Identification of SNP of *VDR* and *VDBP* gene and its Dysregulated pathway through VDR-VDBP interaction network analysis in Vitamin D deficient Infertile Females

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Research Article

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Abstract

Research Question

Is there an association of single nucleotide polymorphism (SNP) of VD Binding Protein (VDBP), VD receptor (VDR) with the dysregulated pathways and gene enrichment of VDR-VDBP in VD defecient unexplained infertile females ?

Design

A cross sectional study was conducted on VD deficient ; fertile and unexplained infertile female subjects. VDBP and VDR were assessed by Enzyme Linked Immuno Assay and genotyping performed. FunRich version 3.1.3 employed for analysis of the identified proteins: VDR and VDBP a with their mapped gene data sets, enrichment and protein–protein interaction (PPI) network.

Results

The mean VD values of infertile females were lower than fertile females (p=0.006). The mean VDR values of infertile females 27.32 ± 7.45 was less than fertile females 41.25 ± 8.1 (p<0.001). Levels of VDBP in infertile females [Median (IQR)]; [296.05 (232.58 – 420.23)] were lower than fertile females [469.9 (269.57 – 875.55)], (p=0.01).

On sequence analysis a mutation rs 4588 SNP (Thr 436 Lys) was found in exon 11 of VDBP gene of infertile females and no mutation was found in exon 8 and 9 of VDR gene, however some intronic variants which were insignificant. The proteins: plasma membrane estrogen receptor signalling pathway (p 0.001) . VDR, SMAD3, NCOR1, CREBBP, NCOA1, STAT1, GRB2, PPP2CA, TP53 and NCOA2 were enriched after biological pathway grouping when VDR was made focused gene and directly interacting with VDBP .

Conclusion

Unexplained infertile females exhibited significantly low VD, VDBP and VDR. Plasma membrane estrogen receptor signaling pathway was enriched in VDD infertile females.

What Is Known About The Topic

The prevalence of female infertility is 22% in Pakistan with Vitamin D deficiency (VDD) discovered as an emerging factor influencing female fertility. Gene variants involved with cholesterol, transport and metabolism of vitamin D (VD) have been allied with VDD. An ongoing argument on global VDD, presence of VDR in reproductive tissues with an increased prevalence of infertility has encouraged us to conduct the study.

What Was Identified In The Study?

We identified the single nucleotide polymorphism (SNP) of VD Binding Protein (VDBP), VD receptor (VDR), the dysregulated pathways and gene enrichment centred on interaction network analysis of VDR-VDBP in in unexplained infertile-females with VD deficiency

Background/introduction

Vitamin D (VD) is involved in calcium-phosphate homeostasis and maintenance of bone mineral density. The active form for VD; 1, 25-dihydroxyvitamin D3 (1, 25-$(OH)_2$D3); metabolized by 1-alphahydroxylase from cholecalciferol (25-OHD), exerts these effects through the vitamin D receptor (VDR). This receptor is present in the intestines, bones, parathyroid glands as well as the ovaries and testes (Hanif et al., 2019; Kinuta et al., 2000). Furthermore, it may be found along the walls of the central organs of reproduction such as the pituitary and hypothalamus and peripheral organs like oviduct, uterus and placenta (Luk, Torrealday, Neal Perry, & Pal, 2012; Nandi, Sinha, Ong, Sonmez, & Poretsky, 2016). VD binds to VDR a transcription factor situated in the nuclei of target cells that facilitates the genomic action of the active form of VD (1,25$(OH)_2D_3$). This transcription factor is circulated to different tissues, functions as an ovarian reserve marker, stimulates production of hormones from the ovaries (Shahrokhi, Ghaffari, & Kazerouni, 2016).

The probable role of VD on impairment of reproductive physiology and the relationship of VD deficiency (VDD), with *VDR* polymorphism and infertility in female subjects has been explored (Azhar, Abid, & Rehman, 2020; Badr, Cassim, & Rehman, 2019; Maha, Masood, & Rehman, 2019; Rehman et al., 2018). The VDR gene is positioned on chromosome 12 that extents around 75 kb of genomic DNA and comprises of 11 exons (Yadav, Kumar, & Rai, 2021).An ongoing argument on global VDD, presence of VDR in reproductive tissues with an increased prevalence of infertility has encouraged us to conduct the study.

Approximately 1 in 8 women of reproductive age pursue advice for infertility issues and 85% of these patients have an underlying cause. Among females, the common causes of infertility are anovulation, tubal disorders, and endometriosis. However, in 15% of infertile women no definite cause is identified which lead to the diagnosis of unexplained infertility (Carson & Kallen, 2021). Unexplained infertility is defined as the inability to conceive despite 12 months of unprotected intercourse, in the absence of known causes of infertility including anovulation, tubal pathology, endometriosis or semen abnormalities (Mol, Tjon-Kon-Fat, Kamphuis, & van Wely, 2018).Many women diagnosed with unexplained infertility may conceive spontaneously over period of time with the rate of 2–4% per menstrual cycle (Gelbaya, Potdar, Jeve, & Nardo, 2014) whereas others need treatment with ovarian stimulation and intrauterine insemination ,if these approaches are not successful in vitro fertilization is considered (Carson & Kallen, 2021). As unexplained infertility is a diagnosis of exclusion, therefore it’s difficult to have any definite explanation of affected fertility potential in these patients.

As far as association of VD and infertility is concerned association of polycystic ovarian syndrome and endometriosis with VDD and infertility has been established (Voulgaris et al., 2017). VD has been linked

with In vitro fertilization (IVF) outcomes as well (Farzadi et al., 2015; Paffoni et al., 2014; Rudick et al., 2012).Similarly, low levels of VD has been correlated with low pregnancy rates due to its harmful effects on conception and endometrial receptivity in women undergoing IVF with single embryo transfer (Polyzos et al., 2014). Therefore, VDD may explain some cases of unexplained infertility or can be a contributing element to other factors that alters fertility potential. However, no conclusive data is available to examine relationship of VD and unexplained infertility or low ovarian reserve.

VDR is widely distributed in almost all human tissues (Holick, 2007) and VD is also involved in regulation of human genome in our body that reflects potential role of VD in various systems including reproduction (Bouillon et al., 2008; Holick, 2007) .Due to VDR presence in ovaries and endometrium, the impact in unexplained infertility could be at multiple levels .In ovaries VD mediated calcitriol has its effects on ovarian steroidogenesis that stimulates hormone synthesis including progesterone, estradiol and estrone (Parikh et al., 2010). Furthermore, in vitro studies have shown association of VD with ovarian reserves markers like anti Mullerian hormone (AMH) (Broekmans et al., 2008) and demonstrated presence of functional VDRE on human AMH promoter (Malloy, Peng, Wang, & Feldman, 2009). One study indicated significant reduction in AMHR-II and FSH receptor mRNA with human granulosa cells by VD3 (Merhi, Doswell, Krebs, & Cipolla, 2014). As AMH has its inhibitory role on folliculogenesis, this can be anticipated that VD treatment can have a beneficial role on folliculogenesis by alleviating inhibitory influence of AMH on the process.

We wanted to identify the single nucleotide polymorphism (SNP) of VD Binding Protein (VDBP), the dysregulated pathways and gene enrichment centred on interaction network analysis of VD receptor (VDR)-VDBP in unexplained infertile females with VD deficiency

Materials And Methods

This study was a cross sectional study and it was conducted from June 2019 to July 2020 after approval from Institutional Ethical Review Committee (ERC) of Aga Khan University (AKU-ERC 2019-0314-5627) in association through Australian Concept Infertility Medical Centre (ACIMC).

Inclusion Criteria: In this study we included fertile females between the age range of 18–45 years from all ethnic backgrounds and having a child with age of less than 2 years. Recruitment of infertile females was based on the criteria of unexplained infertility (12) in the age range of 18–45 years and from all ethnic groups. Infertile females due to male factor causes or due to tubal blockade were also excluded. Furthermore, females' subjects with previous history of artificial reproductive techniques (ART) in preceding pregnancies, recurring miscarriages, thyroid abnormalities, uterine tumours, hypertension and diabetes were excluded. Infertile females with serious general health problem, using contraceptive pills orally and any hormonal treatments or using any contraceptive procedures were also excluded.

We also excluded women (fertile and infertile) who were on VD therapy, calcium supplementation (for the last six months) or exposed to tobacco, gonadotropins or prior chemotherapy.

Blood Collection: For recruitment of subjects, approximately 5 millilitres of venous blood sample was collected from each subject by a pain-free procedure. Serum was extracted by centrifugation of blood samples and quickly stored at − 80°C until estimation of biochemical parameters. **Biochemical Analysis**: VD levels in serum was observed by Human, 1,25-Dihyroxy Vitamin D ELISA Kit (Cat#95503), with an intra and inter assay coefficient of variation (CV) were 2.7% and 4.3% respectively. The lowermost limit of detection was 2.8 ng/ml. After the analysis 40 VDD fertile and infertile subjects were recruited in the study. VDR and VDBP levels were analysed in the comparative groups. VDR levels in serum was measured by commercially available enzyme-linked immunosorbent assay (ELISA Kit (Cat. No: SEA475Hu, Cloud-Clone Corp.) with a detection range of kit 0.625-40ng/ml. The analytical sensitivity was less than 0.225ng/mL, intra and inter assay coefficient of variation were found to be < 10% and 12% respectively. VDBP levels in serum was observed by using commercially available Human VDBP ELISA Kit (Cat. No: 96577, Glory Science Co.Ltd.) using a detection range of kit 8ug/ml -480ug/ml.

Genotyping:

Genotyping of VDR was performed using the SNP genotyping assay and direct DNA sequencing methods.

S. No.	Primers	Primer Sequence
1	Exon8 F – VDR	**GGTGTATACCTGTCAAAGCACTA**
2	Exon8 R – VDR	**CCCTGTTGGTGCCTAACTC**
3	Exon9 F – VDR	**GGGAGTTAGGCACCAACAG**
4	Exon9 R – VDR	**CCCTCAGCAGGTCTTTGTC**
5	Exon 11-F – VDBP	**TAATGAGCAAATGAAAGAAG**
6	Exon 11-R – VDBP	**TGAGTAGATTGGAGTGCATAC**

Polymerase Chain Reaction (PCR) for exon 8 and 9 of VDR gene was performed using 2× PCR Master Mix (Cat# G013, ABM (Applied Biological Materials Inc, Canada) as per the manufacturer's instructions. PCR conditions were initial denaturation was at 95°C for 5 min by 1 cycle then further 35 cycles at 95°C for 30 s, 58°C for 45 s, 72°C for 45 s which is then followed by a final extension at 72°C of 10 min. PCR was performed for VDBP using GoTaq hot start master mix (Cat# M5122, Promega, USA) as per the instructions provided. PCR conditions were initial denaturation at 95°C for 5 min by 1 cycle then further at 95°C for 30 s, 60°C for 1 min, 72°C for 45 s by 40 cycles and then followed by a final extension at 72°C of 10 min. The amplified products were run on gel electrophoresis using 2% agarose gel. Purification of the PCR products was performed by using PCR Clean Up of DNA Sequencing (Cat. No BT5100, Bio Basic Inc, Canada) using the instructions in the protocol.

Sanger sequencing is classical method for sequencing. This method was utilized to sequence the VDR and VDBP gene in samples and PCR products were sent to sequencing company Operon (Canada).

Previously published VDR and VDBP gene sequences data was directly used to compare the resultant sequences using the National Centre for Biotechnology Information (NCBI) database MEGABLAST search engine. Sequence files were viewed by importing them into Chromas Lite, and then analysed by assembling into Molecular Evolutionary Genetic Analysis (MEGA) version 6.0.

Statistical analysis was accomplished by using SPSS version 20 software by performing descriptive statistics and Mann- Whitney U test. Statistical significance was considered at a p-value of < 0.05. Sequences were analysed by using Molecular Evolutionary Genetics Analysis (MEGA) software version 6.0.

VDPB and *VDR* gene interaction pathways were studied in this in silico analysis.

Bioinformatic analysis:

VDPB and *VDR* interacting protein interaction network, mapping of the gene data sets and interaction pathways was obtained by (**FunRich) 3.1.3** http://www.funrich.org which is a Functional Enrichment analysis tool.

Enrichment analysis:

Molecular functions, biological pathways, gene ontology terms and site of expression terms were retrieved by performing enrichment analysis. The depleted and enriched proteins were identified by the fold change for, biological pathways, protein domains and site of expression.

Interaction network analysis:

Biological pathway enrichment of defined nodes was used for visualizing and analysing the PPI network. Only human exclusive datasets were presented in which gene /protein annotations were collected from publicly available protein-centric and gene databases. Human specific FunRich database was selected as the background database for complete analysis. List of genes augmented in specific pathways was highlighted within the interactions network and distinctive sub-network were created for complete analysis. Specific sub-network was analysed by tallying direct neighbours (interacting partners) for mentioned nodes in the sub-network and visualisation. Specific nodes were focused, and the interacting partners of the focused nodes were mapped.

Gene ontology (GO) functional categories, normal and overrepresented and identified pathway associations and significant interactions with datasets were analysed by using BH and Bonferroni tests. The p-value correction was done with the BH and Bonferroni tests and hypergeometric test, p-value <0.05 was taken as the statistical cut off and maintained as default after Bonferroni correction.

Results: Details Of Tables

Description of 80; fertile (n = 40) and infertile (n = 40) females.

Table 1 presents the demographic and biochemical characteristics of fertile and infertile female subjects. The mean age of the female subjects was comparable between fertile and infertile groups. The Body Mass Index (BMI) (mean was ± SD) was greater in infertile females 27.4 ± 3.6 as compared to fertile females 23.5 ± 1.7 ($p < 0.001$). The mean vitamin D values of infertile females 7.45 ± 2.1 were significantly lower than fertile females 15.82 ± 5.2 ($p = 0.006$). The mean VDR values of infertile females 27.32 ± 7.45 were also significantly lower than fertile females 41.25 ± 8.1 ($p < 0.001$). Levels of VDBP in infertile females Median (IQR), 296.05 (232.58–420.23) were lower as compared to fertile females Median (IQR), 469.9 (269.57–875.55), ($p = 0.01$).

Figure 1A & B represents gel electrophoresis images of amplified PCR products for VDR gene (band size = ~ 355bp) and for VDBP gene (band size = ~ 462bp) in infertile and fertile males and females. On sequence analysis a mutation rs 4588 SNP (Thr 436 Lys) was found in exon 11 of VDBP gene of infertile females and no mutation was found in exon 8 and 9 of VDR gene, however there were some intronic variants which were insignificant. Figure 1C & D represents sequencing chromatograms of exon 8 & 9 of VDR gene in infertile samples respectively with highlighted intronic variant (T/C) in exon 9.

Interacting Proteins for VDBP **and** VDR **Gene**:

FunRich version 3.1.3 was employed for the analysis of the identified proteins: VDR and VDBP along with their mapped gene data sets, enrichment and protein–protein interaction (PPI) network.

There were 66 proteins identified interacting directly with *VDR* and *VDBP (GC)* (Fig. 1).

Protein-Protein Interaction (PPI) Analysis of VDBP **and** VDR **Gene**:

FunRich database was used to evaluate the PPI network and envisaging the *VDR-VDBP (GC)* interaction. The interaction network was integrated in the pathway enrichment of identified protein. The differentially controlled interacting proteins of potential retrieved from interaction of *VDR and VDBP (GC)* were recognised in this network shown in Fig. 2. 66 genes were established interacting with *VDR and GC*, all interacts conjointly as shown in Fig. 2. The proteins described the main group mapped along with VDR and VDBP were: CREB-binding protein, nuclear receptor corepressor 1, Mothers against decapentaplegic homolog 3; Receptor-regulated SMAD (R-SMAD), Nuclear receptor coactivator 3 and Nuclear receptor coactivator 1.

The important and associated pathways with their interacting proteins were EGFR-dependent Endothelin signalling events, PDGFR-beta signalling pathway, TRAIL signalling pathway,

Plasma membrane estrogen receptor signalling, validated nuclear estrogen receptor beta network, Retinoic acid receptors-mediated signalling, Androgen-mediated signalling, Regulation of Androgen receptor activity, Glucocorticoid receptor signaling, mTOR signaling pathway, TGF-beta receptor signaling and Regulation of cytoplasmic and nuclear SMAD2/3 signalling,

The proteins enriched in Plasma membrane estrogen receptor signalling were 29 in number with $p < 0.001$ including *VDR, SMAD3, NCOR1, CREBBP, NCOA1, STAT1, GRB2, PPP2CA, TP53 and NCOA2* were enriched in Plasma membrane estrogen receptor signalling pathway when *VDR* was made focused gene and directly interacting with more than 2- fold enrichment as shown in Fig. 3. It is worth mentioning that *VDR, NCOR1 and SMAD3* were enriched in ovarian infertility genes pathway.

Discussion

In addition to low VD, a significant low VDBP and VDR was observed in unexplained infertile females. We have found mutation in exon 11 of VDBP gene of infertile rs 4588 SNP which may alter their protein function (Thr 436 Lys). Plasma membrane estrogen receptor signalling pathway was enriched in VD deficient infertile females. *VDR, SMAD3, NCOR1, CREBBP, NCOA1, STAT1, GRB2, PPP2CA, TP53 and NCOA2* were enriched in plasma membrane estrogen receptor signalling pathway. It is worth mentioning that *VDR, NCOR1 and SMAD3* were found to be enriched in the ovarian infertility genes pathway.

Our results are supported by a study (IsbilenE et al) (Isbilen et al., 2020) which indicated VDR gene polymorphisms as a contributing factor for infertility in patients with for unexplained infertility.It was highlighted that impaired *VDR gene* expression affects the endometrial receptivity and implantation process through unknown underlying mechanisms. The study exhibited VDR gene BsmI and TaqI polymorphisms as a substantial risk for unexplained infertility whereas VDR gene Aa genotype in ApaI polymorphism as a protective factor.Another, study mentioned important effects of vitamin D on endometrial receptivity at normal levels however, due to anti-oestrogenic effect found its detrimental effect on oocyte and embryo quality (Laganà, Vitale, Ban Frangež, Vrtačnik-Bokal, & D'Anna, 2017). Whereas Jeremic A et al suggested measurements of vitamin D in serum and follicular fluid as a complementary tools for routine assessment of embryos in unexplained infertility patients undergoing IVF treatment (Jeremic et al., 2021).

VDR polymorphisms is associated with infertility, decrease in; folliculogenesis, oocyte yield, fertilization and pregnancy rates after controlled ovarian stimulation responses (COS) in assisted reproductive techniques (ART) (Dasgupta, Dutta, Annamaneni, Kudugunti, & Battini, 2015). FokI is one of the most evaluated polymorphisms of the VDR gene. A polymorphic variant (FF) is generated from a change of T to C in the start codon sequence that is reduced by three amino acids and displays amplified transcriptional deficit of the VDR protein in contrast to the long ff allele form (Uitterlinden, Fang, Van Meurs, Pols, & Van Leeuwen, 2004).A study from India has established association of VDR gene (FokI, rs-2228570; C > T) polymorphisms and male factor infertility (Bhakat et al., 2017).

The identification of a VDR polymorphisms specifically related to infertility and response to ovarian stimulation may help in better understanding of processes for unexplained infertility and affected ovarian reserves. Djurovic J et al (Djurovic, Stamenkovic, Todorovic, Aleksic, & Stojkovic, 2020), explored association of VDR gene polymorphisms and haplotypes with unexplained infertility.They examined DNA of 117 patients with unexplained infertility and compared with 130 control fertile women. The results

highlighted those changes in expression and activity of VDR gene affected expression of VD -responsive genes, leading to altered immune effect and possible impact on reproduction. With two identified haplotypes, BAT was associated with increased risk for ability to conceive again infertility whereas haplotype BAT indicated protective role forability to conceive first time ($p < 0.05$).

Presence of low VDBP in infertile females was indicated in a pilot study (Franasiak, Shapses, Sun, Scott, & Wang, 2017). Observed association of (rs 4588 SNP) mutation in exon 11 of VDBP gene of infertile subjects in our study corroborates with mortality due to COVID-19 with VDD and VDBP polymorphisms of rs7041 and rs4588 in literature (Speeckaert, De Buyzere, & Delanghe, 2021).

Given growing concerns over the widespread and uncontrolled use of assisted reproductive technologies (ART) and ICSI, VD3 supplementation may turn out to be a simple and cheap clinical treatment for infertile couples.

Large-scale interaction networks encompass the results of experiments that help to describe different biochemical interactions between genes and their encoding proteins (Leiserson, Eldridge, Ramachandran, & Raphael, 2013).

The Pathway analyses play an important role in appreciating biological steps involved in various disease processes. Hence, more compelling biomarkers can be identified using dysregulated pathway. (Shi et al., 2016).

We used a network-based method to determine the dysregulated pathways developed in VD deficient infertile females which may provide new discernments of the processes leading to infertility in females (Luk et al., 2012). Estrogen facilitates its biological response through various potential cellular mechanisms, are this occurs in mainly in two cellular ways including receptors: genomic activity and rapid nongenomic effects (Hewitt, Winuthayanon, & Korach, 2016).

It was described that the prompt response occur within minutes in the process of therapy. Additionally, inhibition of the MAPK/ERK or AKT signalling pathway, can block nongenomic effects. Initiating Commencement of these signalling pathways activities is closely related to GPR30-mediated plasma-membrane-associated processes (Clark et al., 2014).

In current in silico interaction analysis, plasma membrane estrogen receptor signalling pathway was found to be dysregulated pathway based on close interaction of VDR and VDBP genes. Our research identified several proteins enriched in estrogen receptor signalling pathway involving *VDR, SMAD3, NCOR1, CREBBP, NCOA1, STAT1, GRB2, PPP2CA, TP53 and NCOA2.*

Estrogen receptor protein is considered as a main factor for estrogen action, as it binds estrogens to originate the tissue responses. These receptor proteins are of two types and include ERα and ERβ, both have distinctive expression configurations (Mueller & Korach, 2001).

It is worth mentioning finding in this study that *VDR, NCOR1 and SMAD3* were found to be enriched in ovarian infertility genes pathway also. Fertility attributes in human inhabitants are controlled genetically (Kosova, Abney, & Ober, 2010).

Genomic Wide Association Studies (GWAS) identified 34 genome-wide significant signals for fertility in women with replication in de CODE data for the Icelandic population, and in the Women's Genome Health Study. The signals comprise association with intronic SNPs in the oestrogen receptor 1 (*ESR1*) gene that is also linked with the number of offspring (Day et al., 2016).

With this context, identification of *VDR, NCOR1 and SMAD3* in this study must be evaluated further to explore their role in therapies for Vit D deficient females with infertility.

Study Conclusion

>Significantly low levels of VD, VDBP and VDR was observed in unexplained infertile females. A mutation in VDBP gene at rs 4588 SNP in exon 11which is likely to alter the protein function (Thr 436 Lys) was noticed. No mutation was found in exon 8 and 9 of VDR gene, however there were some intronic variants which were insignificant. Plasma membrane estrogen receptor signalling pathway was enriched in VD deficient infertile females. VDR, SMAD3, NCOR1, CREBBP, NCOA1, STAT1, GRB2, PPP2CA, TP53 and NCOA2 were enriched in plasma membrane estrogen receptor signalling pathway. It is worth mentioning that VDR, NCOR1 and SMAD3 were found to be enriched in the ovarian infertility genes pathway.

Declarations

Conflict of Interest: None to declare

Contribution Statement:

This Study was design by Dr. Zil-e-Rubab and Rehana Rehman. Dr. Sumaira Naz took part in recruitment of patients & collection of data, Mussarat Ashraf have done the bench work, data management & statistical analysis, Saba Shahid have helped in sequencing analysis & all authors took part in write up of manuscript and critical review.

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Data Accessibility Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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Table 1

Table 1
Comparison of Study Variables in Fertile and Infertile Females

	Fertile (n = 40)	**Infertile (n = 40)**	**p-value**
Age (Years)*	29.4 ± 6.2	31 ± 5.7	0.416
BMI (Kg/m^2)*	23.5 ± 1.7	27.4 ± 3.6	< 0.001
Vitamin D (ng/ml)*	15.82 ± 5.2	7.45 ± 2.1	0.006
VDR (ng/ml) *	41.25 ± 8.1	27.32 ± 7.45	< 0.001
VDBP (ng/ml) **	469.9 (269.57–875.55)	296.05 (232.58–420.23)	0.01
Estradiol (pg/ml) *	270.36 ± 275.72	277.76 ± 264.65	0.878

*Values are expressed as Mean ± SD, **Median (IQR). Mann Whitney U test was applied to find p-values, p values < 0.05 was considered statistically significant.

Figures

Figure 1 (A to D): Gel electrophoresis of VDR & VDBP gene and sequencing chromatogram of VDR gene

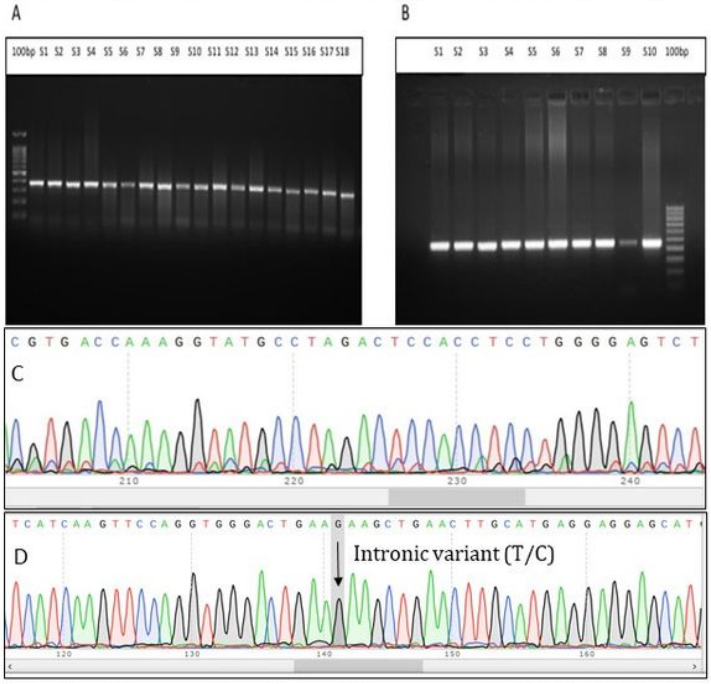

Figure 1 A & B: PCR amplification shown as bands on 2% agarose gel of VDR (355bp) and VDBP (462bp) of fertile and infertile subjects numbered S1-S18 and S1-S10 respectively. C& D shows Sanger sequencing chromatograms of exon 8 and exon 9 of VDR gene in infertile females respectively with highlighted intronic variant (T/C) in exon 9.

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Figure 2: Protein-Protein Interaction (PPI) of *VDR- VDBP*

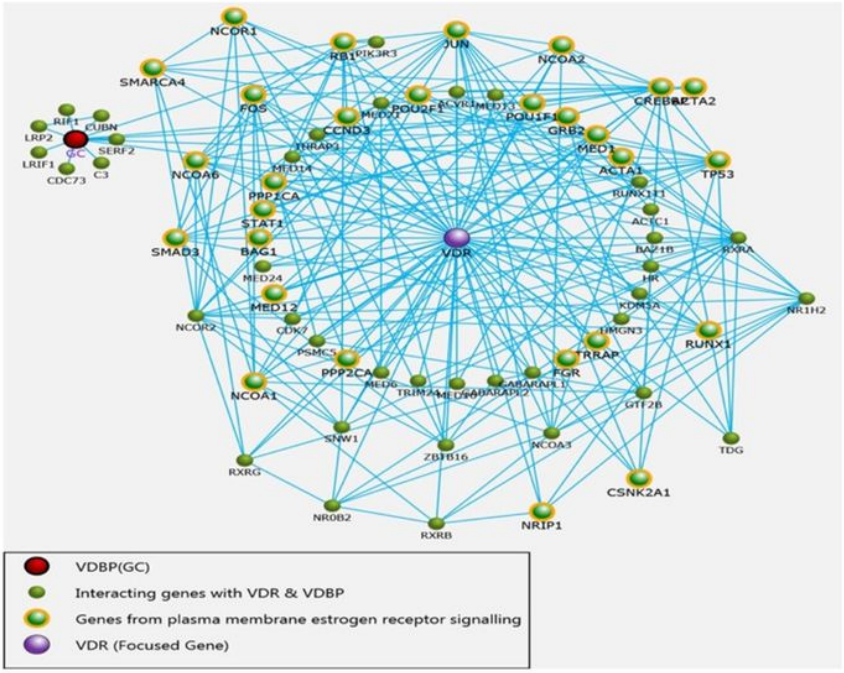

Figure 2

Protein-Protein Interaction (PPI) of *VDR- VDBP*

Figure 3: Genes enriched in Plasma membrane estrogen receptor signalling pathway

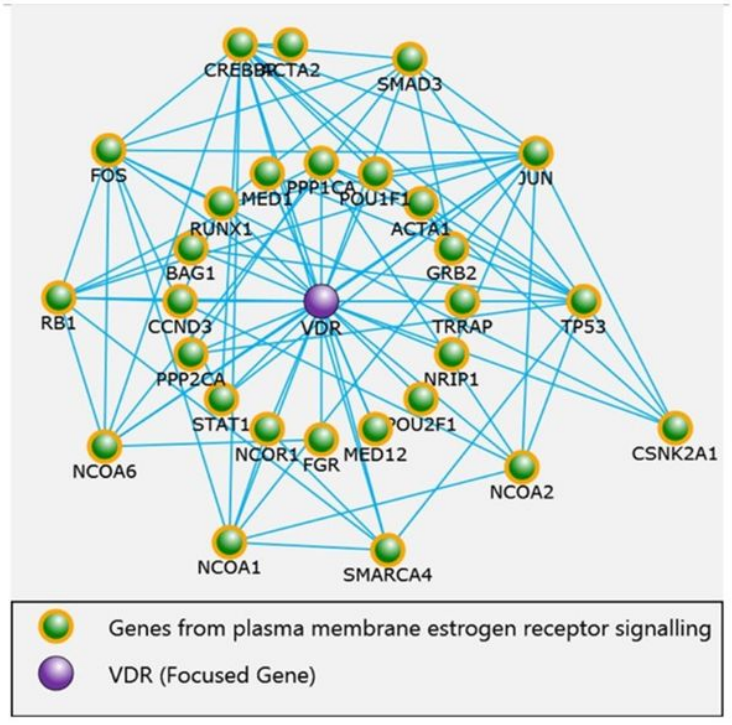

Figure 3

Genes enriched in Plasma membrane estrogen receptor signalling pathway