RESEARCH ARTICLE

Editorial Process: Submission:07/26/2018 Acceptance:12/16/2018

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

Sana Raza[1], Anupam Dhasmana[2], Madan Lal Brahma Bhatt[3], Mohtashim Lohani[4], Jamal M Arif[1]*

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; χ^2=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*

Asian Pac J Cancer Prev, **20 (1)**, 199-206

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

*[1]Department of Biosciences, Integral University, [3]Department of Radiotherapy, King George Medical University, Lucknow, [2]Himalayan School of Biosciences and Cancer Research Institute, Swami Rama Himalayan University, Dehradun, India, [4]College of Applied Medical Sciences, University of Jazan, Saudi Arabia. *For Correspondence: jmarif@gmail.com*

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-α-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 (Díaz 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* polymorph 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 44.472yrs 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 1min, annealing at 60°C for 1 min, and extension at 72°C for 1min. 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, 2500,000, 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) (Mashiach 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

Table 1. Primer Sequences Used for PCR Amplification

Primer	Sequence
Fok1 Forward	5'-GAT GCC AGC TGG CCC TGG CAC TG-3'
Fok1 Reverse	5'-ATG GAA ACA CCT TGC TTC TTC TCC CTC-3'

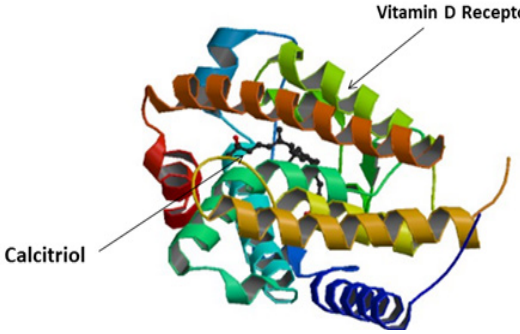

Figure 1. Crystal Structure of Nuclear VDR Complexed to Vitamin D (as obtained from RCSB protein data bank; PDB ID: 1DB1).

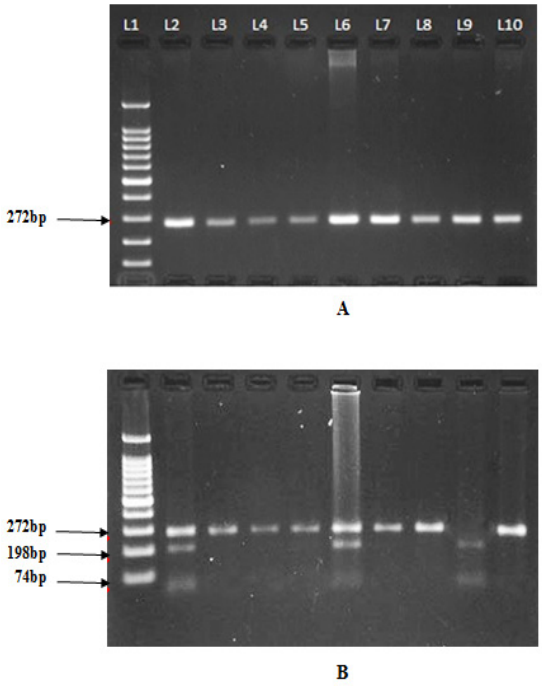

Figure 2. Ethidium Bromide Stained 2% Agarose Gel Picture of Fok1 PCR Amplification (Panel A) and Digestion Products of VDR Gene (Panel B). A) The size of the amplification product for the Fok1 polymorphism was 272bp. Lane 1 shows a 100bp ladder. Lanes 2-10 show single bands corresponding to the 272bp 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.

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

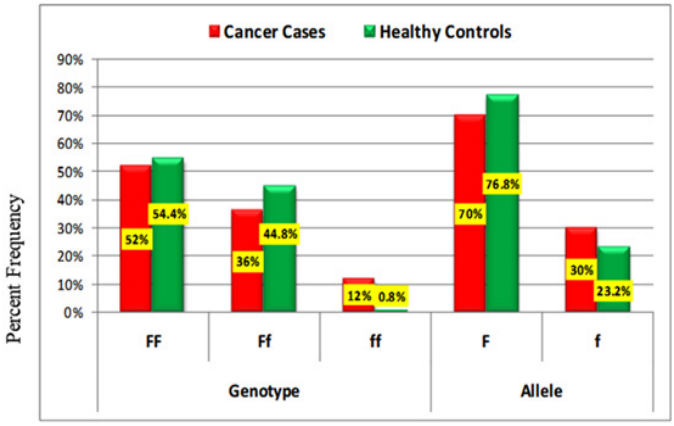

Figure 3. The Genotype, Allele Frequencies, in *Fok1* Cases and Controls

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

Fok1 T/C (rs2228570) SNP		Cancer Cases (n=125)		Healthy Controls (n=125)		OR	95% CI	X^2	p values	Bonferroni corrected p values
		N	%	N	%					
Genotype	FF	65	52	68	54.40	0.908	0.55 - 1.49	0.15	0.704	1
	Ff	45	36	56	44.80	0.693	0.42 - 1.15	2.01	0.156	0.469
	ff	15	12	1	0.80	16.909	2.20 - 130.11	13.09	0.0003	0.001
Allele	F	175	70	192	76.80	0.705	0.47 - 1.05	2.96	0.085	0.171
	f	75	30	58	23.20	1.419	0.95 - 2.11	2.96	0.085	0.171

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

Table 3. Computational Analysis of *Fok1* VDR SNP by the SIFT Tool

SNP	*Fok1*
Coordinates	12,48272894,1,T/C
Region	EXON CDS
DbSNP ID	rs2228570
Prediction	DAMAGING
Gene ID	ENSG00000111424
Gene Name	VDR
SNP type	Non-synonymous
Gene desc	Vitamin D (1,25- dihydroxyvitamin D3) receptor [Source:HGNC Symbol;Acc:12679]
Protein Family ID	ENSFM00630001050580
Protein Family Desc	Vitamin d3 receptor (VDR); 1, 25 dihydroxyvitamin d3 receptor nuclear receptor; subfamily 1 group i member 1.
Transcript status	KNOWN
Substitution effect of SNP on Protein sequence amino acid	M1I
ΔΔG value prediction of VDR protein	-0.40 Kcal/Mol

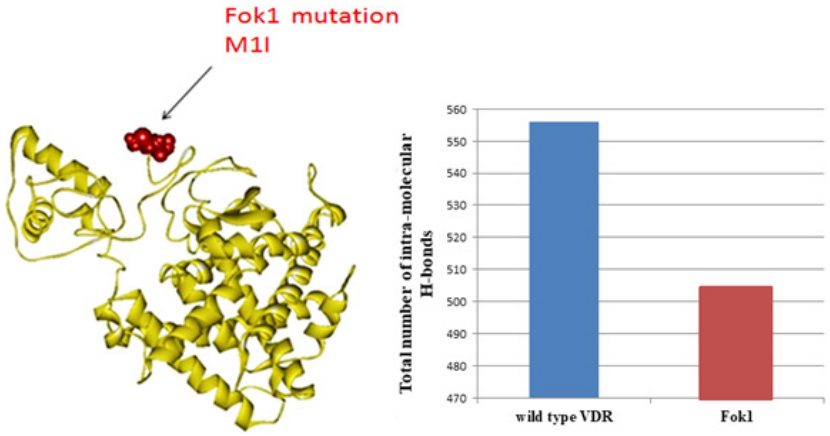

Figure 4. Computational Genomic Analysis of SNP: (A) The position of SNP induced amino acid substitution in *Fok1* variant (Position 1: Methionine to Isoleucine) of VDR (B) Change in number of intra-molecular H-bonds upon point mutation (wild type VDR: 556; *Fok1* variant: 505).

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; χ^2=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 4. Molecular Interaction and Binding Analysis of VDR with Calcitriol

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

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

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

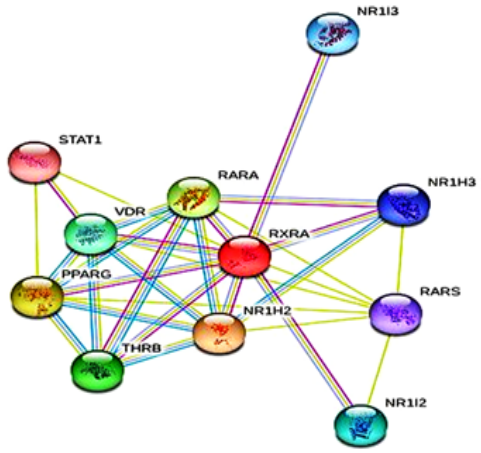

Figure 5. String Database Network Showing Preferential Functional Partners of VDR.

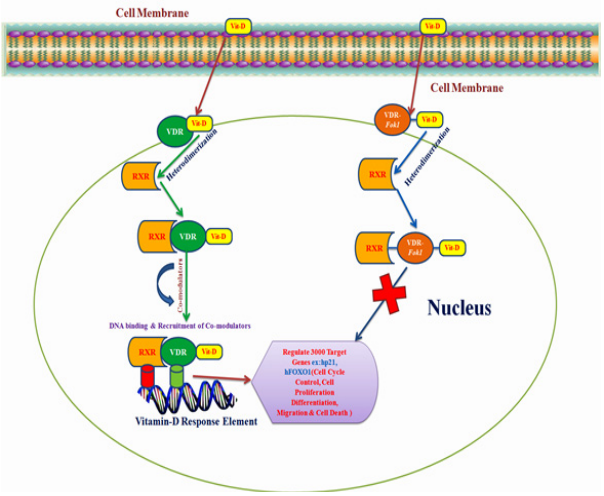

Figure 6. Molecular Mechanism of Cancer Susceptibility Associated with the SNPs of the Nuclear Hormone Receptor VDR

variant showed 505 intra-molecular H-bonds (Figure 4B). The molecular interaction analysis of wild type VDR with calcitriol and *Fok1* variant with calcitriol is illustrated in Table 4. The *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 *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 *Fok1* variant to RXR shows that liganded VDR variant (*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 *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 *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 *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 (ΔΔG) of the SNP variant shows that *Fok1* SNP variant has ΔΔG energy of-0.40 kcal/mol. The output of the predicted free energy change (ΔΔG) classifies the results into one of the three classes i.e. largely stable (ΔΔG>0.5kcal/mol), neutral (-0.5≤ΔΔG≤0.5 kcal/mol) or largely unstable (ΔΔG<−0.5 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. ΔΔG=0 (Du et al. 2016). A significant decrease in total number of intra-molecular H-Bonds was also observed in the *Fok1* variant compared to wild type VDR protein (wild type VDR: 556; *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 *Fok1* variant with calcitriol shows that the SNP causes a loss in affinity of VDR for calcitriol. The *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

binding. Further, 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 *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.

Funding

Financial assistance as Senior Research Fellowship to Miss. Sana Raza, provided by Maulana Azad National Fellowship, University Grants Commission, India, is gratefully acknowledged.

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.

Acknowledgements

The author wishes to thank King George Medical University, Lucknow, for supporting this research. Financial assistance as Maulana Azad National Fellowship (MANF) from University Grants Commission (UGC), India, is gratefully acknowledged. We are thankful to Integral University, Lucknow for providing necessary facilities for research. The manuscript has been approved by competent authority (Assigned Manuscript Communication Number- IU/R and D/2017-MCN000170).

References

Ali I, Wani WA, Saleem K (2011).Cancer scenario in India with future perspectives. *Cancer Ther*, **8**, 56–70.

Anderson LA, Cotterchio M, Cole D (2011).Vitamin D related genetic variants, interaction with vitamin D exposure and breast cancer risk among Caucasian women in Ontario. *Cancer Epidemiol Biomark Prev*, **20**, 1708-17.

Apweiler R, Bairoch A, Wu CHet al (2004). UniProt: the Universal Protein knowledgebase. *Nucleic Acids Res*, **32**, 115–19.

Babu GR, Lakshmi SB, Thiyagarajan JA (2013). Epidemiological correlates of breast cancer in South India. *Asian Pac J Cancer Prev*, **14**, 5077–83.

Balasubramaniam SM, Rotti SB, Vivekanandam S (2013). Risk factors of female breast carcinoma: a case control study at

Puducherry. *Indian Cancer*, **50**, 65–70.
Berndt SI, Dodson JL, Huang WY, Nicodemus KK (2006). A systemic review of vitamin D receptor gene polymorphisms and prostate cancer risk. *J Urol*, **175**, 1613-23.
Bikle DD (2016). Extraskeletal actions of vitamin D. *Ann N Y Acad Sci*, **1376**, 29–52.
Campbell MJ (2014). Vitamin D and the RNA transcriptome: more than mRNA regulation. *Front Physiol*, **5**, Doi: 10.3389/fphys.2014.00181.
Capriotti E, Calabrese F, Casadio R (2006). Predicting the insurgence of human genetic diseases associated to single point protein mutations with support vector machines and evolutionary information. *Bioinformatics*, **22**, 2729–34.
Deeb KK, Trump DL, Johnson CS (2007). Vitamin D signalling pathways in cancer: potential for anticancer therapeutics. *Nat Rev Cancer*, **7**, 684-700.
Díaz L, Díaz-Muñoz M, García-Gaytán AC, et al (2015). Mechanistic effects of calcitriol in cancer biology. *Nutrients*, **7**, 5020–50.
Du X, Li Y, Xia YL, et al (2016). Insights into protein-ligand interactions: mechanisms, models, and methods. *Int J Mol Sci*, **17**, 144.
Dwivedi PP, Hii CS, Ferrante A, et al (2002). Role of MAP kinases in the 1, 25-dihydroxyvitamin D3-induced transactivation of the rat cytochrome P450C24 (CYP24) promoter.Specific functions for ERK1/ERK2 and ERK5.J *Biol Chem*, **277**, 29643–29653.
Eswar N, Webb B, Marti-Renom MA, et al (2006).Comparative protein structure modeling using Modeller. *Curr Protoc Bioinformatics*, **5**, Unit 5.6. Doi: 10.1002/0471250953.bi0506s15.
Fang Y, van Meurs JBJ, d'AlesioA, et al (2005). Promoter and 3'-untranslated region haplotypes in the vitamin D receptor gene predispose to osteoporotic fracture: the Rotterdam study. *Am J Hum Genet*, **77**, 807-823.
Feldman D, Krishnan AV, Swami S, Giovanucci E, Feldman BJ (2014). The role of Vitamin D in reducing cancer risk and progression. *Nat Rev*, **14**, 342-57.
Ferlay J, Soerjomataram I, Ervik M, et al (2012). Cancer incidence and mortality worldwide, IARC Cancer Base No.11.
Finkelmeier F, Kronenberger B, Koberle V, et al (2014). Severe 25-hydroxyvitamin D deficiency identifies a poor prognosis in patients with hepatocellular carcinoma -a prospective cohort study. *Aliment Pharm Ther*, **39**, 1204-12.
Gil A, Plaza Diaz J, Mesa MD (2018). Vitamin D: Classic and novel actions. *Ann Nutr Metab*, **72**, 87–95.
Haussler MR, Jurutka PW, Mizwicki M, Norman AW (2011). Vitamin D receptor (VDR)-mediated actions of 1α, 25 (OH)2 vitamin D3: genomic and non-genomic mechanisms. *Best Pract Res Clin Endocrinol Metab*, **25**, 543-59.
Hollingsworth SA, Karplus PA (2010). A fresh look at the Ramachandran plot and the occurrence of standard structures in proteins. *Biomol Concepts*, **1**, 271–83.
Jurutka PW, Whitfield GK, Hsieh JC et al (2001). Molecular nature of the vitamin D receptor and its role in regulation of gene expression. *Rev Endocr Metab Disord*, **2**, 203-16.
Kaarthigeyan K (2012). Cervical cancer in India and HPV vaccination. *Indian J Med Paediatr Oncol*, **33**, 7–12.
Khokhar A (2012). Breast cancer in India: where do we stand and where do we go?. *Asian Pac J Cancer Prev*, **13**, 4861-6.
Kumar P, Henikoff S, Ng PC (2009). Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. *Nat Protoc*, **4**, 1073–81.
Lopes N, Paredes J, Costa JL, Ylstra B, Schmitt F (2012). Vitamin D and the mammary gland: a review on its role in normal development and breast cancer. *Breast Cancer Res*, **14**, 211.
Macindoe G, Mavridis L, Venkatraman V, Devignes MD, Ritchie DW (2010). HexServer: an FFT-based protein docking server powered by graphics processors. *Nucleic Acids Res*, **38**, 445-9.
Mashiach E, Schneidman-Duhovny D, Andrusier N, Nussinov R, Wolfson HJ (2008). FireDock: a web server for fast interaction refinement in molecular docking. *Nucleic Acids Res*, **36**, 229-32.
Miller SA, Dykes DD, Polesky HF (1988). A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res*, **16**, 1215.
Pace CN, Fu H, Fryar KL, et al (2014). Contribution of hydrogen bonds to protein stability. *Protein Sci*, **23**, 652–61.
Parthiban V, Gromiha MM, Abhinandan M, Schomburg D (2007). Computational modeling of protein mutant stability: analysis and optimization of statistical potentials and structural features reveal insights into prediction model development. *BMC Struct Biol*, **7**, 54.
Pastinen T, Ge B, Hudson TJ (2006). Influence of human genome polymorphism on gene expression. *Hum Mol Genet*, **15**, 9–16.
Penna-Martinez M, Ramos-Lopez E, Stern J, et al (2012). Impaired Vitamin D activation and association with CYP24A1 haplotypes in differentiated thyroid carcinoma. *Thyroid*, **22**, 709-16.
Schneidman-Duhovny D, Inbar Y, Nussinov R, Wolfson HJ (2005). Patch Dock and Symm Dock: servers for rigid and symmetric docking. *Nucleic Acids Res*, *33*, 363–7.
Shaukat N, Jaleel F, MoosaFA,Qureshi NA (2017). Association between Vitamin D deficiency and Breast Cancer. *Pak J Med Sci*, **33**, 645-9.
Sherry ST, Ward MH, Kholodov M, et al (2001). dbSNP: the NCBI database of genetic variation. *Nucleic Acids Res*, **29**, 308–11.
Siegel RL, Kimberly D, Miller,Jemal A (2017). Cancer statistics, 2017. *CA Cancer J Clin*, **67**, 7–30.
Snedecor GW, Cochran WG (1989). Statisitical methods. Ames, IA: Iowa State University Press, 8th edition, pp xx + 503.
Srinath RK, Shah B, Varghese C, Ramadoss A (2005). Responding to the threat of chronic diseases in India. *Lancet*, **366**, 1744–9.
Szklarczyk D, Franceschini A, Wyder S, et al (2015). STRING v10: protein–protein interaction networks, integrated over the tree of life. *Nucleic Acids Res*, **43**, 447-52.
ToptasB, Kafadar AM, Cacina C, et al (2013). The vitamin D receptor (VDR) gene polymorphisms in Turkish brain cancer patients. *Bio Med Res Int*, **12**, 295791.
Uitterlinden AG, Fang Y, Meurs JBV, Pols HA, Leeuwen JPV (2004). Genetics and biology of vitamin D receptor polymorphisms. *Gene*, **338**, 143-56.
Wang H, Chen W, Li D, et al (2017). Vitamin D and chronic diseases. Aging Dis, 8, 346–353.
Yang D, Anderson PH, Wijenayaka AR, et al (2017). Both ligand and VDR expression levels critically determine the effect of 1α, 25-dihydroxyvitamin-D3 on osteoblast differentiation. *J Steroid Biochem Mol Biol*, **177**, 83-90.
Yao S, Zirpoli G, Bovbjerg DH (2012). Variants in the vitamin D pathway, serum levels of vitamin D, and estrogen receptor negative breast cancer among African-American women: a case-control study. *Breast Cancer Res*, **14**, 58.
Zerwekh JE (2008). Blood biomarkers of vitamin D status. *Am J Clin Nutr*, **87**, 1087-91.