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The relationship between vitamin D receptor (VDR) rs2228570 and rs7975232 genetic variants and the risk of recurrent pregnancy loss

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ABSTRACT

Objective: Recurrent pregnancy loss is one of the most common medical events that occur in the first and second trimesters. Hence, this study aimed to evaluate the relationship between vitamin D receptor (VDR) polymorphisms (rs2228570 and rs7975232) and the risk of recurrent pregnancy loss. The effect of rs2228570 polymorphism on protein stability was also predicted via in silico investigation.

Methods: This cross-sectional study was conducted on 52 women with recurrent pregnancy loss and 52 control women without pregnancy loss. We used the polymerase chain reaction technique to amplify the polymorphism regions on the chromosome. The PCR products were cut by *Fok*I and *Apa*I restriction enzymes and the obtained data were analyzed.

Results: Our results showed the case group consisted of 32.7% wild type, 65.4% heterozygote, and 1.9% homozygote genotypes for polymorphism rs7975232.The controls included 48.1% wild type, 42.3% heterozygote, and 9.6% homozygote genotypes. There was a significant difference between polymorphism rs7975232 and recurrent pregnancy loss ($P = 0.034$). These genotypes for rs2228570 polymorphism were53.8% wild type, 38.5% heterozygote, and 7.7% homozygote. However, the control group included 80.8% wild type, 15.4% heterozygote, and 3.8% homozygote. There was a significant difference between polymorphism rs2228570 and recurrent pregnancy loss ($P = 0.014$).

Conclusion: We found a significant difference between VDR rs2228570 and rs7975232 genetic variants with recurrent pregnancy loss. Protein stability was also decreased following single nucleotide polymorphism in VDR rs2228570.

1. Introduction

According to the definition by WHO, pregnancy loss is the termination of pregnancy before 20 weeks of gestation or delivering a fetus weighing less than 500 g. Approximately, a quarter of all women would experience at least one recurrent pregnancy loss during their reproductive age. Those with more than two pregnancy losses consider as recurrent pregnancy loss (Aali et al., 2011; Daher et al., 2012; Yildirim et al., 2019). Some factors such as age, drug abuse, alcohol, infection, immunologic (thrombophilia), and endocrine disturbances can effect recurrent pregnancy loss (Aali et al., 2011; Abbas et al., 2020; Haghighian, 2019; Kaur and Gupta, 2016; Pike et al., 2017; Tsikouras et al., 2019).

Vitamin D is one of four fat-soluble vitamins that is derived from the steroidal hormone family and conducts its activities via the vitamin D receptor (VDR). VDR is a ligand-dependent transcription factor mainly found in cell nuclei and it is the genomic functional mediator of 1,25 (OH)D3 (Daryanto et al., 2020; Pike et al., 2017). These receptors are found in different tissues such as the reproductive system (ovaries, uterus, placenta, and endometrium) and their gene transcription is

Abbreviations: VDR, Vitamin D receptor; RFLP, Restriction fragment length polymorphisms.

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Table 1
The sequence of applied primers to amplifying parts of VDR gene.

Primer	Sequence 5′–3′	Lenght
FokF	AGCTGGCCCTGGCACTGACTC TGCTCT	265 bp
FokR	ATGGAAACACCTTGCTTCTTCTCCCTC	
ApaF	CAGAGCATGGACAGGGAGCAAG	740 bp
ApaR	GCAACTCCTTCATGGCTGAGGTCTCA	

adjusted via interactions on gene promoters (Holick, 2007). VDR could be found in the nuclei and cytoplasm of ovarian granulosa cells that indicate it is a responsible receptor for physiologic actions of 1,25(OH) D3 in ovarian follicles. The presence of VDR in reproductive tissues in women implies that Vitamin D plays an important role in reproductive processes (Shahrokhi et al., 2016).

Structural and functional analyses of VDR protein led to the detection of distinct regions that play an active role in binding to DNA, ligand, receptor dimerization, and gene transactivation. Variations in DNA sequence would commonly happen amongst the general population, referred to as polymorphism. Most studies have concentrated on region 3 of the VDR gene including Apa1, Taq1, BSM1, and Fok1 in region 5. Apa1 polymorphism (rs7975232) that is located in intron 8 leads to the substitution of guanine by thymine (G → T) (Rasoul et al., 2019) whileFok1 rs2228570 polymorphism results in (C → T) transition.

In this study, we investigated the relationship between VDR gene polymorphisms (rs2228570 and rs7975232) and recurrent pregnancy loss. In silico study was also performed to assess the effect of the rs2228570 polymorphism on VDR protein stability.

2. Material and methods

2.1. Relationship between VDR genetic variants and recurrent pregnancy loss

This cross-sectional survey was conducted on 52 women, with spontaneous pregnancy loss syndrome and a history of three or more losses. The study was conducted by the Helsinki Declaration. Kerman University of Medical Sciences ethics committee approved this work (IR.KMU.REC.1397.229). The control groups consisted of 52 healthy women with no history of pregnancy loss. The exclusion criteria included any history of abortion, cardiovascular diseases, hypertension, coagulative and other chronic conditions (renal, hepatic, and rheumatologic), multifetal gestations, molar pregnancies, thrombophilia, lupus, anti-phospholipid antibody syndrome, and other known reasons for spontaneous abortion. A vitamin D level of less than 20 was regarded as deficient.

Genomic DNA of subjects was isolated from leukocytes of the whole blood using the salt-saturation method as previously prescribed (Miller et al., 1988). Polymerase chain reaction (PCR) was used to amplify DNA fragments of the polymorphism regions on chromosomes. To accomplish this, a 265 bp and a 740 bp of DNA fragments encompassing rs2228570 and rs7975232 polymorphisms were amplified using a pair of previously designed primers for each variant (Manzon et al., 2014) (Table 1).

Consequently, by restriction fragment length polymorphisms (RFLP) technique, the PCR products were cut using restriction enzymes of Apa1 and Fok1 to detect the polymorphisms of rs7975232 and rs2228570 of VDR, respectively.

In order to evaluate the results of RFLP, electrophoresis was conducted on an agarose gel to compare the length of bands. According to the results on the agarose gel, digestion of 745 bp rs7975232 PCR products by APa1 enzyme results in225bp and 515 bp DNA fragments, while the rs2228570 polymorphism gives a 69 bp and a 196 bp DNA fragment flowing Fok1 digestion.

Based on the results of RFLP, the patients were divided into three categories regarding VDR gene polymorphism: normal (wild type), heterozygote, and homozygote. After deeply evaluating all individuals regarding heterozygote and homozygote status, data were analyzed using SPSS version 20. The results were then evaluated using chi-square and independent *t*-test. *P*-value of less than 0.05 was regarded as statistically significant.

2.2. Effect of the rs2228570 genetic variant on VDR stability

The computerized analysis was performed to assess the effect of the rs2228570 polymorphism on VDR protein stability. The procedures are described as follows:

2.3. Molecular modeling and refinement

The three-dimensional structures of wild type and mutated VDR proteins (*Homo sapiens*) were simulated. Briefly, the functional domain sequence of VDR protein (*Homo sapiens*, CCDS8757.1) was acquired from the CCDS server and suitable template was recognized by blasting. Crystal structure of template protein was acquired from the RCSB (4nqaI). The target sequence was as follow:

>CCDS8757.1 [P11473-1].

MEAMAASTSLPDPGDFDRNVPRICGVCG-
DRATGFHFNAMTCEGCKGFFRRSMKRKAFTCPFNGD-
CRITKDNRRHCQACRLKRCVDIGMMKEFILTDEEVQRKRE-
MILKRKEEEALKDSLRPKLSEQQRIIAILLDAHHKTYDP-
TYSDFCQFRPPVRVNDGGGSHPSRPNSRHTPSFSGDSSSSCSDH-
CITSSDMDSSSFSNLDLSEEDSDDPSVTLELSQLSML-
PHLADLVSYSIQKVIGFAKMIPGFRDLTSEDQIVLLKSSAIEVIMLR-
SNESFTMDDMSWTCGNQDYKYRVSDVTKAGHSLELIE-
PLIKFQVGLKKLNLHEEEHVLLMAICIVSPDRPGVQDAA-
LIEAIQDRLSNTLQTYIRCRHPPPGSHLLYAKMIQKLADLRSL-
NEEHSKQRCLSFQPECSMKLTPLVLEVFGNEIS.

The conformations of wild type and mutated VDR proteins were simulated by I-TASSER server and checked with Ramachandran plot at (http://servicesn.mbi.ucla.edu/Verify3D/) (Tavakkoli et al., 2019).

2.4. Molecular dynamics simulation

Molecular dynamics simulation was performed using the GROMACS 5.4.1 package to assess the stability of simulated VDR structure. In brief, the simulated VDR protein was subjected into the OPLS force field and the water model SPC/E was used. The solvated system was defined into a charge neutralized system by adding sodium ions. The cubic solvent box with box sizes of 12.43512, 12.43512 and 12.43512 was considered and solvated by explicit water using the gmx solvate algorithm. The system was minimized for 50,000 steps using the steepest descent algorithm and then run at 300 K and 1 bar under periodic boundary conditions. The v-rescale and Parrinello-Rahman algorithms were used for temperature and pressure coupling, respectively. Electrostatic interactions were measured by the particle-mesh Ewald method. The stability of the VDR protein during the simulations was investigate by calculating the radius of gyration using gmx gyrate tool.

The effect of rs2228570 polymorphism in protein stability was also predicted via mCSM server http://biosig.unimelb.edu.au/mcsm/. The rs2228570 variation type is a single nucleotide variation, which was located at chr12:47879112. The molecular structure of simulated VDR proteins (wild type and mutated) were uploaded onto mCSM software to evaluate their stability by measuring the change in Gibbs free energy (ΔΔG).

3. Results

3.1. Relationship between VDR genetic variant and recurrent abortion

In this study, the relationship between rs2228570 and rs7975232 variants of VDR gene and spontaneous abortion on 52 individuals with a history of at least three or more abortions and 52 patients with no

Table 2
Clinical indices and fertility information of patients with recurrent spontaneous abortions and normal individuals.

Variables	Control	Case	*P* value
	Mean ± SD	Mean ± SD	
Age	31.40 ± 6.19	33.53 ± 5.72	0.550
Gravid	2.53 ± 0.17	5.55 ± 0.23	0.001
Para	2.50 ± 0.18	1.77 ± 0.21	0.013
Vitamin D level	19.22 ± 5.62	19.12 ± 5.83	0.929

Table 3
Regression analysis for examine the relationship between variants and vitamin D levels.

	B	t	*P* value
VPA	−0.095	−0.958	0.34
FOK	−0.083	−0.803	0.42
Groups	−0.019	−0.183	0.85

similar history was evaluated. The mean age of all participants was 33.53 ± 5.72 years. The mean number of gravida was higher amongst cases compared to the normal individuals (5.55 ± 0.23) which were statically significant. Moreover, the mean number of para was also significantly higher amongst cases (2.50 ± 0.18) (Table 2). The regression analysis was performed to examine the relationship between variants and vitamin D levels, but no difference was seen (Table 3).

Fig. 1(A) demonstrates the PCR results of detected rs7975232 polymorphism. In this figure, the first column shows people with 740 bp homozygote wild type allele (GG)while the second and third ones show heterozygote (GT) and homozygote (TT) of the polymorphism alleles, respectively (Fig. 1A). Amongst cases, we detected 32.7% wild type, 65.4% heterozygote, and 1.9% homozygote genotypes for rs7975232 polymorphism. On the contrary, while 48.1% of the controls had wild type genotype, 42.3% and 9.6% were heterozygote and homozygote, respectively. The genotype of rs7975232 polymorphism in the VDR gene was different amongst cases and controls (Table 4).

Fig. 1B is presenting a sample of RFLP results of detected rs2228570 polymorphism. In this figure, the first column represents individuals with 265 bp homozygote wild type allele (CC) while the second and third ones represent heterozygote (CT) and homozygote (TT) individuals, respectively (Fig. 1B). Amongst patients, we found 53.8% wild type, 38.5% heterozygote, and 7.7% homozygote genotypes for rs2228570 polymorphism. In contrast, 80.8% of the controls had wild type genotype while 15.4% and 3.8% were heterozygote and homozygote, respectively. The genotype of rs2228570 polymorphism in the VDR gene was different between cases and controls. There was a

Table 4
The evaluation of rs7975232 polymorphism amongst cases with recurrent spontaneous abortion and healthy individuals.

rs7975232 polymorphism (G → T)	Control	Case	*P* value
	No (%)	No (%)	
Normal (GG)	25 (48.1)	17 (32.7)	0.034
Heterozygous (GT)	22 (42.3)	34 (65.4)	
Homozygous (TT)	5 (9.6)	1 (1.9)	
Total	52 (100)	52 (100)	–

Table 5
The evaluation of rs2228570 polymorphism amongst individuals with spontaneous recurrent abortion and normal individuals.

rs7975232 polymorphism (C → T)	Control	Case	*P* value
	No (%)	No (%)	
Normal (CC)	42 (80.8)	28 (53.8)	0.014
Heterozygous (CT)	8 (15.4)	20 (38.5)	
Homozygous (TT)	2 (3.8)	4 (7.7)	
Total	52 (100)	52 (100)	–

Table 6
The evaluation of vitamin D deficiency amongst individuals with a history of recurrent abortion and healthy ones.

Vitamin D deficiency	Control	Case	*P* value
	No (%)	No (%)	
Yes	34 (65.4)	31 (59.6)	0.543
No	18 (34.6)	21 (40.4)	
Total	52 (100)	52 (100)	–

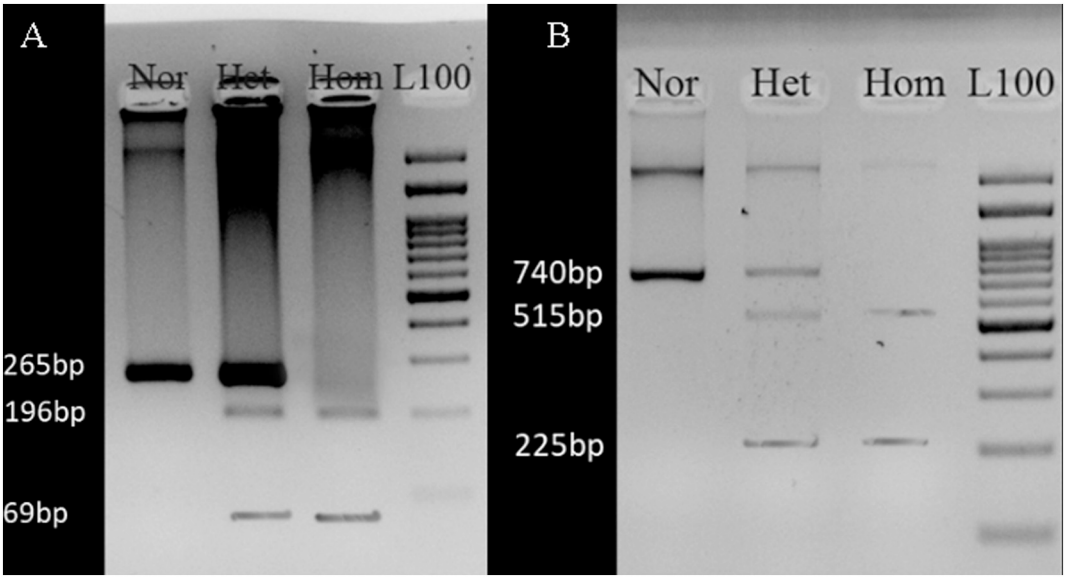

Fig. 1. RFLP Gel electrophoresis assay using (A) ApaI enzyme to present rs7975232 polymorphism, cut the 740 bp PCR products into 225 bp and 515 bp DNA fragments. (B) FokI enzyme to detect rs2228570 polymorphism. Homozygous, cut the 265 bp PCR products into 196 bp and 69 bp DNA fragments. Homozygous: Hom, Heterozygous: Het, Normal: Nor, Pair: bp, Marker: L100 Blank: test tube without DNA as control.

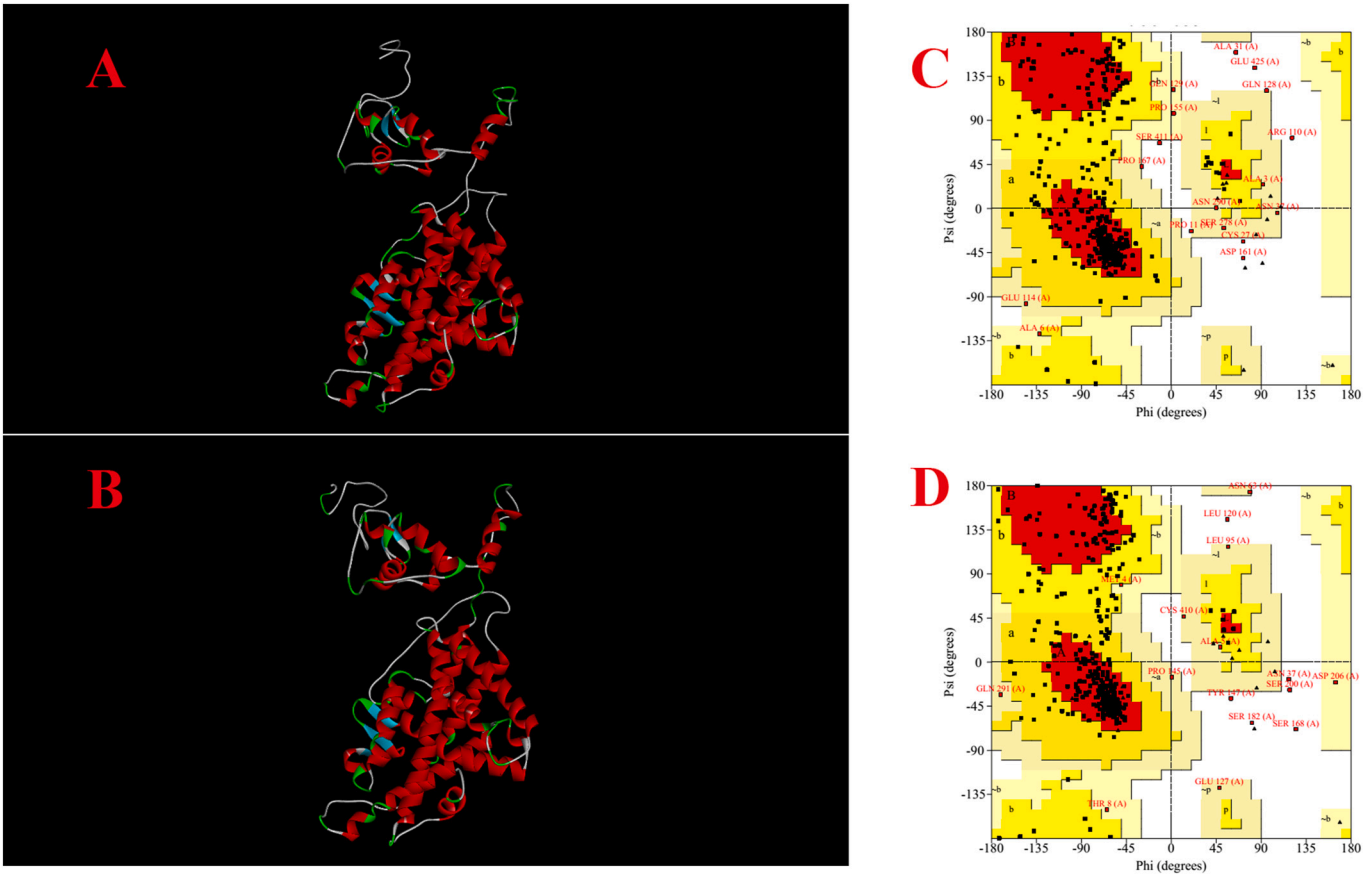

Fig. 2. The structures of simulated proteins. (A and B) Models of wild type and mutated VDR proteins (*Homo sapiens*), respectively. (C and D) Ramachandran plots for wild type and mutated VDR proteins, respectively.

significant difference between this polymorphism and recurrent abortion ($P = 0.014$) (Table 5).

Furthermore, 59.6% of the cases and 65.4% of the controls were vitamin D deficient. Such a difference was not statistically significant ($P = 0.543$) (Table 6).

3.2. Structural simulation of VDR proteins

The 3D structures of wild type and mutated VDR proteins (Homo sapiens) were simulated (Figs. 2A and B). The qualities of simulated proteins was investigated by Ramachandran plots which are presented at Figs. 2C and D. PROCHECK results, for wild type and mutated VDR proteins showed that 75.2% and 74.7% of the residues were in most favored regions, 21.1% and 21.4% of the residues were in additional allowed regions, 1.8% and 2.3% of the residues were seen in generously allowed regions, and 1.8% and 1.6% of residues were in disallowed regions, respectively. These data showed that good quality models would be expected. The structural parameters of wild type VDR protein were as follows: number of amino acids = 427, molecular weight = 48,315.26, the total number of negatively charged residues (Asp + Glu) =60, the total number of positively charged residues (Arg + Lys) =53, and the total number of atoms = 6719.

The structural parameters of mutated VDR protein were as follows: number of amino acids = 427, molecular weight = 48,289.18, total number of negatively charged residues (Asp + Glu) =60, total number of positively charged residues (Arg + Lys) =53 and total number of atoms = 6711.

The modeled proteins were tested for their conformational stability using MD simulations. The simulation box is presented in Fig. 3A. By calculating the radius of gyration, it is revealed that the proteins remain stable, in their compact (folded) form throughout 1000 ps at 300 K (Figs. 3B–E).

The impact of rs2228570 genetic variant on protein stability was also accessed via mCSM server. The result showed that protein stability decreases following single nucleotide polymorphism. The predicted numerical value of change in Gibbs free energy (ΔΔG) was −1.355 Kcal/mol.

4. Discussion

Pregnancy loss is referred to termination of pregnancy before 20 weeks of gestation. Some studies reported that the relationship between the polymorphisms of the VDR gene and the risk of pregnancy loss was not significant (Mahdavipour et al., 2017; Mofidimanesh et al., 2017).

Here we 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. Some studies have demonstrated the relationship between the polymorphisms of this gene and some other diseases such as type I and II diabetes, metabolic syndrome, femoral neck, and lumbar vertebral bone density. Furthermore, there have been some other studies demonstrating the relationship between recurrent abortion and polymorphism of other genes (Li et al., 2012; Mahdavipour et al., 2017; Mofidimanesh et al., 2017; Rasoul et al., 2019).

For instance, Li et al. in 2012 evaluated the relationship between (rs1544410) and *Apa* I (rs7975232) polymorphism in the VDR gene and femoral neck and lumbar vertebral bone density. The results of their study did not reveal any relationship between such a polymorphism and femoral neck and lumbar vertebral bone density (Li et al., 2012).

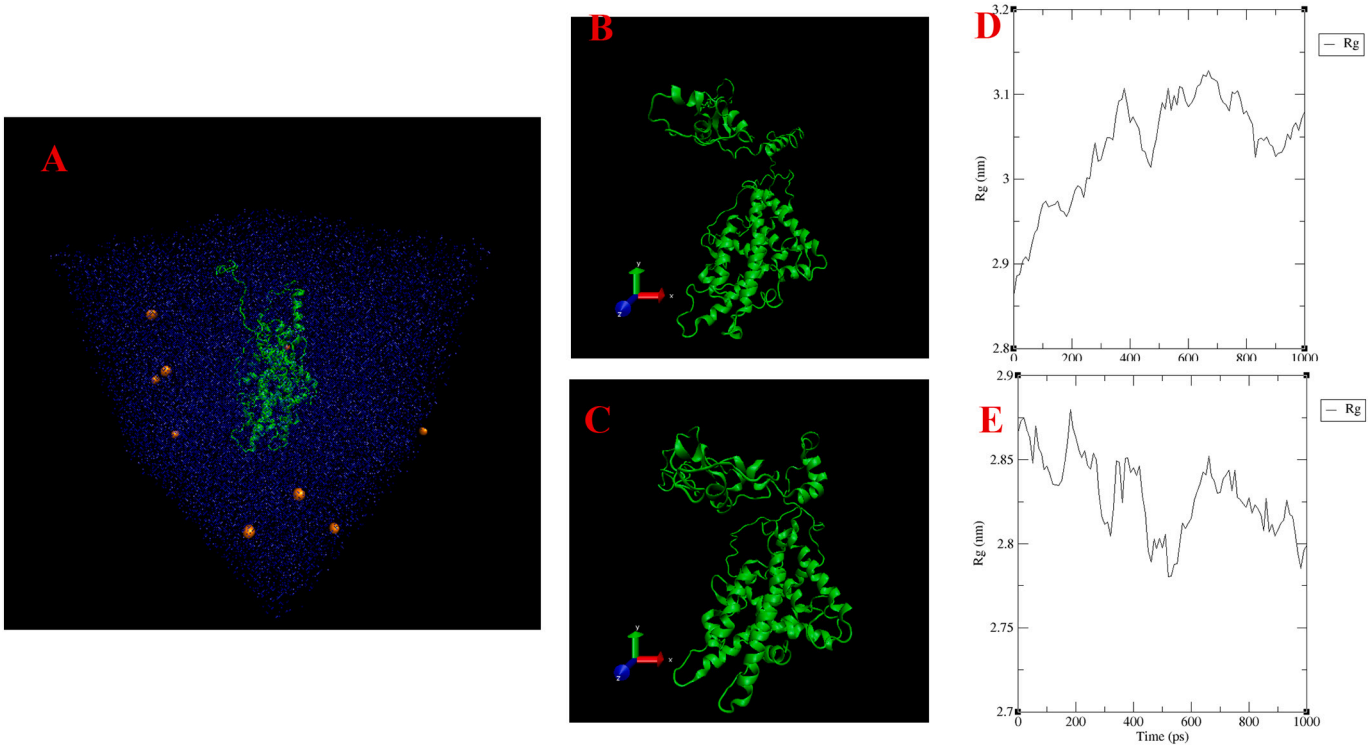

Fig. 3. Molecular dynamics simulations to investigate impact of rs2228570 genetic variant on protein stability. (A) Simulated VDR protein solvated in SPC/E water and sodium ions. (B and C) Final models of wild type and mutated VDR proteins after molecular dynamics simulations, respectively. (B and C) Radius of gyration values for wild type and mutated VDR proteins, respectively.

Moreover, the study by Hong et al. did not show the relationship between VDR gene polymorphisms and metabolic syndrome and type II diabetes in the Korean population (Hong et al., 2015).

The study conducted by Soltanghoraee et al. presented the 4G/4G allele in PAI-1 as the crucial factor in recurrent abortion amongst Iranian patients (Soltanghoraee et al., 2007). Pineda et al. have also introduced the polymorphism of rs2234693 (C>T, defined by restriction enzyme *Pvu*II) and rs9340799 (A>G, defined by restriction enzyme *Xba*I) in intron 1 of the ESRα gene as the risk factors of recurrent abortions (Pineda et al., 2010). Another study was conducted by Courtney Hanna and their colleagues in 2010. Their study evaluated 35 polymorphisms of 20 different genes including rs1256049 and CA repeat in estrogen receptor gene, beta type, using sequencing technique. (Hanna et al., 2010).

In this study, vitamin D deficiency was more commonly observed amongst healthy individuals; however, such a difference was not statically significant. We did not find a difference between variants and vitamin D levels in regression analysis. Since vitamin D deficiency is very common in pregnant women in the study area, it is not surprising to detect the lack of difference in vitamin D levels between the two groups. However, a study by Li et al. showed expression of VDR was significantly decreased amongst women with recurrent pregnancy loss resulting in lower levels of vitamin D. (Huang et al., 2017).

As the studies have revealed that a myriad number of reasons would lead to recurrent abortion, various studies have applied different control groups which could affect the results. Another reason behind controversies could be the differences in genetic and environmental histories. Since the expression of a certain disease is strongly related to its polymorphisms in the populations and environmental factors, racial differences play an important role in genetic linkage studies. Allele frequencies could differ in different nations. For example, it is possible that polymorphism would correlate with recurrent pregnancy loss in Indian and Korean populations but not Japanese or Iranians. Moreover, the sample sizes of these studies were different from each other. In order to confirm or rule out a relationship between polymorphism of a gene and etiologies of certain diseases, a higher number of patients and healthy individuals, as controls, are needed. Accordingly, a limited number of women cannot lead to definite and precise results which could be generalized to the whole population.

5. Conclusion

This study showed a significant relationship between rs2228570 and rs7975232 variants of the VDR gene and recurrent abortion. Analysis of the effect of gene polymorphisms on recurrent abortion should be studied in different cases. Detection of the association between these polymorphisms and recurrent abortion could provide better strategies for further treatments.

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CRediT authorship contribution statement

Zohreh Salari: Conceptualization, Writing - original draft. **Nasrollah Saleh-Gohari:** Supervision, Writing - original draft, Writing - review & editing. **Monire Rezapour:** Methodology. **Ahamad Khosravi:** Methodology. **Hadi Tavakkoli:** Software, Formal analysis. **Ehsan Salarkia:** Methodology. **Fatemeh Karami-Robati:** Software, Writing - original draft.

Declaration of competing interest

There is no conflict of interest in the work presented.

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