

Correlating Vitamin D Receptor Gene Polymorphism in Vitamin D Deficient Premenopausal Pakistani Females via PCR/RFLP and Breast Cancer Risk

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Abstract

Objective: Vitamin D due to its non-proliferative, non-angiogenic properties which are the hallmarks of some cancers like breast, colon, lung and prostate has changed the direction of Research towards finding out the association of Vitamin D deficiency, VDRGP and breast cancer.

Methods: Asymptomatic, pre-menopausal women (20 to 40 years) were inducted for VDR gene polymorphism. PCR –RFLP for Fok1 and Apa1 was done. Serum vitamin D levels were done twice before and after supplementation.

Results: Women (n- 294) with minimum age of 20 and maximum age of 40 were inducted. Shapiro-Wilk normality test was done. The data was non-parametric. The mean \pm SD (30.6 \pm 6.01) median and IQR were also computed. The median age was 30 years and IQR for age was 10.33 The mean \pm SD of Vitamin D baseline was 9.87 \pm 7.29 and 7(6.63) as IQR. Both genes Fok1 and Apa1 were prevalent in the population. The p-values i.e. 0.58 & 0.259 showed that there was no significant mean difference observed in vitamin D at baseline level for Fok1 PCR and Apa1 PCR. Similarly, Fok1 & Apa1 RFLP showed no statistically significant difference in the mean values of vitamin D at baseline level; p-values were 0.759 and 0.44 respectively.

Conclusions: VDR genotypes vary widely. There was equal distribution of Fok1 and Apa1 genes. The important observation was the absence of homozygous group in Apa1. In comparison to other studies both Fok1 and Apa1 were not significantly related to vitamin D deficiency, which concludes that VDR gene polymorphism is not significantly related to breast cancer.

Keywords: Polymorphism, Heterodimer, Exons, Intron, Calcitriol, Apoptosis, Angiogenesis, SNPs (Single nucleotide polymorphisms), Electrophoresis.

Introduction:

In Asian countries, including Pakistan, the rise in breast cancer is alarming. The incidence of breast cancer in Pakistan is 2.5 times more than in other countries of Asia [1]. Fifty out of 100,000 in Pakistan as compared to Indian women (19 out of 100,000) were affected with breast cancer [2]. It is said that, out of every nine women, one is likely to be diagnosed with breast cancer and this ratio increases as the age increases [3].

In the United States breast cancer has caused quite a stir and the rise in morbidity and mortality has molded the research in the direction of prevention rather than early detection. At the top of the list are

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modifiable risk factors like vitamin D and breast density, both are associated with reduced breast cancer risk.

In Pakistan vitamin D deficiency is reported in the range of 89%-92% [4-5] which is quite alarming as the country is flooded with sunlight.

The initial observations linking vitamin D with breast cancer were deduced from ecological studies [6]. Since ultraviolet B (UVB) rays are essential for cutaneous production of vitamin D, sunlight exposure is mandatory for optimum vitamin D levels.

Ecological studies have associated increased sunlight exposure with low breast cancer incidence and mortality [7-10]. The above-mentioned studies laid the foundation for examining the hypothesis that vitamin D deficiency increases cancer risk and mortality, including breast cancer. Keeping this heightened interest regarding this association in mind, a number of recent observational studies, as well as clinical trials of vitamin D supplementation were observed, which examined the relationship between vitamin D status and breast cancer. Vitamin D deficiency is often defined as a serum 25-hydroxy vitamin D (25(OH) D) less than 20ng/mL or 50nmol/L (1ng/mL = 2.5nmol/L). This has become a shared concern among physicians, many of whom now routinely screen for vitamin D deficiency and/or recommend supplementation both in healthy women as well as breast cancer patients. The upper safety limit in healthy individuals was raised from 2000 IU to 4000 IU daily. However, the IOM also raised concerns about negative health effects for circulating 25(OH) D levels above 50ng/mL (or 125nmol/L).

After a thorough review of the literature, it was concluded that there was insufficient evidence to recommend vitamin D supplementation both for replacement and maintenance for cancer prevention or treatment ([http:// www.IOM.edu/vitaminD](http://www.IOM.edu/vitaminD)).

The current knowledge about the anti-cancer properties of vitamin D, VDR and VDR gene polymorphism in different ethnicities was observed and also evaluating its association with breast cancer.

Vitamin D performs by binding with vitamin D receptor (VDR) which is a potent transcription factor but is ligand-dependent. The receptor has two zinc finger structures with a characteristic DNA-binding domain and a carboxyl terminal ligand-binding domain [11]. When bound to its ligand, calcitriol (1,25(OH)₂D), VDR dimerizes with the retinoid X receptor (RXR), a conformational change occurs, that allows the Heterodimer to translocate into the nucleus, where it binds to vitamin D response elements (VDRE) in the promoter regions, responsible for transcriptional regulation of target genes. The important functions of VDR include apoptosis, cell proliferation, differentiation, angiogenesis and metastasis [12].

The main impact of Vitamin D deficiency in the human body or VDR dysfunction can cause poor bone development and health, as well as increase the risk of developing many chronic diseases, including cancer [13]. The non-bone effects of vitamin D in the humans are mediated by its receptor, VDR. This nuclear receptor is influenced by the presence of several

genetic polymorphisms [14-15]. The VDR gene, located on chromosome 12q12-q14, includes eight protein coding exons (exons 2-9) and one untranslated exons (exons 1a-1f) [16-17]. By screening with different restriction enzymes, only some restricted areas of VDR gene could be analyzed to verify DNA sequence variations [18]. The genes which were frequently investigated in the VDR polymorphisms are Fok1, located at exon 2 of VDR, BsmI and ApaI located at intron 8, and TaqI located at exon 9 of VDR [16]. These are single nucleotide polymorphisms (SNPs), where FokI is located at the 5' end of VDR gene and the other three SNPs are at the 3' end of the gene [14,17]. We investigated the presence two genes Fok1 and Apa 1 and studied their presence in our population.

Materials and Methods

Study participants: Women between 20 to 40 years of age, asymptomatic and premenopausal were inducted as study participants from the breast clinic of Patel Hospital Karachi (Pakistan) from June 2013-June 2014. All participants underwent measurement of serum vitamin D levels and the buffy coat was saved for DNA extraction and later DNA amplification through PCR and restriction fragment length polymorphism (RFLP).

All those females with history of breast cancer, breast surgery, pregnant or lactating, unknown menopausal status and on oral contraceptives were excluded. The study was approved by Institutional Review Board of Ziauddin University Hospital. Written and informed consent was obtained.

Serum 25 (OH) D analysis: The serum samples from patients were obtained from median cubital vein and serum 25 (OH) D were assayed via Chemiluminescence immunoassay.

Holick's protocol: Weekly 50,000 IU of vitamin D3 for a period of 8 weeks given to the participant to replenish the deficient vitamin D. In the maintenance phase 50,000 IU vitamin D3 was administered every 2 weeks. The serum level of 25 (OH) D was measured after 6 months of treatment; and if the level was above 30 ng/ml the maintenance therapy was given [19].

Data collection

Study participant's anthropometric measurements, age at menarche, parity, number of pregnancies, number of children, age at first birth and breast-feeding history were inquired and recorded on a structured format by a single trained physician.

DNA extraction: Venous blood was collected from the study participants in a 10 mL EDTA tubes. Genomic DNA was extracted from the collected blood via pure link Tm Genomic DNA mini Kit (cat no. k1820-01). (Ref Invitrogen lot no 1398040). DNA was stored at -80°C.

Polymerase chain reaction: The genotype for the 2SNPs of the VDR gene was determined by the digestion pattern of the amplified DNA fragments using the restriction enzymes for Apa1 and Fok1. DNA was extracted by using a thermal cycler (The PCR conditions were 5

min at 940°C for initial denaturation, 30 secs at 940°C, 30 secs at 600°C, followed by 5 mins at 720°C for final extension). This was done to amplify the VDR gene. The exon 2 was amplified to study the Fok1 polymorphism, and exon 9 was amplified for the Apa1 polymorphisms. The genomic DNA was amplified using specific primers (Apa1, primer 1-5'-CAGAGCATGGACAGGGAGCAA-3'), Fok 1 primer(5'AGCTGGCCCTGGCACTGACTCTGCTCT-3') the working solution for the PCR reaction of the exons 2 and 9 were made with a final volume mix of 25 µL, 5µg of genomic DNA was added to the final mix.

Restriction fragment length polymorphism: The digestion of the amplified fragments was done with specific enzymes under specific time and temperature conditions, allowing the assessment of the genotype of each participant used in the study. Two VDR polymorphisms were studied: The Fok1 (rs10735810 T>C) and Apa1 (rs7975232 G>T). The respective enzymes for Fok1 and Apa1 were used for the amplified fragments. 10 µL of PCR product was used to proceed with the respective fragment digestion by adding 5 µL containing 4 Units of Fok1 enzyme the digestion occurred in an incubator under temperature conditions of 37°C for 16 hours. After the digestion of VDR exon 2 with Fok1 enzyme, three genotypes could be distinguished: the homozygous wild type FF, the homozygous ff genotype and the heterozygous Ff genotype. The homozygous ff genotype resulting from this digestion produced 2 fragments (70 and 197 bp), and the FF genotype produced a single fragment (267 bp) due to absence of digestion. The heterozygous Ff genotype produced 3 fragments (70, 197 and 267 bp). To study the Apa1 polymorphisms, the amplified PCR product of VDR exon 9 was used. For the Apa1 polymorphism, a 5 µL mix containing 5 units of Apal enzyme was added to the PCR product. The digestion of Apa1 occurred under temperature conditions of 25°C for 16 hours, producing three distinct genotypes. The homozygous aa genotype produced 2 fragments (217 and 528 bp), and homozygous AA genotype produced 1 fragment (745 bp). Heterozygous Aa genotype produced 3 distinct fragments (217, 528 and 745 bp).

Electrophoresis: To study the PCR amplification and enzymatic digestion, we performed electrophoresis; this technique was used to verify the migration of DNA fragments in 2% agarose gel and stained with Green Safe Buffer (1 µL/mL) (NZY tech Lda., Lisbon, Portugal). For electrophoresis of enzymatic digestion, 3% agarose gel was prepared with the total volume of enzymatic digestion products loaded in the gel. To compare the molecular weight of the DNA fragments, a molecular weight marker 100 bp Plus DNA Ladder was used. For agarose gel visualization, UV light Hero Lab was used.

Statistical analyses

The data was analyzed by SPSS (Statistical software for social sciences) version: 20. Shapiro –Wilk’s normality test was applied over the whole data to check the assumption of normality. The hypothesis of normality of data was rejected i.e. the data were not normally distributed since p-value was less than 0.05.

Quantitative variables i.e. age, vitamin D level baseline, were presented by median and IQR instead of mean±SD due to the violation of normality assumption.

Spearman Correlation co-efficient was computed (level of significance = 0.05) to determine the association between quantitative variables.

Results

The study was carried out from June 2013 till June 2014. Among 350 women, from 20 to 40 years were recruited into the research study. Of these, 235 women underwent the investigation and for VDR gene polymorphism fok1 & Apa1 the number of participants recorded were 154 and 152, respectively. All patients were found to be vitamin D deficient (Table 1). The ages varied between twenty to forty years and since all of them were vitamin D deficient keeping the age group under consideration as young females are more active and more exposed to the external environment.

As in this case sunlight is the most pertinent external factor to naturally increase their vitamin D levels. This age group is also more concerned about their diet and if married and breast feeding they have an added advantage to see a physician during this process and must have been guided regarding their vitamin D levels.

The response of vitamin D supplementation was significant (Figure 1). The level increased from deficient (Table 1) to sufficient (Figure 1). Wilcoxon signed rank test was done to assess difference between baseline and at 12 months’ vitamin D level. There was a significant increase from the base line of 9.87 to 68.3 (Table 1). This showed that the patients responded to the supplementation of Vitamin D with p value <0.001(Figure 1).

Table 1: Descriptive demographics.

Variables	Mean + SD	Median (IQR)	Minimum	Maximum
Age of Patients(n=294)	30.6 ± 6.01	30(10.3)	20	40
Vitamin D level Baseline (n=235)	9.87 ± 7.29	7(6.63)	03	29.9

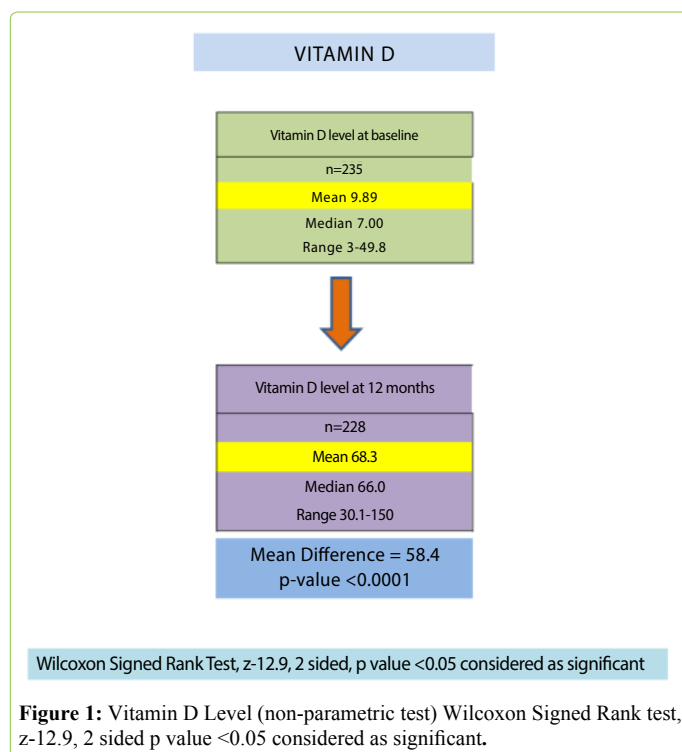


Figure 1: Vitamin D Level (non-parametric test) Wilcoxon Signed Rank test, z=12.9, 2 sided p value <0.05 considered as significant.

The Proforma filled by the patients had all the anthropometric measurements and Spearman’s correlation co-efficient was computed for the age of patients with different parameters in our data (Table 2). It was observed that only vitamin D level at baseline was significantly correlated with age of the patients. Vitamin D level at baseline was low and correlated weakly positive with the age of the patients with the r value of 0.181 (p<0.05). This also brings us to a weak correlation regarding vitamin D levels; the data suggesting as the age of the patient increases vitamin D level also increases but this was a weak correlation.

The genes which are associated with vitamin D receptor are varied and the two which were tested in our population were Fok1 and Apa1 and recorded as (154, 52.4%) and (152, 51.7%) respectively (Figure 2). Once the gene was confirmed, VDRGP was done and the wild type, Homozygous and heterozygous were studied by the process of RFLP (Figure 3).

The two genes Fok1 and Apa1 were correlated with vitamin D at baseline only (Table3), due to shortage of resources could not be repeated with sufficient vitamin D levels and the results were not significant. Once the genes are there may be the change would be in polymorphism.

There was no significant mean difference observed in vitamin D at baseline level for Fok1 PCR and Apa1 PCR respectively. In the same way, Fok1 RFLP and Apa1 RFLP with their three different polymorphisms showed no statistically significant difference in the mean values of vitamin D at baseline level. Their P-values were 0.759 and 0.44 respectively (Table 3).

The polymorphism of VDR genes showed that the homozygous group was predominant i.e. 46.7% in Fok1 while 71.7% heterozygous were found in Apa1 which was the highest among all polymorphism of Apa1. As our retrospective analysis showed a lot of ethnic differences and a column of ethnicity was in the Proforma so that we could correlate different ethnicities, but this will be taken up in another study. The interesting and important observation was the homozygous group which was observed highest in fok1 was absent in Apa1 (Figure 3). There was significant association observed between the polymorphism of Fok1 and Apa1 as P-value<0.001 (Figure 4).

Discussion

How and why breast cancer develops is a million-dollar question, which leads to limited opportunities pertaining to disease prevention. This has shifted the direction of research towards prevention rather than early detection. There are factors which cannot be modified like genetic, molecular signaling pathways and environmental, however certain factors are modifiable. Once the modifiable risk factors linked with this disease are recognized only then we can accurately monitor and prevent breast cancer which is again a question mark. Keeping this in mind we took the task to investigate two factors of significant importance to breast cancer, Vitamin D level and VDR gene polymorphism and its correlation with breast cancer [20].

Vitamin D one of the modifiable risk factor, is also protective for certain cancers, and there is consistent epidemiologic evidence that increased Vitamin D intake is associated with reduced risk of colorectal [21] and breast cancer [22]. The level of this vitamin has never been approached in a manner we would expect to see for any other vitamin. Unlike other vitamins, we have never had dietary intakes of vitamin D as a reasonable reference point for deciding on how much of this vitamin people should be consumed [23].

Along with other third world countries, the deficiency of this vitamin is quite striking in Pakistan. What is the exact time period during which vitamin D levels may affect breast cancer occurrence or survival is currently not clear? Therefore, the ideal method is to diagnose treat, and prevent

Table 2: Spearman’s Correlation between Age of patients and other lab parameter.

Variables	Vitamin D level Baseline	eulav-p
Age	0.181*	<0.05

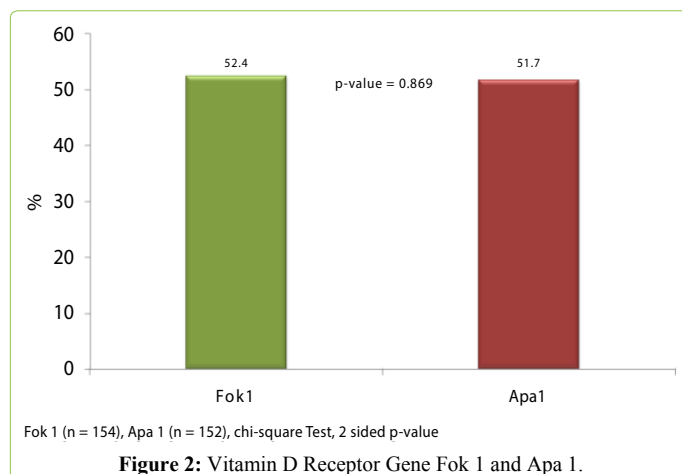


Figure 2: Vitamin D Receptor Gene Fok 1 and Apa 1.

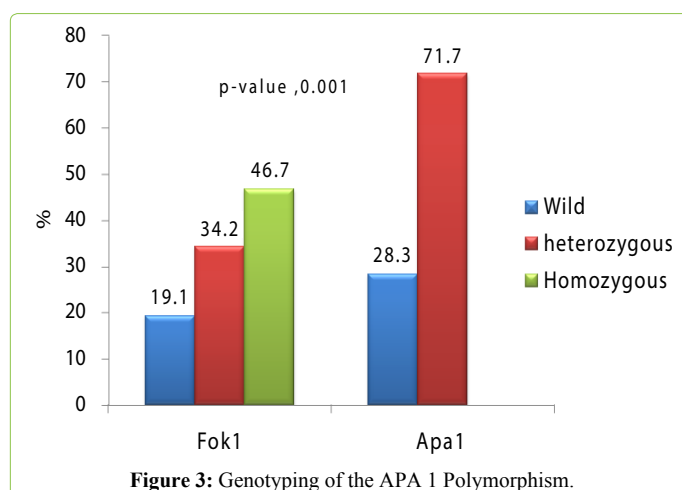


Figure 3: Genotyping of the APA 1 Polymorphism.

Table 3: Vitamin D at Baseline with Fok1 and Apa1.

(n = 235)	p-value	Statistical Test
Fok1 PCR	0.58	Mann Whitney Test
Fok1 RFLP	0.759	Kruskal Wallis Test
Apa1 PCR	0.259	Mann Whitney Test
Apa1 RFLP	0.44	Kruskal Wallis Test

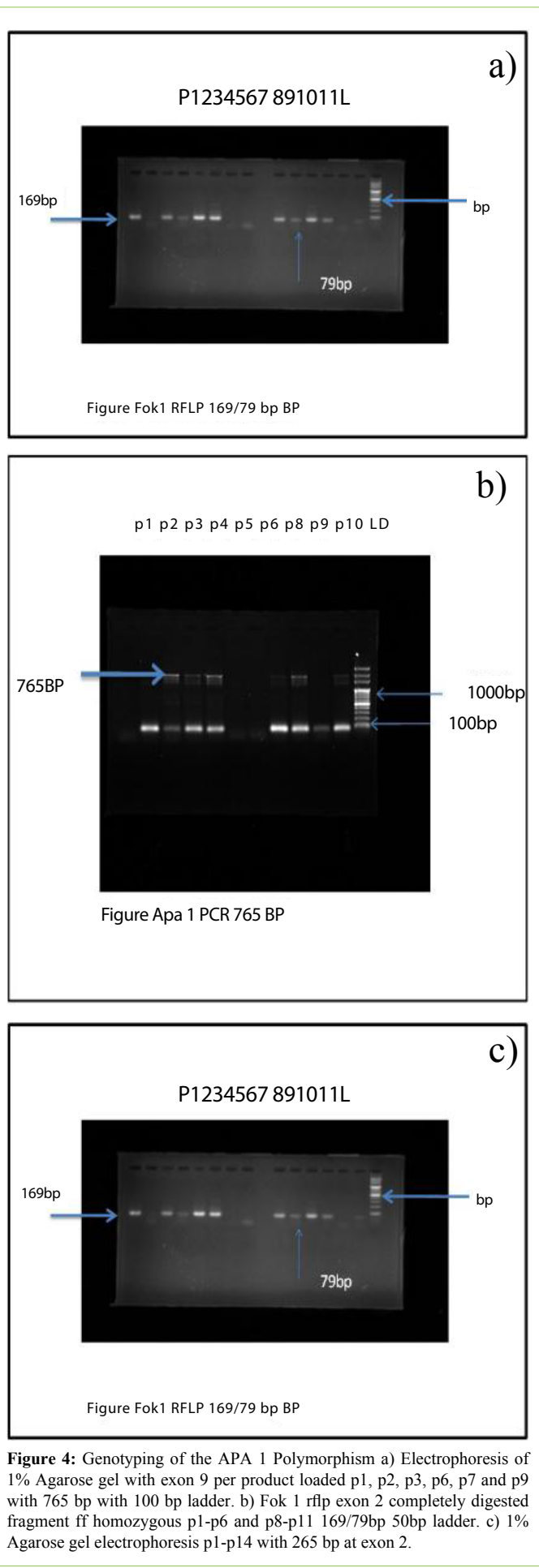


Figure 4: Genotyping of the APA 1 Polymorphism a) Electrophoresis of 1% Agarose gel with exon 9 per product loaded p1, p2, p3, p6, p7 and p9 with 765 bp with 100 bp ladder. b) Fok 1 rflp exon 2 completely digested fragment ff homozygous p1-p6 and p8-p11 169/79bp 50bp ladder. c) 1% Agarose gel electrophoresis p1-p14 with 265 bp at exon 2.

vitamin D deficiency. A large number of comparisons were made, and the collection of statistically significant interactions suggest that sunlight exposure and vitamin D may be most important for women who are genetically susceptible, and that breast tissue is more susceptible to insult. As more studies confirm similar results, dietary vitamin D and adequate sunlight exposure will be among the modifiable risk factors for breast cancer patients i.e. by just improving their Vitamin D levels they can prevent from such a morbid pathology [24].

Our study participants comprised of females of 20-40 age group, vitamin D was in the range of less than 20ng/ml. Cross sectional multicenter study done in Karachi, Pakistan, showed hypovitaminosis D, mainly due to inadequate intake and avoidance of sunlight. As a result, the affected population was of females as compared to males and vitamin D was in the deficient range [24].

The other important factor was VDR and its gene polymorphism. A Meta-analysis done on prospective studies of VDR gene polymorphism found no association between fok1 and Apa1 gene [20]. There was some association between ff genotype of Fok1 with breast cancer [25]. In our participants there was equal distribution of folk1 and Apa1 genes. In the polymorphism we saw three groups in Fok 1; wild type, heterozygous and homozygous. The predominant one was the homozygous variant. On the other hand, there were only two variants of VDR in Apa1 gene i.e. the wild type and the heterozygous variant and the predominant one was heterozygous.

As compared to other studies both Fok 1 and Apa 1 were not significantly related to vitamin D deficiency, which brings us to the conclusion that VDR gene polymorphism is not significantly related to breast cancer.

VDRGP and breast cancer risk with Fok1 gene: There was no association found in 7 studies done on fok1. In Apa 1, only 2 studies comprised of 95% Caucasians showed no significant association between Apa 1 and breast cancer risk. What causes breast cancer is not well understood, it is thus limiting opportunities for disease prevention. Therefore, it is extremely important to pursue research focused on breast cancer etiology. Only through the identification of modifiable risk factors associated with this disease can effective breast cancer prevention be realized.

One of the modifiable risk factor which is also protective for certain cancers is vitamin D [26]. Fok1 polymorphism showed no association with breast cancer risk and also showed no association between Bsm1, Taq1, and Apa1 polymorphisms and breast cancer risk in mixed races. It is well established that VDR genotypes vary widely with ethnicity and also there is no definite association observed between Fok1, Apa1 and Bsm1 polymorphisms and breast cancer risk [27].

In our study there was equal distribution of folk1 and Apa1 genes. The other interesting aspect of VDRGP was that in Fok1 the homozygous group was predominant and in comparison, it was absent in Apa1, also like other studies both Fok1 and Apa1 were not significantly related to vitamin

D deficiency, which brings us to the conclusion that VDR gene polymorphism is not significantly related to breast cancer risk [28-29].

Conclusion

In conclusion, we would like to comment that the current study provides the evidence that VDR polymorphism (Fok1, and Apa1) was not associated with the risk of breast cancer in general population. Further studies are necessary to clarify these results.

Conflict of Interest

The authors report no conflict of interest.

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Patient Consent

The study was approved by the Institutional Review Board of Ziauddin University and Patel Hospital. Written informed consent was obtained from all the study participants prior to recruitment in the study, and anonymity and confidentiality of the data was maintained throughout the research.

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