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The University of Texas Rio Grande Valley

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Molecular Mechanism of Cancer Susceptibility Associated with Fok1 Single Nucleotide Polymorphism of VDR in Relation to Breast Cancer

Sana Raza1, Anupam Dhasmana2, Madan Lal Brahma Bhatt3, Mohtashim Lohani4, Jamal M Arif1*

Abstract

Breast cancer is the leading cause of death among women worldwide. It is a multi-factorial disease caused by genetic and environmental factors. Vitamin D has been hypothesized to lower the risk of breast cancer via the nuclear vitamin D receptor (VDR). Genetic variants of these vitamin D metabolizing genes may alter the bioavailability of vitamin D, and hence modulate the risk of breast cancer. Materials and Methods: The distribution of Fok1 VDR gene (rs2228570) polymorphism and its association with breast cancer was analysed in a case–control study based on 125 breast cancer patients and 125 healthy females from North Indian population, using PCR-RFLP. An In silico exploration of the probable mechanism of increased risk of breast cancer was performed to investigate the role of single nucleotide polymorphisms (SNPs) in cancer susceptibility. Results: The Fok1 ff genotype was significantly associated with an increased risk of breast cancer (p=0.001; χ²=13.09; OR=16.909; %95 CI=2.20 - 130.11). In silico analysis indicated that SNPs may lead to a loss in affinity of VDR to calcitriol, and may also cause the impairment of normal interaction of liganded VDR with its heterodimeric partner, the retinoid X receptor (RXR), at protein level, thereby affecting target gene transcription. Conclusion: Breast cancer risk and pathogenesis in females can be influenced by SNPs. SNPs in VDR may cause alterations in the major molecular actions of VDR, namely ligand binding, heterodimerization and transactivation. VDRE binding and co-activator recruitment by VDR appear to be functionally inseparable events that affect vitamin D-elicited gene transcription. This indicates that breast cancer risk and pathogenesis in females may be influenced by SNPs.

Keywords: Vitamin D- vitamin D receptor- polymorphism- breast cancer- Fok1

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Introduction

Breast cancer is the most common type of female cancer worldwide which represents about a quarter (23%) of all cancers in women (Ferlay et al., 2012), and its rate of incidence and mortality are annually increasing. About 63,410 cases of female breast carcinoma in situ and 74,680 cases of melanoma in situ were expected to be diagnosed in the United States in 2017 (Siegel et al., 2017). The incidence of breast cancer in India has surpassed cervical cancer, which was earlier considered to be the most common cancer among Indian women. Breast cancer has now become the leading cause of cancer death among Indian women (Kaarthigeyan, 2012). A significant increase in the incidence of cancer and cancer-associated morbidity and mortality has been observed in the Indian subcontinent, as reported by a number of studies (Srinath et al., 2005; Ali et al., 2011; Babu et al., 2013; Balasubramaniam et al., 2013). A 13.82% increase in the rate of mortality and 11.54% increase in the rate of cancer incidence have been seen in India, due to breast cancer during 2008–2012 (Ferlay et al., 2012). This observed hike in mortality may be attributed to lack of adequate strategies for breast cancer screening, diagnosis of the disease at an advanced stage and inappropriate medical facilities.

Vitamin D has classically been associated with the maintenance of calcium and phosphorous homeostasis in the body. Besides the classical biological effects of vitamin D on calcium and phosphorous homeostasis, calcitriol, the active form of vitamin D, is known to exert a broad variety of actions including various anticancer effects which may be mediated either transcriptionally and/or via non-genomic pathways (Haussler et al., 2011). Vitamin D is involved in cell cycle regulation, induction of apoptosis, promoting cell differentiation and also known to act as an anti-inflammatory factor within
the tumor microenvironment (Deeb et al., 2007). The protective action of vitamin D has also been implicated as a suppressor of cancer cell invasion, angiogenesis and metastasis (Lopes et al., 2012). Like other cancers, breast cancer is a multistep process with multifactorial involvement of genetic and environmental factors. Environmental exposure to lower level of UV radiation, which is essential for the synthesis of vitamin D, may be a risk factor for higher cancer incidence of many types including breast cancer (Finkelmeier et al., 2014). Biosynthesis of vitamin D starts with the non-enzymatic conversion of 7-dehydrocholesterol (pro-vitamin D) to cholecalciferol (pre-vitamin D). Cholecalciferol is transported to the liver where it gets hydroxylated by the enzyme 25-hydroxylase (in liver) to form 25-hydroxyvitamin D, also known as calcidiol, the most abundant and stable vitamin D metabolite. Calcidiol is further hydroxylated by the enzyme 1-a-hydroxylase (in kidney) to 1, 25-dihydroxyvitamin D or calcitriol (Khokhar, 2012), the most active metabolite of vitamin D (Penna-Martinez et al. 2012) and binds nuclear vitamin D receptor (VDR) in target organs. A number of researchers have shown an association between vitamin D levels and breast cancer (Zerwekh, 2008; Yao et al., 2012; Shaukat et al., 2017). It is possible that the genetic variants of vitamin D metabolizing genes can alter the bioavailability of vitamin D and thus modulate the risk of breast cancer.

Vitamin D, a steroid hormone, exerts most of its biological activities by binding to a specific high-affinity receptor, the VDR. VDR allows the body to respond to vitamin D in an appropriate manner (Deeb et al., 2007). VDR belongs to the superfamily of nuclear receptors (NRs) for steroid hormones and regulates gene expression by acting as a ligand activated transcription factor (Diaz et al., 2015; Yang et al., 2017) and, therefore, represents an important drug target which may directly be linked to a number of severe diseases. VDR is a member of superfamily of proteins which are known to contain amino acid homologies within two separate functional domains and exerts the transcriptional activation and repression of a number of target genes by binding to nuclear VDR (Feldman et al., 2014). The genomic mechanism of transcriptional activation by VDR involves recognition and binding to the vitamin D response elements (VDREs) in promoter regions of target genes, through the DNA-binding domain (Feldman et al., 2014). Genetic polymorphisms of VDR may modulate the risk of breast cancer by altering its expression as well as function in breast cell. The gene encoding the human VDR is located on the long arm of chromosome 12 i.e. 12q.13.11 (NCBI GeneID: 7421) and harbours several polymorphisms in the coding and non-coding regions. A number of single nucleotide polymorphisms (SNPs) have been identified to be located in the VDR gene promoter region, in and around exon 2-9 and in the 3’ UTR region (Uitterlinden et al., 2004; Fang et al., 2005). Functional genetic polymorphisms that lead to an alteration in the regulation of gene expression are predicted to have a significant influence on disease pathogenesis (Pastinen et al., 2006). The allelic variants of the human VDR gene may, therefore, be risk factors for a variety of diseases including breast cancer.

One of the VDR polymorphisms to be commonly found is the 5’ Fok1 site (rs2228570) in exon 2 (Berndt et al., 2006) resulting in thymine (T) to cytosine (C) substitution. In the present study, we analysed the distribution of Fok1 VDR gene polymorphism in the North Indian population and its association with breast cancer. An in silico exploration of the probable mechanism of increased risk of breast cancer with the Fok1 polymorphism of the VDR was performed to explore the mechanism underlying breast cancer susceptibility.

Materials and Methods

In-vitro analysis

Ethics statement

Ethical approval for the study was obtained from the Institutional Ethics Committee, King George Medical University, Lucknow (Ref. no. 78th ECM IIB-Ph.D./P2/2016). All participants signed an informed written consent prior to providing blood samples.

Subjects

We investigated the prevalence of VDR Fok1 (rs2228570) T/C gene polymorphism in 125 breast cancer patients and 125 age matched healthy control subjects who were in follow up at the Department of Radiotherapy, King George Medical University, Lucknow. Selection of patients was mainly done using the following criteria: Any patient who had been histologically diagnosed for breast cancer with no concurrent chronic disease, had no infection with HIV, HBV or HCV, and aged between 18 and 70 years. The age-matched controls were randomly selected from among a pool of healthy volunteers attending general health check-ups, and blood bank donors at King George Medical University. The sample size was determined using statistical approach (Snedecor et al., 1989). The mean age of cases and control groups were 44.47yrs and 40.88yrs respectively.

Blood sample collection and DNA isolation

After a careful review of the inclusion and exclusion criteria, blood specimens (2ml) were collected in tubes containing ethylene diamene tetra acetic acid (EDTA) as anticoagulant. The blood specimens were collected after obtaining informed consent after queries related to study and benefits expected would be given to participants. A peer reviewed, well drafted study proforma was filled for each subject providing patient personal, familial and clinical details. Blood samples were stored at -80°C. Frozen blood samples were later thawed at room temperature and high molecular weight DNA was extracted by using the salting out procedure (Miller et al., 1988).

Genotyping of Fok1 (T>C rs2228570)

Genotyping was performed by using polymerase chain reaction (PCR) and restriction fragment length polymorphism analysis (RFLP). The primer pair used for amplification of genomic DNA for genotyping of Fok1 polymorphism is shown in Table 1. The reaction conditions used for PCR amplification were as follows:
Initial denaturation step of 4 min at 94°C followed by 30 cycles of 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min. A final extension step at 72°C for 4 min was also performed (Toptas et al., 2013).

The 272bp PCR product was digested with 1U of Fok1 restriction enzyme (New England Biolabs) and incubated at 37°C (Toptas et al., 2013), overnight digestion was allowed to minimise partial digests. 6µl of the digested reaction mixture was electrophoresed for 1 hour at 100V and visualised on 2% agarose gels, stained with ethidium bromide. The size of the restriction endonuclease digested products was determined using a 100bp DNA ladder (G Biosciences). The presence of a given restriction site was assigned by lower case (f) and absence by upper case (F).

**Statistical method**

To evaluate the relationship between gene and genotype frequencies, Hardy-Weinberg equilibrium (HWE) was tested. The difference in distribution of the genotypes or alleles between cases and controls was tested using the chi-square statistic. Odds ratios (ORs) and 95% confidence intervals (CI) were calculated to estimate the risk of breast cancer. The association between genetic polymorphisms and breast cancer risk was evaluated using multivariate unconditional logistic regression analysis and p value <0.05 was considered as statistically significant. Statistical analysis was performed on SPSS Software (SPSS Inc., version 17.0).

**Computational genomic analysis**

The information of SNPs [SNP ID, amino acid position] of the human VDR gene was procured from the National Center for Biotechnology Information (NCBI) SNP database [http://www.ncbi.nlm.nih.gov/snp/] (Sherry et al., 2001). An *in silico* exploration of the Fok1 SNP of VDR was performed using the Sorting Intolerant From Tolerant (SIFT) bioinformatics online tool (http://sift. bii.a-star.edu.sg/www/SIFT_seq_submit2.html). SIFT predicts the effect of coding variants on protein function, based on sequence homology and physical properties of amino acids (Kumar et al., 2009).

**Retrieval of the primary sequence of VDR**

Primary FASTA sequence of VDR was procured from the Uniprot database (Uniprot ID-P11473), a comprehensive resource for protein sequence and annotation data (Apweiler et al., 2004).

**Procurement and generation of the 3D structural model of VDR, Fok1 variant and calcitriol**

The 3D structure of VDR (Figure 1) was procured from the RCSB protein Data Bank (Pdb.ID 1DB1). Due to absence of the experimental 3D structures of Fok1 variant, MODELLER 9.10 was used for the Homology Modeling and generation of the 3D structure of VDR variant (Eswar et al., 2006). Ramachandran Plot (RAMPAGE) was used for the validation and selection of the generated 3D structures (Hollingsworth et al., 2010). The structure for calcitriol (the active form of Vitamin D) was generated using the ChemSketch Software (ACD Lab Version 12.0) and CORINA online server. Chimera was used for energy minimization and removal of steric collision.

**Protein stability (ΔΔG) prediction**

To examine the effect of point mutation on the change in protein stability, the difference in folding free energy was calculated using the I-mutant (I-mutant suite 3.0) program. I-mutant is a support vector machine (SVM) based tool used for the automatic prediction of protein stability changes upon single point mutation (Capriotti et al., 2006).

**Molecular docking studies**

Molecular interaction and binding analysis was done using Autodock (Version 4.0) suite and Cygwin interface in the Microsoft Windows Professional Version 2002, Intel® i7 processor, 3.30Ghz and 16 GB RAM DELL Machine. Molecular docking simulation methods were followed by searching of the best conformation of the wild type and mutated VDR protein and calcitriol complexes. Water molecules were deleted from the protein structures before docking simulation and hydrogen atoms were added to both wild type and mutated proteins. Kollman united charges and salvation parameters were added to the proteins. Gasteiger charge was added to the chemical compounds. The values were set to 55×61.25×70.835 Å in X, Y and Z axis of grid point. The default grid points spacing was 0.375 Å. Lamarckian Genetic Algorithm (LGA) was used for wild type and mutated proteins and calcitriol for docking calculations. The LGA parameters like population size (ga_pop_size), energy evaluations (ga_num_generation), mutation rate, crossover rate and step size were set to 150, 25000, 27,000, 0.02, 0.8 and 0.2 Å, respectively. The LGA runs were set to 10 runs. All obtained 10 conformations of proteins and calcitriol complexes, were analyzed for the interactions and binding energy of the docked structure using Discovery Studio Visualizer version 2.5.5 and PyMol. The analyses were performed in Microsoft Windows 7 professional Version 2002, Intel (R) i7 processor, 3.30 GHz CPU and 16.0 GB RAM DELL Machine.

**Protein-protein interaction analysis**

String database was used to identify the preferential functional partners of VDR (Szklarczyk et al., 2015). The protein-protein interaction analysis was performed using Hex version 6.3 (Macindoe et al., 2010), PatchDock Beta version 1.3 (Schneidman-Duhovny et al. 2005), and Fast Interaction Refinement in Molecular Docking (FireDock ref.1 server) (Mashiahi et al., 2008) bioinformatics tools.

**Results**

**In-vitro**

The size of the amplification product for the Fok1 polymorphism was 272bp (Figure 2A). An intact amplification product indicated the absence of Fok1 restriction site (F), while the presence of the Fok1 site (f) was indicated by two or three fragments.

The undigested, single 272bp bands were genotyped as FF genotype (homozygote of common allele) in the agarose gel. The ff genotype (homozygote of infrequent...
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allele) generated two fragments of 198bp and 74bp while the heterozygotes (Bb) displayed three fragments (272bp, 98bp, and 74bp). Figure 2B depicts the distribution of VDR Fok1 polymorphism in cases and controls.

The analysis included 125 breast cancer cases and 125 healthy controls. Table 2 depicts genotype and allele frequencies in breast cancer cases and healthy controls. 36% of the patients were found to be heterozygous (Ff) for the Fok1 polymorphism, 52% were homozygous (FF) and 12% were homozygous (ff). The respective frequencies of these genotypes in the control group were 36%, 52%, and 12%, respectively. The respective allelic frequencies were 70% for F and 30% for f.

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Table 1. Primer Sequences Used for PCR Amplification

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>Fok1 Forward</td>
<td>5'-GAT GCC AGC TGG CCC TGG CAC TG-3'</td>
</tr>
<tr>
<td>Fok1 Reverse</td>
<td>5'-ATG GAA ACA CCT TGC TTC TTC CTC-3'</td>
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</table>

Table 2. Genotype and Allele Frequencies in Breast Cancer Cases and Healthy Controls

<table>
<thead>
<tr>
<th>Fok1 T/C (rs2228570)</th>
<th>Cancer Cases (n=125)</th>
<th>Healthy Controls (n=125)</th>
<th>OR</th>
<th>95% CI</th>
<th>(X^2)</th>
<th>p values</th>
<th>Bonferroni corrected p values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FF</td>
<td>65</td>
<td>52</td>
<td>68</td>
<td>54.40</td>
<td>0.908</td>
<td>0.55 - 1.49</td>
<td>0.15</td>
</tr>
<tr>
<td>Ff</td>
<td>45</td>
<td>36</td>
<td>56</td>
<td>44.80</td>
<td>0.693</td>
<td>0.42 - 1.15</td>
<td>2.01</td>
</tr>
<tr>
<td>ff</td>
<td>15</td>
<td>12</td>
<td>1</td>
<td>0.80</td>
<td>16.909</td>
<td>2.20 - 130.11</td>
<td>13.09</td>
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<tr>
<td>Allele</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>175</td>
<td>70</td>
<td>192</td>
<td>76.80</td>
<td>0.705</td>
<td>0.47 - 1.05</td>
<td>2.96</td>
</tr>
<tr>
<td>f</td>
<td>75</td>
<td>30</td>
<td>58</td>
<td>23.20</td>
<td>1.419</td>
<td>0.95 - 2.11</td>
<td>2.96</td>
</tr>
</tbody>
</table>

Average power, 0.816; OR, odds ratio; CI, confidence interval.

Figure 1. Crystal Structure of Nuclear VDR Complexed to Vitamin D (as obtained from RCSB protein data bank; PDB ID: 1DB1).

Figure 2. Ethidium Bromide Stained 2% Agarose Gel Picture of Fok1 PCR Amplification (Panel A) and Digestion Products of VDR Gene (Panel B). A) The size of the amplification product for the Fok1 polymorphism was 272bp. Lane 1 shows a 100bp ladder. Lanes 2-10 show single bands corresponding to the 272bp Fok1 PCR product. B) Ethidium bromide stained 2% agarose gel picture of Fok1 digested amplification products of VDR gene: The upper bands represent F (T allele), the lower bands represent f (C allele). Lane 1 shows a 100bp ladder. Lanes 2, 6, and 9 show FF heterozygotes; lanes 3,4,7,8 and 10 show FF homozygotes; lane 5 shows ff homozygote.

Figure 3. The Genotype, Allele Frequencies, in Fok1 Cases and Controls

Table 2. Genotype and Allele Frequencies in Breast Cancer Cases and Healthy Controls

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<td></td>
<td></td>
</tr>
<tr>
<td>FF</td>
<td>65</td>
<td>52</td>
<td>68</td>
<td>54.40</td>
<td>0.908</td>
<td>0.55 - 1.49</td>
<td>0.15</td>
</tr>
<tr>
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<td>45</td>
<td>36</td>
<td>56</td>
<td>44.80</td>
<td>0.693</td>
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<td>16.909</td>
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<td>13.09</td>
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<td>Allele</td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>0.705</td>
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<td>58</td>
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<td>0.95 - 2.11</td>
<td>2.96</td>
</tr>
</tbody>
</table>

Average power, 0.816; OR, odds ratio; CI, confidence interval.
44.8%, 54.4% and 0.8%. The VDR Fok1 ff genotype was significantly increased in breast cancer patients as compared to controls (Figure 3). The Fok1 ff genotype was significantly associated with an increased risk of breast cancer (p=0.001; χ²=13.09; OR=16.909; %95 CI=2.20 - 130.11).

**In-silico**

The computational genomic analysis and exploration of the Fok1 variant shows that Fok1 is a non-synonymous SNP and lies in the coding/exonic region (Table 3). The Fok1 polymorph persists its genomic alteration at protein level and shows amino acid substitution at position 1 (M1I; Methionine to Isoleucine) (Figure 4A). Point mutation in VDR caused a loss of protein stability, and the difference in free folding energy (ΔΔG) of the SNP variants was analysed using I mutant program. Fok1 SNP showed ΔΔG energy of -0.40 kcal/mol. A significant decrease in the total number of intra-molecular H-bonds was observed in the Fok1 SNP variant compared to the wild type VDR protein. Wild type VDR showed 556 intra-molecular H-bonds while the Fok1

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Protein</th>
<th>Ligand</th>
<th>Binding Energy (kcal/mol)</th>
<th>Ki (μM)</th>
<th>Hydrogen Bond</th>
<th>Distance (Å)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>VDR(wild type)</td>
<td>Calcitriol</td>
<td>-7.39</td>
<td>214.66</td>
<td>:LYS264:HZ3 - :Calcitriol:O28</td>
<td>2.01639</td>
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<tr>
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<td></td>
<td></td>
<td>:Calcitriol:H71 - :PRO344:O</td>
<td>1.8552</td>
</tr>
<tr>
<td>2</td>
<td>VDR(Fok1 variant)</td>
<td>Calcitriol</td>
<td>-5</td>
<td>3.82</td>
<td>:HIS397:HE2 - :Calcitriol:O9</td>
<td>2.23125</td>
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<td>:Calcitriol:H70 - :SER278:OG</td>
<td>2.15288</td>
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<td></td>
<td>:Calcitriol:H71 - :TYR236:OH</td>
<td>1.93697</td>
</tr>
</tbody>
</table>

Figure 4. Computational Genomic Analysis of SNP: (A) The position of SNP induced amino acid substitution in Fok1 variant (Position 1: Methionine to Isoleucine) of VDR (B) Change in number of intra-molecular H-bonds upon point mutation (wild type VDR: 556; Fok1 variant: 505).
variant showed 505 intra-molecular H-bonds (Figure 4B). The molecular interaction analysis of wild type VDR with calcitriol and \textit{Fok1} variant with calcitriol is illustrated in Table 4. The \textit{Fok1} variant (-5.0 kcal/mol) has a lower binding energy in comparison to wild type VDR protein (-7.39 kcal/mol).

The total number of binding (amino acid) residues found between wild type VDR and calcitriol were 19, however, a decrease in the total number of binding residues was seen upon point mutation, and only 10 binding residues were seen to be formed between the \textit{Fok1} variant and calcitriol.

The retinoid X receptor (RXR) is a well-established functional partner of VDR (Orlov et. al. 2012). String database network depicting most preferential functional partners of VDR (Figure 5). The protein-protein interaction analysis of liganded wild type VDR (VDR+Calcitrol) to RXR (Patch Dock: 15456; HEX: -727.3; FireDock: -36.75) and liganded \textit{Fok1} variant to RXR shows that liganded VDR variant (\textit{Fok1}) has a lower binding score (PatchDock-15064; HEX: -717; Firedock: -10.10) compared to wild type VDR (Table 5). Both forms of the RXR (RXR alpha and RXR beta) were analysed.

**Discussion**

The \textit{Fok1} polymorphism lies in exon 2, and alters the start codon ATG, leading to the substitution of methionine by isoleucine at position 1 (M1I). This polymorphism leads to a substitution from T to C (ATG to ACG) at the first initiation codon (ATG) leading to an altered translation start site. The presence of \textit{Fok1} restriction site (denoted f) results in expression of the full isoform of VDR protein (427 residues). The shorter isoform (424 residues long) is produced in the absence of \textit{Fok1} site (denoted F) (Jurutka et al., 2001). The production of two differently sized proteins may affect VDR function. VDR is known to heterodimerize with RXR, recruiting other transcriptional co-activators that regulate target gene transcription.

Our study shows that SNPs may hamper the normal interaction of liganded VDR with RXR at protein level. Statistical potential algorithms are often used for prediction of changes in stability of proteins, upon point mutation (Parthiban et al., 2007). The analysis of difference in free folding energy ($\Delta\Delta G$) of the SNP variant shows that \textit{Fok1} SNP variant has $\Delta\Delta G$ energy of 0.40 kcal/mol. The output of the predicted free energy change ($\Delta\Delta G$) classifies the results into one of the three classes i.e. largely stable ($\Delta\Delta G>0.5\text{kcal/mol}$), neutral ($-0.5\leq\Delta\Delta G<0.5\text{kcal/mol}$) or largely unstable ($\Delta\Delta G<-0.5\text{kcal/mol}$). This indicates a decrease in stability of VDR upon point mutation, as a protein prefers to stay in its lowest energy conformation i.e. $\Delta\Delta G=0$ (Du et al. 2016). A significant decrease in total number of intra-molecular H-Bonds was also observed in the \textit{Fok1} variant compared to wild type VDR protein (wild type VDR: 556; \textit{Fok1}: 505). Hydrogen bonds contribute favourably to protein stability (Pace et al., 2014). A reduction in the total number of intramolecular H-bonds also indicates a decrease in the overall protein stability, upon point mutation. The binding affinity analysis of wild type VDR and \textit{Fok1} variant with calcitriol shows that the SNP causes a loss in affinity of VDR for calcitriol. The \textit{Fok1} SNP variant shows lower binding energy with calcitriol (-5.0 kcal/mol) compared to the wild type VDR protein (-7.39 kcal/mol). The more negative the energy, the more effective

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**Table 5. Protein-Protein Interaction Analysis of Liganded Wild Type VDR and Liganded SNP Variants of VDR with RXR (Alpha and Beta)**

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Receptor</th>
<th>Ligand</th>
<th>Binding Score by Patch Dock</th>
<th>Binding Score by HEX</th>
<th>Binding Score by Fire Dock</th>
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<tr>
<td>1</td>
<td>WT-VDR+ Calcitriol</td>
<td>RXR-Alpha</td>
<td>15456</td>
<td>-727.3</td>
<td>-36.75</td>
</tr>
<tr>
<td>2</td>
<td>Fok1-VDR+ Calcitriol</td>
<td>RXR-Alpha</td>
<td>15064</td>
<td>-717</td>
<td>-10.1</td>
</tr>
<tr>
<td>3</td>
<td>WT-VDR+ Calcitriol</td>
<td>RXR-Beta</td>
<td>16558</td>
<td>-837.6</td>
<td>-22.22</td>
</tr>
<tr>
<td>4</td>
<td>Fok1-VDR+ Calcitriol</td>
<td>RXR-Beta</td>
<td>16080</td>
<td>-715.8</td>
<td>-21.69</td>
</tr>
</tbody>
</table>

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Figure 5. String Database Network Showing Preferential Functional Partners of VDR.

Figure 6. Molecular Mechanism of Cancer Susceptibility Associated with the SNPs of the Nuclear Hormone Receptor VDR
binding. Further, the protein-protein interaction analysis of liganded wild type VDR (VDR+Calcitriol) to RXR (Patch Dock: 15456; Hex: -727.3; Firedock: -36.75) and liganded Fok1 variant to RXR shows that liganded VDR Fok1 (PatchDock: 16080; Hex: -715.8; Firedock: -21.69) and RXR alpha have lower binding score as compared to wild type VDR and RXR alpha. A similar trend was observed with RXR beta form. The Fok1 SNP (PatchDock: 16080; Hex: -715.8; Firedock: -21.69) variant showed a lower binding score to RXR-Beta as compared to the wild type VDR protein (PatchDock: 16558; Hex: -837.6; Firedock: -22.22). A significant reduction in the number of intermolecular H-Bonds was also observed between the liganded Fok1 SNP variant of VDR and RXR. This indicates that SNP may hamper the normal interaction of liganded VDR with the RXR at protein level. The aetiology of any specific cancer may probably be associated with a set of genetic variants, some of which could adversely interact with certain environmental factors. VDR regulates gene expression in a ligand-dependent manner (Dwivedi et al., 2002). It is also involved in micro-RNA directed post-transcriptional mechanisms (Campbell, 2014).

VDR has also been reported to be involved in estrogen related pathways, immunomodulation, insulin-like growth factor signaling and known to affect gene expression in a ligand-dependent manner (Yang et al., 2017).

Based on the above observations, we tried to elucidate the mechanism of cancer susceptibility associated with the SNPs of VDR at protein level (Figure 6). The binding affinity analysis of liganded wild type VDR and liganded Fok1 variant to RXR shows that the liganded Fok1 variant has a lower binding energy as compared to wild type VDR and RXR. This indicates that the SNPs may cause an impairment of the normal interaction of VDR with its heterodimeric partner RXR at protein level. The liganded VDR-RXR heterodimerization is functionally linked to VDRE binding and recognition in the DNA sequence of vitamin D regulated genes. The SNPs may not only cause a loss in affinity of VDR to calcitriol, but also lead to the impairment of the normal interaction of VDR to RXR at protein level. VDR is known to regulate about 3,000 genes in the human genome, including some genes like hp21 and hFOXO1, which are involved in cell cycle control (proliferation, differentiation, migration and death) and apoptosis (Anderson et al., 2011). The other genetic polymorphisms of VDR may also have a role in modulating the risk of breast cancer by affecting gene splicing, transcription factor binding, etc.

Over the past decades, extensive research has shown that low sunlight exposure and deficiency of vitamin D are associated with increase in risk of extra-skeletal diseases like cancer (Bikle et al., 2016; Wang et al., 2017). VDR has been suggested to control the expression of a number of genes that are associated with cell proliferation and differentiation. This indicates that VDR may play a key role in prevention of cancer (Gil et al., 2018). To date, very few studies have shown the importance of gene variants as prognostic markers unequivocally. However, a small percentage of the known genes have been adequately studied till yet, therefore, the investigation of SNPs still remains active. SNP association studies targeting cancer may be divided into two broad categories i.e. investigation of susceptibility and investigation of outcomes. The outcomes seek to determine the prognostic information for survival, response to pharmacological intervention, or complications. SNP variants may be associated to outcome and, hence, could be applied to making clinical decisions. Further research is needed to explore the functional mechanisms behind the observed effects of these polymorphisms. The assessment of VDR polymorphisms is essential for identification of the groups at risk and to develop strategies to target it. Thus, SNPs in VDR may cause alterations in the major molecular actions of VDR, namely ligand binding, heterodimerization, and transactivation. Breast cancer risk and pathogenesis in females can be influenced by SNPs and the analysis of SNPs in breast cancer research has pleiotropic implications for clinical and public health issues, as well as cancer biology. Computer-based structural & genomic analysis of SNPs may play a significant role in cancer management.

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Conflict of Interest

We have no conflict of interest with anybody working in the area and among authors in the manuscript. The authors alone are responsible for the content and writing of the paper.

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