing the pre-incubated, released vitamin D serum, controls and standard with anti-25(OH)-vitamin D antibody incubated over night at 4-8^{0}C. In the incubation step, 25(OH)-vitamin D in the sample and a fixed amount of 25(OH)-vitamin D bound to the micro titer compete for the binding of the antibody. The micro titer plate was washed out to clean from an unbound compound like proteins etc. An enzyme labeled (peroxidase-conjugated) antibody was added into each microplate we. A complex of 25(OH)-vitamin D - anti-25(OH)-vitamin D antibody – peroxidase conjugate was formed. A coloring

substance tetramethylbenzidine (TMB) were used as a peroxidase substrate. After 10-15 minute incubation at 25C, an acidic stop solution is added to stop the reaction. The color changes from blue to yellow. The optical density of which can be measured at 450nm light. The intensity of the yellow color is inversely proportional to the concentration of 25(OH)-vitamin D. A curve was made between the absorbance of at 450nm and the concentration of the standards. The results were verified using the control values and the value of analyte was recorded. The patients were grouped into severely deficient, deficient, normal and toxic level.

3.6.2 Measurement of total calcium:

The ready to use kit was used to measure serum calcium level in the serum. The kit contained Arsenazo-III dye which in the presence of acidic solution reacts with calcium and form blue-purple complex. The intensity of the color is measured at 660 nm which is proportional to the calcium concentration in the sample.

3.6.3 Dexa Scan:

The bone mineral densities were carried out using Dexa Scan.

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3.7 STATISTICAL ANALYSIS

The obtained data was analyzed using different statistical tools. All the tests were applied by using different online software's.

4. RESULTS

4.1 Description of samples:

Seventy-eight clinically diagnosed bone disease patients in whom 25 were male and 53 were female, the mean age for the male were 45.04 years and the females were 52.45 years Seventy-eight healthy controls were also enrolled in the study in whom 37 were male and 41 were females the mean age were 44.04 years for the male and 51.05 years for the female. Standard technique phenol chloroform technique was performed to isolate DNA in batches. The serum was obtained using centrifugation. The DNA samples were analyzed for VDR Fok1 genetic polymorphism. The serum samples were analyzed for serum vitamin D and calcium level. All the patients and controlled were assessed. The age and sex wise distribution are shown in the figure 4.1

4.2 Results of Fok1 Polymorphism:

This shows a deletion at position 2549 of nucleotide adenine (A) results in the combination of Fok1 allele. The amplified PCR products were followed by digestion with restriction enzyme. The digested products were run by 3% agarose for the separation of band sand interpretation of results. The 150bp fragment 110bp fragment and 40 bp fragment. If there is only one band of 150bp the genotype will be FF, if there is 150 and 110 the genotype will be Ff (heterozygous) and if there is band 110 and 40 bp the genotype will be ff homozygous mutant as shown in figure 4.3

4.3 Results of serum calcium:

Calcium is important for many bodily functions. The normal concentration of

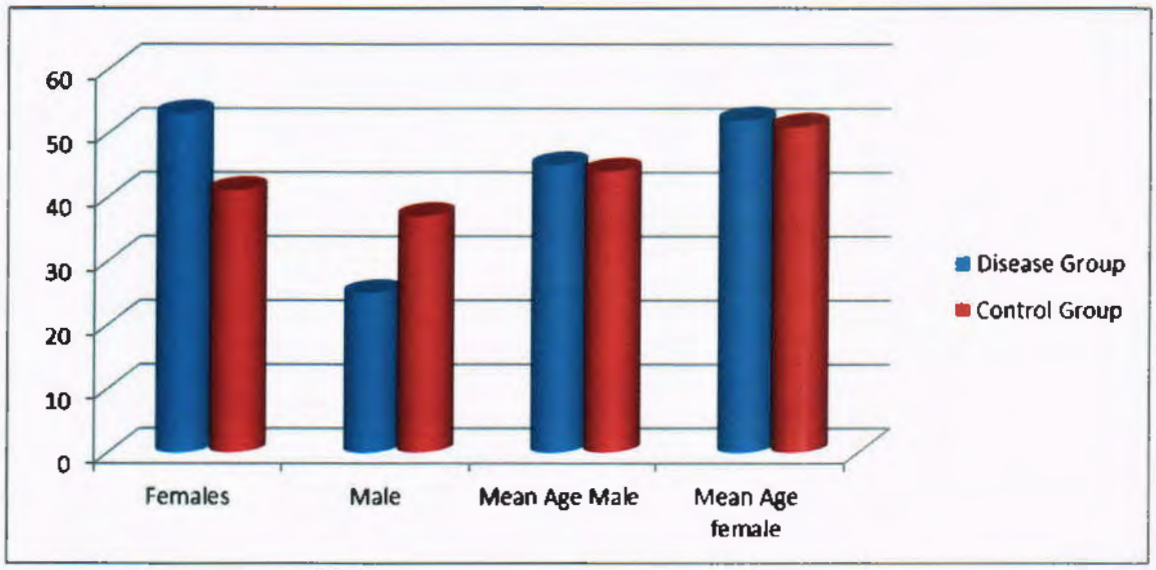

Fig 4.1: Distribution of patients and control age and gender wise

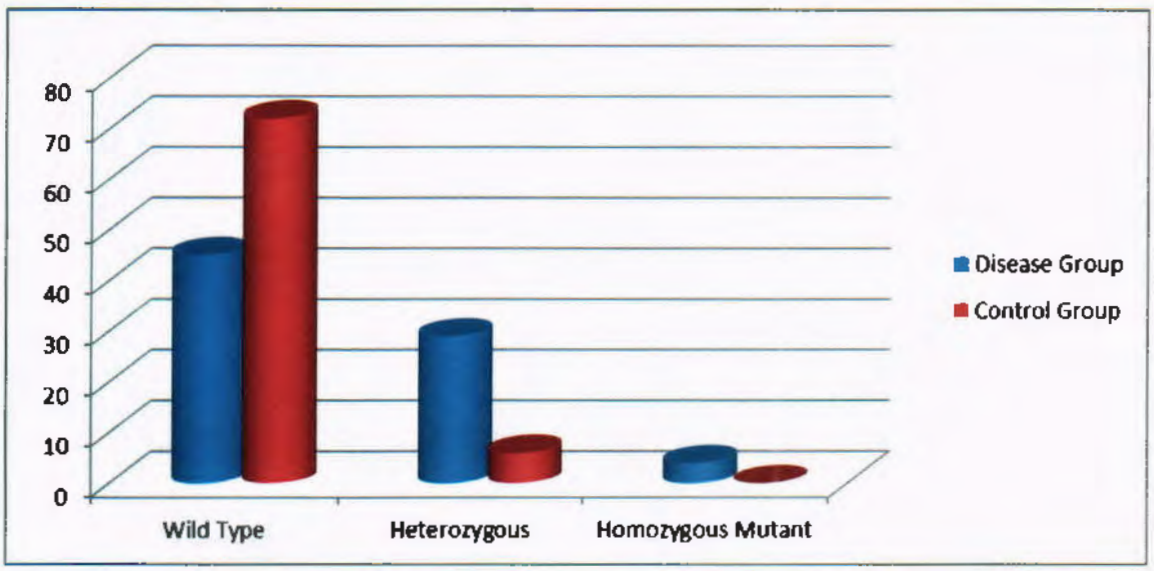

Fig 4.2: Frequency of polymorphism in patients and control

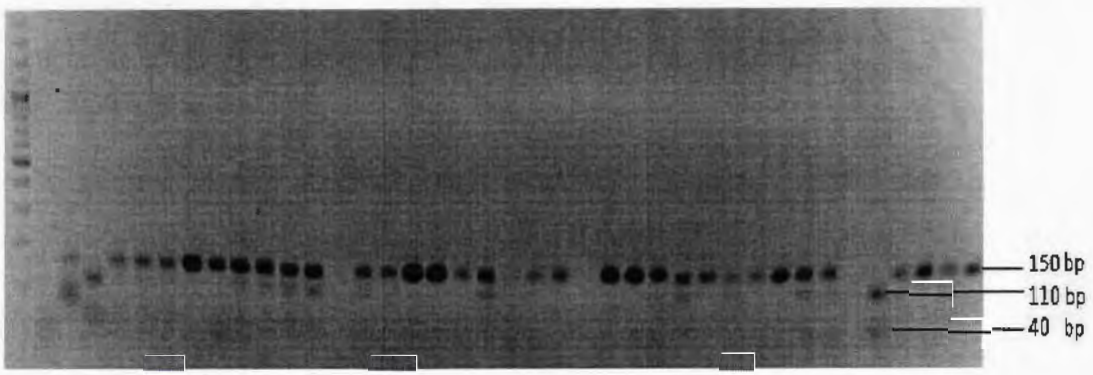

Fig 4.3: Electropherogram showing the digestion with FokI endonuclease. The 150 bp band shows the FF (Homozygous for wild type) genotype. The 150 and 110 band shows Ff genotype (Heterozygous) and the 110 and 40bp allele shows ff genotype (Homozygous for minor/mutant allele)

Serum calcium is 8.4-10.2 mg/dL. In the serum there are free and bound calcium the bound calcium usually ranges from 40-45% of the total calcium. The calcium uses protein to bind with them. The free calcium is also known as ionized calcium. The normal range of free calcium is 4.8-5.2 mg/dL.

Serum calcium was measured for all the patients and control group. The hypocalcaemia 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 control was found to be hypocalcemia.

4.4 Results of Vitamin D:

Vitamin D is fat soluble vitamin. It is mainly involving in the bone health. It means that it affects the bone mineral density. It is responsible for the absorption of dietary calcium, magnesium and other cementing elements. There are mainly two types of vitamin D in human that is vitamin D_3 (also known as cholecalciferol) and vitamin D_2 (ergocalciferol). Both of these vitamins can be ingested from dietary sources. Few food items have vitamin D. The majority of vitamin D is synthesized in the skin in the presence of sun light. Therefore, as a major contributor to the bone health 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 hypovitaminosis D for the control 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.

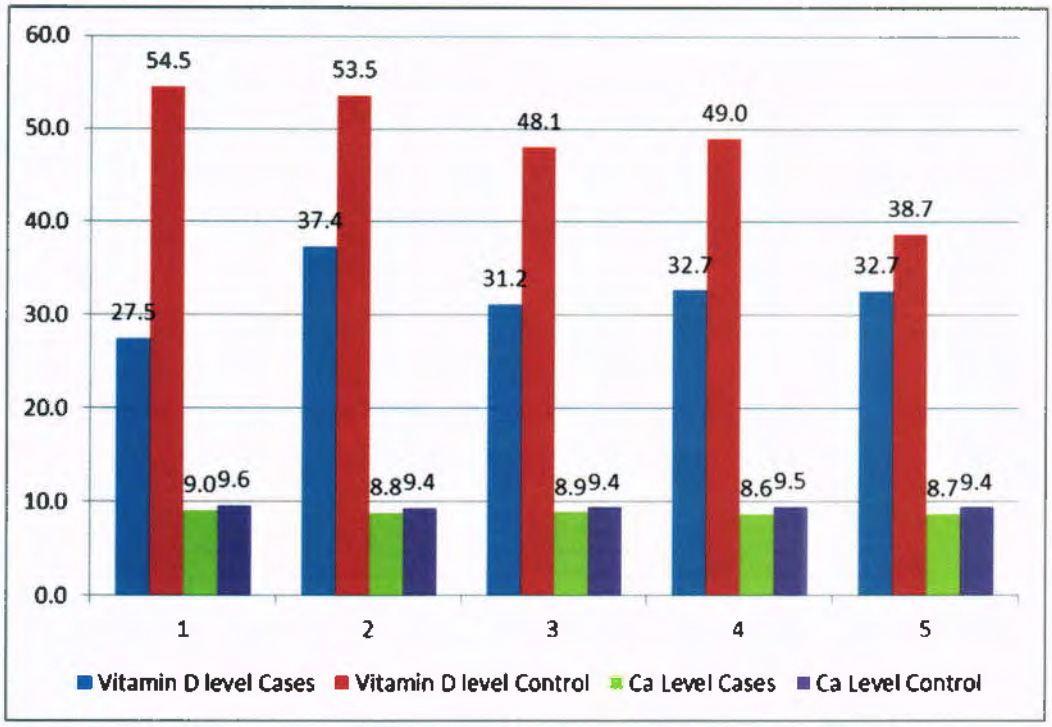

Fig 4.4: Comparison of vitamin D and calcium in patients and controls

4.5 Results of Dexa scan:

The mineral content of the bone can be screened by using dual-energy X-ray absorptiometry (DEXA-Scan) or bone densitometry. It is an advance form of x-ray technology which measure bone decay. DEXA Scan is a standard method for measuring bone mineral density (BMD). DEXA scan is most often performed on the lower spine and hips. The whole body is scanned in advance stages of the disease and mostly in patient's arthritis. Sometime ultrasonography is accompanied to estimate the bone health at real time in children and some adults. Peripheral devices that use x-ray or ultrasound are sometimes used to screen for low bone mass. In some communities, a CT scan with special software can also be used to diagnose or monitor low bone mass (QCT). This is accurate but less commonly used than DEXA scanning.

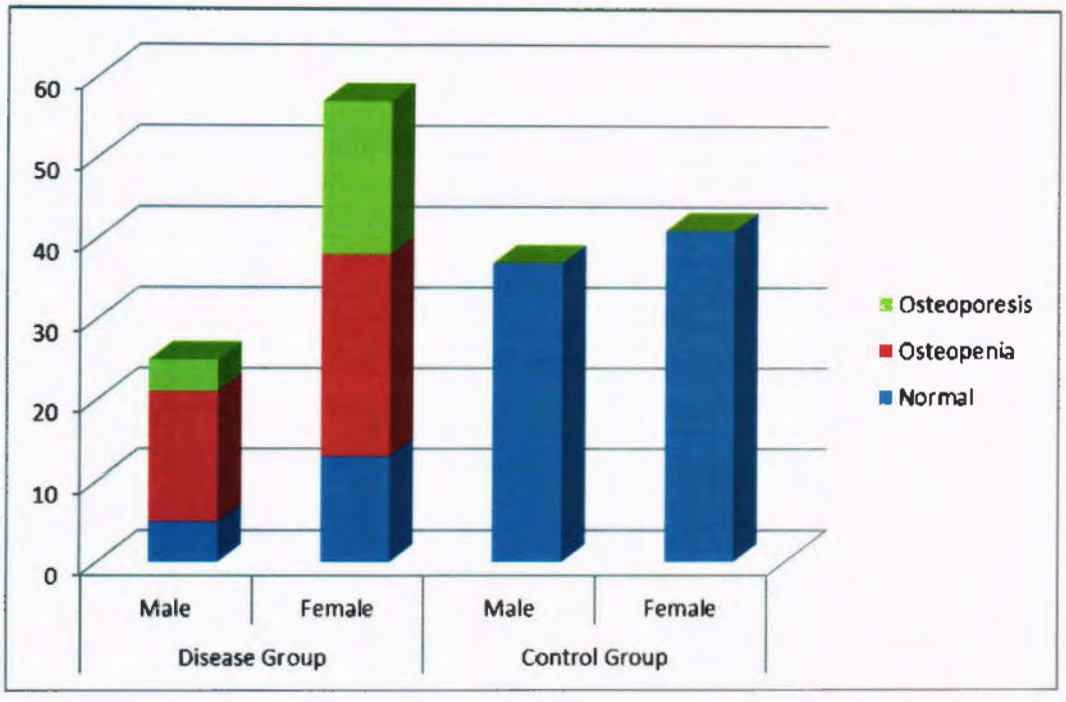

Fig 4.5: BMD in diseased and control gender wise

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 were n=16(20.51%) and female were n=25(32.05%). The Osteoporosis for the patients were n=19(24.35%) in which male were 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.

4.6 Comparison of Vitamin D & Ca level among Patient & healthy control group:

Serum Calcium and vitamin D were compared between the patients and control group and it was observed that vitamin D and calcium level were significantly lower in the diseases group as compared to control group as shown in table 4.1

4.7 Association of serum calcium & vitamin D level and Fok1 polymorphism with bone mineral density:

Serum Calcium Vitamin D and Fok1 polymorphism were compared between the patients and control group and it was observed that vitamin D calcium level and fok1

5. Discussion

Vitamin D receptor is responsible for the efficient receiving and metabolism of the Vitamin D. If there is defective receptor there will be less receiving and less metabolism of Vitamin D. So the deficiency of D vitamin will lead to insult in the mineral metabolism. In this study, a significant association was find out between Fok1 polymorphism of Vitamin D receptor, calcium metabolism and the bone density in bone diseases patients. This is the first study from Pakistani population demonstrating the association between the Polymorphic VDR receptor serum vitamin D and serum calcium level.

The genotypes of Vitamin D receptor were significantly associated to bone and bone associated minerals (Calcium). The patients of the FF genotypes showed higher calcium level as compared to Ff and ff genotypes. The VDR genotype and serum calcium were also compared to control group. It was found that there are 7.69% heterozygous (Ff) genotypes in control but there were no homozygous for minor allele in control groups moreover the calcium and vitamin D was also normal in control group.

In addition, we found that patient with FF genotype the bone mineral density is high as compared to the other genotypes. Moreover, the control group has major genotype and their bone mineral density was normal. The results of our study is consistent with the study in Japanese population they found a significant association between the Fok1 polymorphism and BMD (Arai *et al.*, 1997) Similarly our results are also consistent with Mexican-American population, (Gross *et al.*, 1996) and US white women population. (Harris *et al.*, 1997)

We found 57.69%, of the Pakistani population in our study, homozygous for allele FF, 37.18% heterozygous for Ff allele and 5.13% homozygous for ff allele. The distribution of genotype is almost same as reported by Harris *et al.*, (1997) who reported FF= 65%, Ff=31% and ff=4%. It is fascinating to guess, in any case, that higher frequency of FF genotype more ideal (twice that found for Mexican Americans and Caucasians) could partly explain the increase in bone mineral density seen among

African-Americans, if the frequencies of alleles are considered similar with greater populations studied.

From the last 20 years it is evident that in osteoporosis and the status of BMD the genetic also play a role (Kelly *et al.,* 1993). To reach some conclusion that in the development of osteoporosis genetics is involved is remain controversial. Especially the role of vitamin D receptor is still controversial (Civitelli and Ziambaras, 1998). In the present study, we assessed these connections in the developing osteoporosis, in which the effect of VDR gene polymorphism is not or may be affected less likely by environmental and associated factors. By inspecting the calcium energy and bone density amid this dynamic time of bone development, hereditary components that eventually impact Bone Mineral Density can be more obvious than in the older population where osteoclastic activity is an important element influencing bone mineral density.

The association of genetics and bone health was studied in Mexican-American girls, (Sainz *et al.,* 1997). The results of the study showed significant association between the VDR polymorphism and bone mineral density. On the other hand, in this study, another polymorphism the Bsm1 was investigated and their role in calcium retention was analyzed, Ferrari *et al.,* (1998) studied Fok1 genotype association with Bone Mineral Density at high admissions of calcium in osteoporotic patients, which is steady with our outcomes. They likewise subject for polymorphisms Fok1 Bsm1 and cross-genotyped and found no confirmation of linkage disequilibrium between these loci. Another group studied the association of VDR genotype and calcium absorption in postmenopausal women. (Dawson-Hughes *et al.,* 1995) The authors have reported a relationship of the polymorphism Bsm1 VDR (BB) to a decrease in calcium absorption, but only for calcium intake of less than 300 mg / day. While we did not study the other polymorphism and their association with calcium vitamin D and BMD but we studied the VDR Fok1 polymorphism with the Calcium Vitamin D and bone mineral density scan and find an association between these parameters. VDR molecules encoded by the allele f initiate translation from an upstream ATG (at the polymorphism site Fok1) and three amino acids more than the product of the allele C. Arai *et al.,* 2001 exhibited that the F allele encoded protein produced 1.7 times larger transcriptional transactivation of a

promoter containing a member of the vitamin D-responsive fact that the allele of the product f (Arai *et al.*, 1997).

Reliable with these outcomes indicating utilitarian contrasts between F and f alleles in vitro, this study shows the association of the F allele with increased calcium absorption and bone mineral density of the population. The nature of the relationship between the genotype and calcium metabolism in osteoporotic patients VDR of Pakistan's population requires further longitudinal studies, particularly those that include examining the effects of BMI, and origin ethnic.

6. Conclusion

We studied the Fok1 polymorphisms with BMD serum calcium and serum vitamin D level. The bone health depends on the mineral density and deposition in the bone. Vitamin D helps the deposition of minerals in the bone. The absorption of vitamin D occurs through receptors. Mutated receptor leads to the aberration in the absorption of vitamin D effecting the deposition of the minerals in the bone, thus decreasing the bone density although the serum vitamin D level may be normal.

Vitamin D receptors also suffer from other polymorphism which affects the bone, skin, and even the metabolism of neurotransmitters in the brain. The effect of other polymorphism of vitamin D receptor will be studied in future.

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