

# Vitamin D Receptor Microsatellite Repeats in the 3' untranslated region: Relation to Early Onset Breast Cancer among Pakistani Women

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## ABSTRACT

### Objective

To investigate whether VDR variable-length microsatellite repeat poly (adenylate) sequence (Poly (A)) gene is associated with early onset breast cancer (BC) risk among Pakistani females.

### Methodology

This study is a case control study consisting of 111 BC cases and 108 controls with age range of 20 to 40 years. DNA was isolated from leukocytes and genotyping of VDR poly (A) was performed with the help of Sanger Sequencing.

### Results

The 3'-UTR VDR poly (A) is significantly associated ( $\chi^2=22.366$ ,  $p<0.05$ ) with BC risk; However, Poly (A) LL genotype (OR=4.35, 95% CI=1.593-12.024) and Poly (A) L allele (OR=2.496; 95% CI=1.600-3.895) increases the risk of early onset BC among cases and controls. Moreover, the disease free survival (DFS) reduces significantly with those having poly (A) SL genotype ( $p<0.05$ ).

### Conclusion

Hence, it is concluded that a positive and statistically significant association was found between VDR Poly (A) variant and early onset BC.

**Key words:** Vitamin D Receptor, VDR, microsatellite repeat, poly (A), Breast Cancer, 3' Untranslated region, early onset.

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## INTRODUCTION

Nearly one in nine females of Pakistan suffer from breast cancer (BC) which shows the highest rate of occurrence of BC among Asians.<sup>1</sup> Even though the principal causes for BC development are not clearly understood, however, it is expected that the genetic mutations can escalate the BC risk. The imbalance in hormone levels can also affect BC, therefore mutations or polymorphisms in the genes involved in the regulation of hormonal activity might be a potential contestant for BC. Moreover, the genes involved in the signaling cascade of steroid hormone might perform action together to increase the BC incidence. Among these genes, vitamin D receptor (VDR) involves in the transcriptional regulation of multiple hormone-responsive genes. The circulating form and the most preferred biomarker for VDR is Vitamin D<sub>3</sub>, inhibits proliferation and induces apoptosis in BC cells.<sup>2-4</sup> VDR along with vitamin D is a crucial mediator for proliferation and differentiation.<sup>5</sup> Therefore, polymorphisms in the VDR gene may have an impact on BC risk.

VDR encoding gene is recognized to show several polymorphic variations. A polymorphic site at the 3' UTR of gene is the poly (adenylate) [poly (A)] sequence repeats. Poly (A) is responsible to form the variable length VDR by the addition of adenine nucleotide at 3' end of the gene and can be separated into two groups; addition of 18 to 24 adenine repeats in VDR is the long (L) poly (A) and addition of 13 to 17 adenine repeats is short (S) Poly (A) VDR.<sup>6</sup> Poly (A) is positioned in close proximity with Bsm1, Apa1, Taq1 in the VDR gene, that's why poly (A) has a strong linkage disequilibrium (LD) with these polymorphisms. Therefore, 2 haplotypes are prevalent among Caucasians: baTL and BATs.<sup>7</sup> Many reports investigated relationship between Poly (A) and BC among population other than Pakistan, however, the functional data is inconclusive.<sup>8-10</sup> A recent study evaluating Poly (A) reported that Poly (A) 'LL' genotype has an effect on BC risk.<sup>8</sup> Similar results were obtained from other studies<sup>9,11-13</sup> whereas other has shown no association.<sup>10</sup> In conclusion, controversies regarding the relationship of poly (A) with BC was present, however, data shown

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that 'LL' variant may be a marker of BC development. Therefore, this study evaluates the association of poly (A) in the development of early onset BC among women in a genetically homogenous population.

## METHODOLOGY

This study is a case/control study conducted within the Department of Physiology, University of Karachi (UoK), was covered most of the areas of Karachi, Pakistan carried out exclusively on Karachiites, the metropolitan city of Pakistan where all ethnic groups are present.

All partakers were given with a written informed consent to be filled before drawing a blood sample. All procedures performed according to the guidelines of the Board of Advance Study and Research (BASR), University of Karachi (UoK) with Approval # (10(27) 28032012) and with Helsinki Declaration 1975". The sample size was calculated using OpenEpi<sup>®</sup> software with a 95% confidence interval, 80% statistical power and considering case Subjects between the ages of 20 and 40 years, parous, nulliparous, with or without a positive family history of breast cancer were included. Postmenopausal women and women over age 40 were excluded from the analysis.

Using self-structured questionnaire in English and Urdu, all participants were interviewed by the trained personnel.

One hundred and eleven (111) BC cases were recruited between the dates September 2012 to September 2013 through the tertiary hospitals of Karachi. Cases consisted of all ethnic groups women having breast cancer aged between 20 and 40 years and recognized through observing the hospital admissions and histopathology reports. The sample size is small because the present study was done on early onset breast cancer women and the prevalence of early onset BC is around 5% (hospitals data, not documented) because of lack of comprehensive cancer registries/databases in Pakistan. Nowadays, the early stage BC cases have been rising rapidly and the pathophysiology of this disease remains unclear.

Control subjects (n=108) with age range of 20 to 40 years were healthy females enrolled randomly from general population from different areas of Karachi, Pakistan. All control females had no evidence of having BC and other disorders. One control was matched (1:1) to each BC case based on the age and blood collection time.

For all subjects, a 3-cc of whole blood was taken into an EDTA tube for DNA extraction. 50–100ng of DNA extracted from whole blood in accordance with the directions of manufacturer (Gene JET Genomic DNA Purification Kit, Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania).

Genetic analysis of VDR Poly (A) gene was done by polymerase chain reaction (PCR) described previously<sup>14</sup> using the following primer set: 5'-GTGTAGTGAAAAGGACACCGGA-3' (forward) and 5'-GACAGAGGAGGGCGTGACTC-3' (reverse). The amplification profile from denaturation to extension was also previously described.<sup>14</sup> 425bp products were amplified which was separated on 1.5% agarose gel, and was visualized by ethidium bromide

staining. Purification of PCR products was done using kit method in accordance with the manufacturer instruction (Promega, Wizard<sup>®</sup> SV Gel and PCR Clean-Up System). The resulting products were then sequenced from out sourcing (Macrogen Inc., Korea). The interpretation of VDR Poly (A) was performed blindly without knowing subjects' status; a 100% concordance rate was obtained to those samples which had been consigned between three independent individuals. In case of non-agreement, the entire process of amplification along with the sequence analysis were repeated and reexamined independently again.

Survival analysis of all BC patients was also done using Kaplan Meier (KM) method.<sup>15</sup> The KM method may help in the estimation of recovery rates, survival rates, mortality rates and the effectiveness of treatment. All patients were followed for three years median. The recruitment of BC cases was started at Sept 2012. After three years, all recruited cases were checked if they died, survived or treated.

Women were categorized into three groups on the basis of genotype (SS, SL and LL). Poly a SS was taken as reference category for the analysis. The Hady Weinberg Equilibrium (HWE) was computed to compare the deviation of each group from a normal distribution. Pearson's  $\chi^2$  test was used to associate the VDR poly (A) gene polymorphism with breast cancer. ORs along with 95%CI were calculated to estimate the BC risk among Pakistani population using binary logistic regression. DFS rate among BC was also assessed using KM curve analysis and compared it with VDR poly (A) gene using log rank test. Mean survival time along with standard deviation of all BC participants was also calculated.

The data were analyzed using statistical tool IBM-SPSS software version 23.0 considering nominal  $p < 0.05$  is significant.

## RESULTS

### Characteristics of study participants

This study included 111 cases and 108 controls with poly (A) genotype data. Demographic characteristics are given in Table 1. The average age of BC cases and controls at the time of diagnosis was 35.6 and 35.2 respectively.

Parameters	Cases	Controls
Mean age at the time of sample collection (Yrs)	35.6	35.2
Mean age at menarche (Yrs)	12.4	12.5
Mean Body mass Index (kg/m <sup>2</sup> )	25.46	24.36
Mean Age at first birth (Yrs)	25.1	24.9
Family history of breast cancer (%)	12.2	12.1

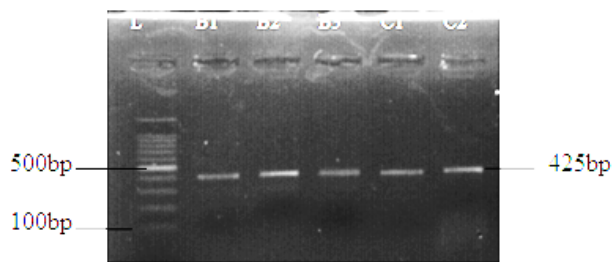
**Table 1:** Characteristics of Study participants

### Poly (A) gene variant and early onset BC risk

VDR Poly (A) polymorphism was genotyped using PCR followed by sequencing. 425bp long product was obtained by amplification (Figure 1A and 1B) which undergoes purification and sequencing. The varying length poly (adenylate) sequence was separated into

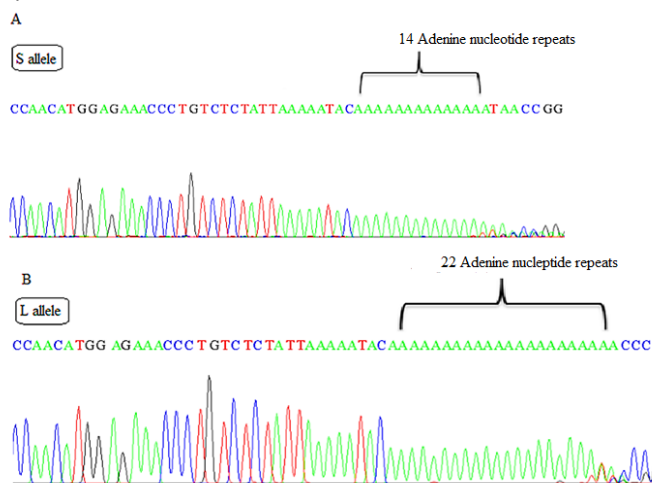
two sets of adenine nucleotide sequence; long (L) Poly (A) tail

contains 18 to 24 adenine nucleotides whereas short (S) poly (A) sequence comprises 13 to 17 adenine nucleotides. However, the Poly (A) genotypes were then grouped into short variant (SS), long variant (LL) and heterozygous variant (SL) in the analysis.



**Figure 1A:** Gel electrophoresis of Amplified product of VDR [Poly (A)] gene variant. The amplified products were 425bp long PCR products

Key: L= Ladder, B1-B3 = Breast cancer cases, C1-C2 = Controls



**Figure 1B:** DNA electropherogram of poly (A) repeat variant in 3'-UTR of VDR gene.

(A) Short allele possesses 8-17 adenine nucleotides (B) long allele possesses 18-24 adenine nucleotides.

The genotype distribution in controls of the Poly (A) polymorphism obeyed HWE ( $P=0.400$ ). Present study observed a positive and statistically significant association ( $\chi^2=22.366$ ,  $p<0.05$ ) between VDR Poly (A) gene with early onset breast cancer. However, Poly (A) LL genotype (OR=4.35, 95%CI=1.593-12.024) and Poly (A) L allele (OR=2.496; 95% CI=1.600-3.895) allele showed statistically significant risk of early onset breast cancer among cases and controls (Table 2).

#### Disease free survival (DFS) analysis

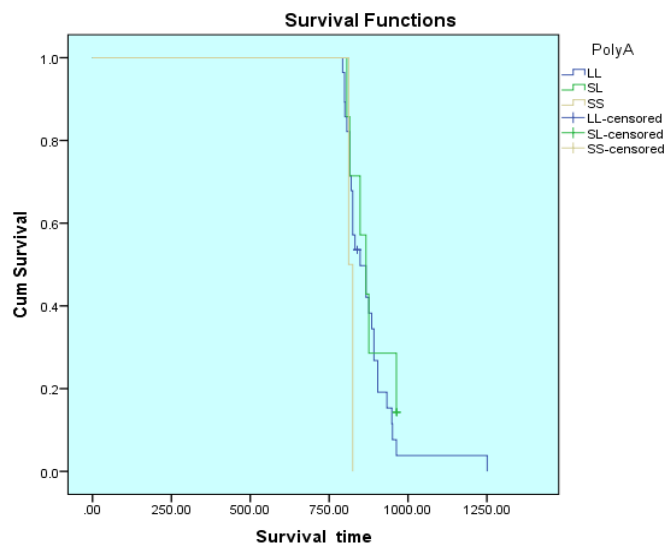
KM survival curves show the distribution of BC patients' survival time in relation with the VDR Poly (A) genotype (Figure 2). The KM analysis shows the statistically significant ( $\chi^2=9.246$ ,  $p<0.05$ ) survival distributions of the different poly (A) genotypes are not equal in the population (Table 3). So, our study suggested that poly

(A) heterozygous SL group showed increase survival time with an

average of 877 days. Moreover, the DFS reduces significantly with the poly (A) SL variant ( $p>0.05$ ).

SNPs	Genotypes	Cases (n)	Controls (n)	$\chi^2$	p-value	OR
						(95%CI)
Poly (A)	SS	6	15	22.4	0.00	1
	SL	21	45			1.16 (0.39-3.43)
	LL	84	48			4.35 (1.59-12.02)
	Total	111	108			
	S-allele	39	75	16.7	0.00	1
	L-allele	183	141			2.496 (1.60-3.89)
	Total	222	216			

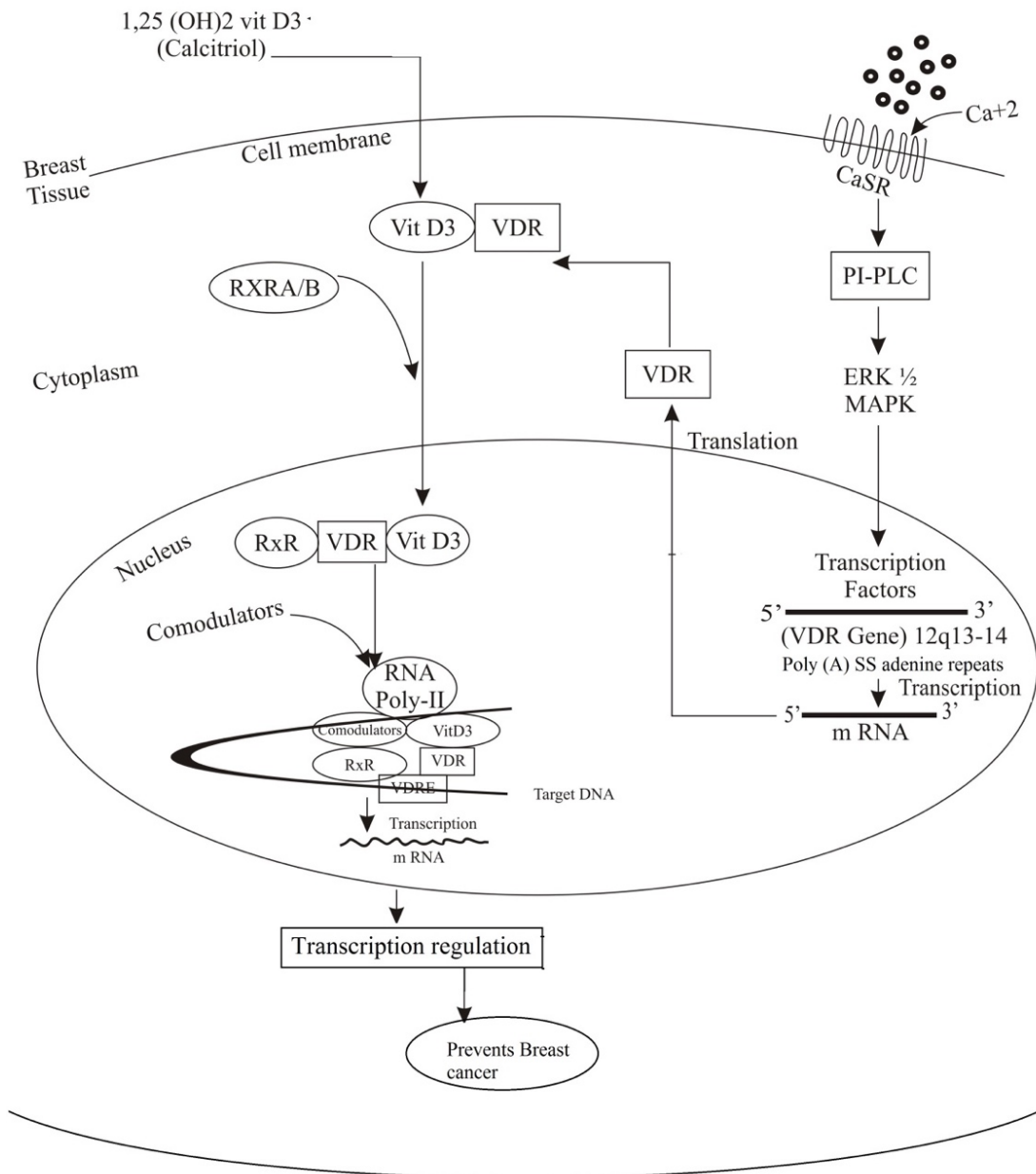
**Table 2:** VDR Poly (A) frequency and their association with premenopausal Breast cancer risk



**Figure 2:** Kaplan Meier curve of overall survival of BC cases by Poly (A)

## DISCUSSION

Breast cancer is a the diverse group of disease and more challenging, devastating and mortifying cancer among females all over the world and Pakistan as well. This cancer demands the lives of many females each year and affects countries at all levels. The prevalence of BC increases day by day in the third world countries just because of increase life expectancy, increase urbanization and adoption of sedentary lifestyle.<sup>16-18</sup> But still, the main reason of occurring BC among young females remains indeterminate. There are many BC risk factors including hormone exposure, being aged, BC family history, early menarche (first menstrual period), late menopause (cessation of menstrual cycle), late first live birth (after 30 years), hormone contraceptives (estrogen progesterone



**Figure 3 - Signaling pathway indicating the relationship between Poly (A) VDR and breast cancer risk..**

CaR = Calcium receptors, PI-PLC = Phosphotidyl inositol—phospholipase C, ERK ½ = Extracellular signal regulated kinases 1 and 2, MAPK = Mitogen activated protein kinases, RXR A and B = Retinoid X receptor A and B, VDRE = vitamin D receptor response element.

pills), radiotherapy and female adiposity. The familial BC is not common; approximately 10% of BC can be caused by having the high risk genes.<sup>19</sup> Hormones exposure <sup>20</sup> also influences the BC among which estrogen which is the most potent mammogen has a principal role in the BC development and progression.<sup>21</sup>The early BC prognosis among Pakistani females is controversial because a valid cancer registry system is not present in the country, which becomes a major hindrance in the exploration of leading facts of BC occurrence among Pakistani females.

In this study, the interplay between VDR poly (A) microsatellite repeats and early onset BC among Pakistani women were investigated. This study is first of its kind to investigate the role of Poly (A) microsatellite repeats among Pakistani population. This study showed the statistically significant association of Poly (A) variants of VDR gene with early onset BC risk. The present study also showed that early stage BC risk with Poly (A) 'LL' genotype and Poly (A) L allele which is in agreement with the Colagar, Firouzjah.<sup>8</sup> However, Ingles, Garcia <sup>12</sup> reported the

opposite observations. A meta-analysis also suggested that poly (A) may increase the susceptibility of BC<sup>22</sup> 3'-UTR are often perilous for the determination of mRNA stability. The internal poly (A) in VDR might be an essential component for regulating mRNA stability. A probable mechanism could be the presence of extraordinarily long and highly conserved adenylate/uridylate-rich elements in the 3'-UTR of VDR which contain multiple copies of the AUUUA motifs. The adenylate/uridylate rich motifs act as destabilizing elements facilitate the degradation of mRNA. VDR gene has 3 AUUUA motifs and variation in the size of motifs sequence may influence the binding poly (A) binding proteins. The binding proteins may bind differently with the long and short sequence and produce different physiological consequences<sup>23</sup> (Figure 3). Poly (A) tail involves in positive regulation of mRNAs stability after binding with binding proteins through interacting with the 5' end of genes.<sup>24</sup>

Results also showed that the survival time of VDR poly (A) variation was statistically significant among population. Therefore, this study also found increased survival time of BC patients having poly (A) SL variants may involve in the tumor progression. Previous study regarding disease free survival was done on other VDR genotyping including Fok1, Bsm1, Apa1 and Taq1 which showed the reduced disease free survival with VDR Fok1 genotype.<sup>25</sup>

The study has the strength that it attempts to answer the probable contribution of VDR poly (A) gene in breast cancer among Pakistani females. Present investigation has the limitation of relatively small sample size along with the single SNP of VDR gene. These limitations could be improved by enrolling more subjects, studying more VDR-SNPs, evaluating serum vitamin D3 levels, and assessing various clinic-pathological parameters and other risk factors.

## CONCLUSION

It is concluded that Poly (A) LL genotype may be associated with early onset breast cancer among Pakistani females. VDR which is expressed in the mammary gland is involved in vitamin D signaling pathway requires a continuous monitoring during treatment for better prognosis. However, the functions and role of VDR cannot be neglected during BC treatment.

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### Disclaimers

This research is part of Ph.D. thesis submitted to Higher Education Commission (HEC), Pakistan in 2017

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