International Journal of Medical Science and Clinical Invention 9(7): 6175-6182, 2022
DOI:10.18535/ijmsci/v9i07.01
https://valleyinternational.net/index.php/ijmsci

Research Article,

Vitamin D receptor gene Polymorphisms (fok1 and taq1) among Sudanese women with recurrent miscarriages

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Abstract:

Background: Vitamin D endocrine system was formally known as a key player in calcium and phosphatehomoeostasis and in regulation of bone remodelling. It is influences maternal and fetal cell differentiation and cell growth immune regulation, insulin secretion and anti-proliferative processes

Methods: A case control study was conducted at the National Center of Neurological Sciences (NCNS), Khartoum, Sudan from 2021 to 2022. All patients attending obese and gaina unit at Ibraheim Malike teaching hospital and diagnosed with unexplained recurrent spontaneous abortion during the aforementioned period were included as a cases. Apparently healthy women with no history of abortion at a reproductive age were selected as control. Genomic DNA was extracted from blood, conventional PCR machine was used to amplify the VDR gene. PCR productswere then sequenced.

Results

A219 bp of VDR (FokI) allele was detected with gel electrophoresis after PCR. The PCR result shows that all of cases were positive100%forVDR (FokI) allele, while34% of controls were positive. When the cases sequencing results were compared with the normal reference the following single Base Exchange were found A>C, G>T and G>A. While when the controls were compared with normal reference, no any single base exchange was found among the all control groups. Mutation taster was used to confirmed the mutations which revealed; A>C Base Exchange polymorphism was predicted, that is confirms the presence of rs2228570polymorphism. Also two another Base Exchange polymorphism was predicted G>A and G>T. For the Taq1 alleles; 242 bp of VDR (TaqI) allele was detected with gel electrophoresis after PCR. The PCR result shows that 97.7% of cases were positive for VDR (TaqI) allele and14% of controls group were positive. The sequencing analysis detected three single Base Exchange polymorphisms C>T, G>C and C>A. Mutation taster was used to confirmed the mutations which revealed; C>T Base Exchange polymorphism was predicted, that is confirms the presence of rs731236 polymorphism. In addition to prediction of G>C and C>A which changed the amino acid sequence and the spliced sit.

Conclusion: The study showed a significant association between fok1 rs2228570 and taq1 rs731236 variants of the VDR gene and recurrent recurrent miscarriage among Sudanese women

Keywords: Vitamin D gene, sequencing, recurrent miscarriage

Introduction:

Vitamin D endocrine system was formally known as a key player in calcium and phosphate homoeostasis and in regulation of bone remodelling.[1] Also takes part in many non-classical pathways such as; influences maternal and fetal cell differentiation and cell growth, immune regulation, insulin secretion and anti-proliferative processes .[2] In humans, only a few amount of vitamin D is obtained through dietary

intake, whilst vitamin D is largely created in the skin with exposure via photochemical conversion of 7 dehydrocholesterol to pre-vitamin D3, and the latter is sequentially metabolized in the liver and kidneys.[3]Vitamin D activity is mediated by the vitamin D receptor (VDR), a nuclear receptor which acts as a high affinity ligand-activated transcription factor.[4,5]

VDR gene present on the chromosome 12q12–14 is highly expressed in various human tissues including skin epithelium, osteoblasts and chondrocytes, muscles, cells from the immune system and placenta [6]. Which recognizes vitamin D response elements (VDRE) in the promoter regions of vitamin D target genes and recruits cofactors to modulate gene transcription. Recently some researches proposed the possibility of association between VDR polymorphisms and the risk of adverse pregnancy outcomes, such as PTB, LBW and SGA births. BsmI (rs1544410), ApaI (rs7975232), FokI (rs2228570) and TaqI (rs731236) polymorphisms are the most commonly investigated.[7] Recurrent Spontaneous abortion (RSA) is one of the largest causes of neonatal death globally. The pathophysiology of RPL is complicated and poorly understood. RSA is difficult to understand due to the involvement of various environmental factors. VDR gene expression in placenta regulates various genes linked with implantation, maturity of fetus and bone formation. The gene polymorphisms may affect its expression, activity and subsequently downstream biological activity of vitamin D. Therefore, it could be a potent genetic marker for prematurity in pregnant women. Several polymorphic sites have been described within VDR gene for different disease, therefore, *VDR* gene polymorphism need to be investigated in the Sudanese women with recurrent Spontaneous abortion.

Material and methods:

A case control study conducted at the National Center of Neurological Sciences (NCNS), Khartoum, Sudan from 2020 to 2021. All patients attending obese and gaina unit at IbraheimMalike teaching hospital and diagnosed with unexplained recurrent spontaneous abortion during the aforementioned period were included as a cases. Apparently healthy women with no history of abortion and areproductiveage were selected as control. Patients with recurrent spontaneous abortion are eligible for inclusion criteria their age (20-40years). Participants were eligible for inclusion as controls if women are without a self-reported history of abortion also their age (20-40years).In sterile containers with EDTA five ml of venous blood was gathered from each subject, then the standard phenol chloroform extraction method was used to isolate the Genomic DNA.

Primers were designed using Prime3 software. The forward primer for FOK1 (rs2228570) (A>C) was designed as "5-CAGGGCCAGTTTCTTTACCA -3"and reverse as "5- TCTGTTTTGGCTGCCTTCTT-3"with product size of 219bp fragment. For Taq 1(rs731236) (C>T) the forward primer "5-CTGCCGTTGAGTGTCTGTGT -3"and reverse as "5- TCGGCTAGCTTCTGGATCAT -3"with product size of 242bp fragment. for the polymerase chain reaction PCR:14 ul double distilled water was placed in PCR tube, then 4 ul of master mix, 1 ul of forward primer, 1 ul of reverse primer and 2 ul of DNA sample was added then vortex. The PCR tube containing this mixture was place in commercial thermal cycler (SwiftTMMaxPro SWT-MXP-BLC-4) at following condition: Denaturation temperature 94oC for 30 secs, annealing temperature at 61oC for 30 sec and extension temperature at 72oC for 30 secs, the final elongation was adjusted for 5 minutes at 72 °C. PCR reaction was regulate at 35 cycles. Products of PCR were sent for sequencing to Macrogen Europe Laboratory. The study was confirmed by the ethical committee of the University of Medical Science and Technology.

Results:

FokI (rs2228570) polymorphism

In the present study 219bp of VDR (**FokI)** allele was detected with gel electrophoresis after PCR (Figure 1).The PCR result shows that all of cases were positive100%forVDR (**FokI)** allele. For the control group34% were positive and the 66 % were negative (Table 1) (Figure2). When compared between the case and control group for the PCR result there wassignificantassociation (P= 0.000)by the Chi square sample (Table2)

The sequencing results were analyzed using different bioinformatics soft-wares and tools. The obtained sequences aligned using BioEdit-ClustalW software with a normal sequence from GenBankgene (accession number NC_000012.121in NCBI).

When the cases were compared with the normal reference the following single Base Exchange

were found A>C,G>T and G>A. While when the controls were compared with normal reference, no any single base exchange was found among the all control groups (Figure 3, 4,5) Mutation taster was used to confirmed the mutations which revealed; A>C Base Exchange polymorphism was predicted, that is confirms The presence of rs2228570polymorphism. Also G>T and G>ABase Exchange polymorphisms were predicted which may change the amino acid sequence and the spliced sit.

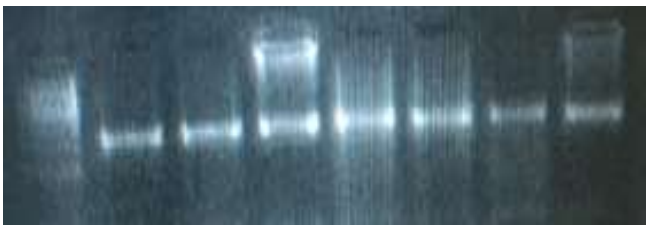

Figure 1:219 bp of factor of VDR (FokI) allele detected with gel electrophoresis

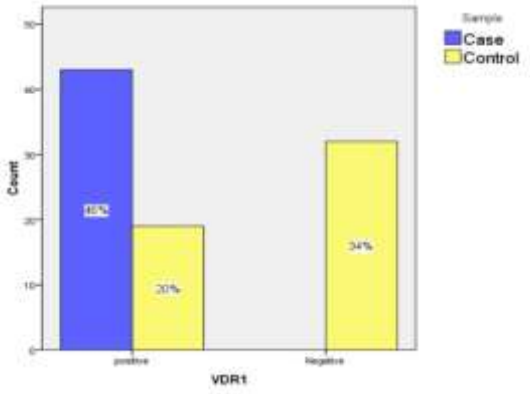

Figure (2): VDR (Fok1) among case and control study groups

Table (1): Distribution of VDR (Fok1) among case and control groups

VDR Fok1	Case	Positive	43	100%
		Negative	0.00	0.00
		Total	43	100%
	Control	Positive	17	34%
		Negative	33	66%
		Total	50	100%

Table (2): Cross tabulation of PCR result between the cases and controls

Crosstab: Chi-Square test				
Gene		Case	Control	P. value
VDR fok1	Positive	43	18	0.00
	Negative	0	32	
Total		43	50	

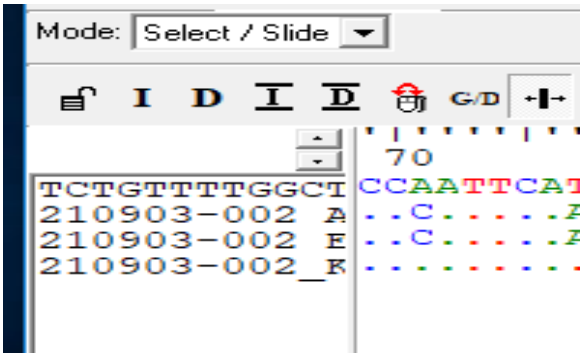

Figure 3: Multiple sequence alignment using Bio-Edit clustal W for cases group with reference gene sequence of FOK1 gene.

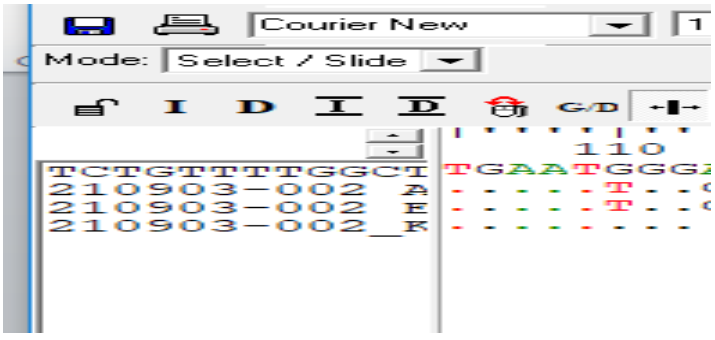

Figure 4: Multiple sequence alignment using Bio-Edit clustal W for cases group with reference gene sequence of FOK1 gene.

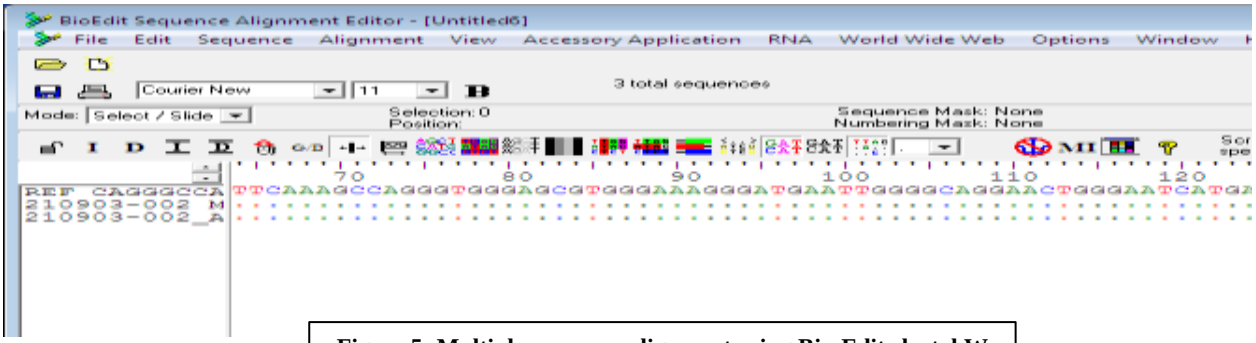

Figure 5: Multiple sequence alignment using Bio-Edit clustal W for control group with reference gene sequence of FOK1 gene.

TaqI (rs731236) polymorphism:

In the present study 242 bp of VDR (**TaqI)** allele was detected with gel electrophoresis after PCR (Figure 6).The PCR result shows that about 97.7% of cases were positive for VDR (**TaqI)** allele and only 2.3% were negative (Table3). For the control group14% was positive and the 86 % were negative (Table 3) (Figure7). When compared between the case and control group for the PCR result there wassignificantassociation (P= 0.000) by the Chi square sample (Table4)

The sequencing results were analyzed using different bioinformatics soft-wares and tools. The obtained sequences aligned using BioEdit-ClustalW software with a normal sequence from Gen Bankgene (accession number NC_000012.121in NCBI). When the cases were compared with the normal reference the following single Base Exchange were found C>T, G>C and C>A. While when the controls were compared with normal reference, no any single base exchange was found among the all control groups (Figure 8,9 ,10)

Mutation taster was used to confirmed the mutations which revealed; C>T Base Exchange polymorphism was predicted, that is confirms the presence of rs731236 polymorphism. Also G>C and C>A Base Exchange polymorphisms were predicted, in addition to present of G>A polymorphism which was Base Exchange polymorphism, protein features might be affected and alteration location was at chromosome 12, alteration type was single base exchange, c DNA changes position was 1332 G>A with reference ID rs 4967032.

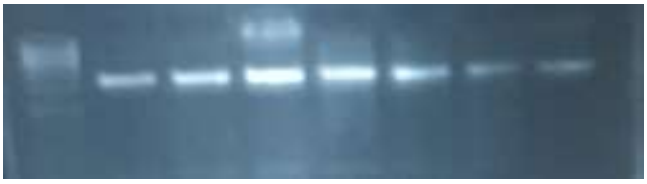

Figure (6) :219 bp of factorof VDR (TaqI)allele detected with gel electrophoresis

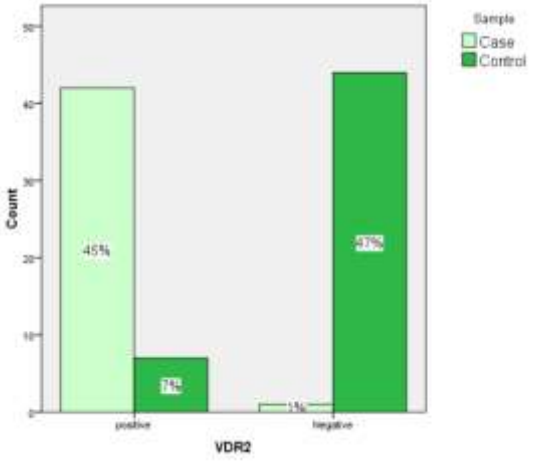

Figure (7): VDR (TaqI) among case and control study groups

Table (3) Distribution of VDR (TaqI) among case and control groups

	Case	Positive	42	97.7
VDR TaqI		Negative	1.00	2.3
		Total	43	100
	Control	Positive	7	14
		Negative	43	86
		Total	50	100

Table (4): Cross tabulation of PCR result between the cases and controls group

Crosstab: Chi-Square test				
Gene		Case	Control	P. value
VDR TaqI	Positive	42	7	0.00
	Negative	1	43	
Total		43	50	

Figure (8): Multiple sequence alignment using Bio-Edit clustal W for cases group with reference gene sequence of TaqI gene.

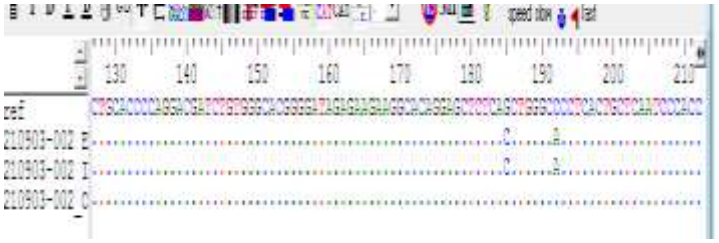

Figure (9): Multiple sequence alignment using Bio-Edit clustal W for cases group with reference gene sequence of TaqI gene.

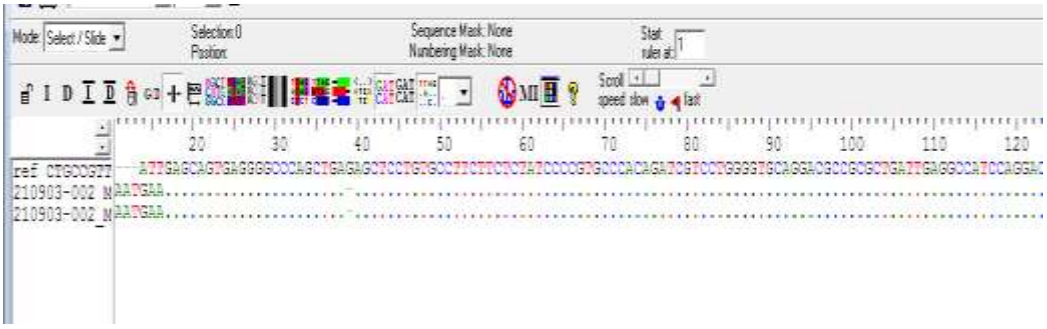

Figure (10): Multiple sequence alignment using Bio-Edit clustal W for control group with reference gene sequence of TaqI gene.

Discussion:

Maternal vitamin D status varies across different pregnancy trimesters, and the risk of vitamin D deficiency increases due to the high maternal and fetal demands, in addition the stores of vitamin D in newborns are dependent on maternal vitamin D status, and the deficiency of it during pregnancy has been associated with several maternal and offspring complications that persist later in life. [8,9]Moreover, recent studies indicate association of vitamin D deficiency with infertility, polycystic ovary syndrome, in vitro fertilization outcomes and male gonadal function [10]. Besides, vitamin D and its components may have a significant

influence on fetoplacental development and expression of multiple placental hormones (human chorionic gonadotropin, human placental lactogen, estradiol, progesterone) and proinflammatory cytokines [11].

This study aimed to detect VDR alleles (FokI and taqI) (rs2228570, rs731236) among Sudanese women with recurrent miscarriage. The PCR result showed that all of cases were positive (100%) for VDR (**FokI)** allele while in the control group only 17 (34%) were positive. When compared between the case and control group for the PCR result there was significant association (P= 0.000). The sequencing results were analyzed using different bioinformatics soft-wares and tools. When the cases were compared with the normal reference alignment the following single Base Exchange were found A>C,G>T and G>A. While when the controls were compared with normal reference alignment, no any single base exchange was found among the all control groups. In addition mutation taster software was used to confirmed the mutations which revealed; A>C Base Exchange polymorphism was predicted , that is confirms The presence of rs2228570polymorphism, also tow another Base Exchange polymorphisms were predicted and changed the amino acid and the spliced sit G>T and G>A.

Coşkun et al reported The *FokI*SNP (rs2228570), It is uniquely different from the other SNPs for two reasons. First, it is the only polymorphism that is not linked to any of the other VDR variants. Second, it is the only known VDR polymorphism, in contrast to other variants, that is translated into two different VDR protein products, thus affecting the VDR protein structure and function.[12,13]

Also Kananetal revealed; the coding region of the VDR gene creates a second upstream start site due to a codon change from ACG → ATG, this start codon change causes the expression of a VDR protein that is 3 amino acids longer and has been shown to reduce transcriptional activity and to be less responsive to 1,25(OH)2D [14]

One of study carried out by Cai*et al.* Which showed VDR *FokI*genotype was associated with increased risk of preterm delivery. Moreover,A new Danish study indicates that genetic variations are associated with low levels of vitamin D in the blood leads to premature death [15,16]. Furthermore a high attribution of RPL patients have vitamin D deficiency, and low concentrations of vitamin D have been associated with raised risk of first trimester miscarriage [8] Also ZohrehSalari etal. Evaluated the relationship between rs7975232 and rs2228570 variants in the vitamin D receptor gene and the risk of recurrent abortion. The results of this study showed these genetic variants in VDR could be risk factors for recurrent pregnancy loss. [17]

In the present study for the VDR (TaqI)**,** The PCR result shows that about 42(97.7%) of cases were positive for VDR (TaqI) allele and only 2.3% were negative. For the control group7 (14%) were positive and the 43(86 %) were negative. When compared between the case and control group for the PCR result there was significant association (P= 0.000)

The sequencing results; When the cases were compared with the normal reference the following single Base Exchange were found C>T, G>C and C>A. Mutation taster was used to confirmed the mutations which revealed; C>T Base Exchange polymorphism was predicted, that is confirms the presence of rs731236 polymorphism. Also G>C, C>A and G>A (ID rs 4967032) single Base Exchange polymorphism were predicted, which may changed the amino acid sequence, spliced sit and protein features.

Indeed, the TaqIlocated in 3' untranslated region should be related to VDR activity and expression [18]. Our result finding was totally differed from other results present in the literature such as Zohreh Salariet al. which found; *TaqI*polymorphism is located in the non-coding region of the *VDR* gene, and they do not have any effect on the final protein product. The *TaqI*genotype/allele did not yield any significant results for the PTB [17, 19,20]

Also Manzon etal. They concluded that women who carried the A allele of FokI were at higher risk of PTB than those who carried the G allele. By contrast, women who carried the mutant allele of TaqI polymorphism exhibited a lower risk of PTB compared to those with non-mutated allele [21]. On the other hand Baczynska-Strzechaet al. revealed that; SNPs of the VDR gene, namely the TaqI, BsmI and ApaI polymorphisms, had no effect on preterm birth. [22]Furthermore one of study done in the women with RM from Slovenia and Croatia to assess the relationship of *VDR* polymorphisms (*FoIk*rs222857, *Cdx2* rs11568820, and *Taq1* rs731236); The authors observed higher frequency of mutated variants of *VDR*

*FokI*polymorphism and associated with the RM while the other polymorphisms were not associated with RM development[23]

Conclusion:

The study showed a significant association between fok1 rs2228570 and taq1rs731236variants of the VDR gene and recurrent miscarriage in the Sudanese women, according to this result we recommend to used vitamin D as basic supplemented treatment during the pregnancy. Further studies involving exonsequencing and large sample size are required to evaluate the direct effect of VDR gene polymorphism in the RM. Adopting the measurement of VD level inthe serum and VDR polymorphisms as one of the most important investigation to diagnosis of unexplained recurrent miscarriages Sudanese women.

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