



Association of FSHR missense mutations with female infertility, *in silico* investigation of their molecular significance and exploration of possible treatments using virtual screening and molecular dynamics



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ABSTRACT

This study investigated the association of A419T (rs121909661) and T449I (rs28928870) with infertility among Iranian women and possible treatments by agonizing the mutated receptor. 151 women were genotyped at A419T and T449I sites. Homology modeling, pharmacophore modeling, virtual screening, docking and molecular dynamics (MD) were performed. A419T and T449I indicated a significant and a weak association with infertility among Iranian women ($P = 0.005$ and $P = 0.03$, respectively). Significant differences found among three genotypes of A419T with FSH ($P = 0.01$) and LH ($P < 0.0001$). G-allele carriers of A419T had susceptibility to display higher FSH and LH serum levels. *In silico* results revealed the most potent agonists among 3041 similar compounds and MD supported this finding. Altogether, genotyping of A419T and T449I as potential markers might be helpful in prognosis and treatment of infertility. Also, a new series of potent FSHR agonists were identified for future drug development and treatment of infertility related to FSHR dysfunction.

1. Introduction

Follicle stimulating hormone (FSH) plays an essential role in gonadal development and sexual maturation at puberty [1]. Also, several studies have demonstrated the importance and vital role of FSH in gamete production during the fertile period recruitment and maturation [2,3]. FSH binds to the N-terminal, extracellular domain of its receptor (FSHR) [4]. *FSHR* gene is located on chromosome 2p21 and consists of 10 exons and 9 introns [5,6]. Missense polymorphisms such as Ala307Thr, Arg524Ser, Ala665Thr and Ser680Asn on exon 10 showed remarkable impact on FSHR structure and/or its function [7,8].

Ala419Thr (A419T) was first described in a Finnish female with primary amenorrhea and hypergonadotropic ovarian failure. Ala419 was found to be located in the second α -helix of the FSHR transmembrane domain [9]. It has been reported that A419T does not impact binding affinity of the hormone but impairs FSH-dependent cAMP production [10]. Another structural variant, Thr449Ile (T449I), seems to display a decreased ligand's specificity and was linked with spontaneous ovarian hyperstimulation syndrome (OHSS) in a case report of

Moroccan family [11].

It is more suitable for infertile individuals to use small molecule agonists of glycoprotein hormones as oral therapy than steroidal drugs. Also, compared to the steroidal drugs, a negative allosteric modulator of FSHR can lead to find a highly specific oral contraceptive with decreased side effects. Oral contraceptives have been linked to the increased risk of some types of cancer like breast and endometrial cancer. Besides, estradiol-based oral contraceptives can increase the risk of cardiovascular thrombosis. Small molecules, in this respect, generally display a wider safety profile for adjusting the function of endocrine system than steroidal modulators [12–14].

According to the literature and to the best of our knowledge, there is no case-control study of A419T and T449I based on SNP genotyping and molecular modeling among infertile women; thus, the goal of the current study was to investigate the association of A419T and T449I with susceptibility to infertility among Iranian women and computational studies were designed to reveal how A419T missense mutation impairs the normal activity of FSHR receptor. Furthermore, a virtual screening was organized to identify the most potent agonists of this

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receptor based on previous studies. MD simulation was performed to validate the results of virtual screening and to investigate the interactions of ligands with the receptor.

2. Methods

2.1. Subjects and genotyping

In this study, 151 women (77 infertile and 74 fertile women) were examined. The age range was between 20 and 35 years old. Each healthy control had at least one normal pregnancy and one child. Infertile people were under the supervision of a specialist physician and the cause of their infertility was the lack of ovulation due to primary and secondary amenorrhea. Infertility for female patients were defined as at least 12 months of infertility despite having a regular sexual intercourse (without using any form of contraception). For male partners, a normal semen analysis were recorded to avoid unrelated infertility due to partners' problems. To achieve this goal, a total motile sperm count of at least 15 million per ejaculation was determined as the minimum of active sperms for male partners. Patients with infertility due to surgery (vasectomy for males and tubal ligation for females), Turner syndrome and hysterectomy were excluded for this study. Additionally, women with infertility due to deformity in sexual organs were omitted. Informed consent documents were obtained from all subjects prior to sampling. The present study was conducted in accordance with the declaration of Helsinki regarding the use of human samples.

Genomic DNA was extracted from white blood cells with the Blood and Cell Culture DNA kit (Sinacolon, Iran) according to manufacturer's protocol. Then, DNAs were analyzed by electrophoresis on 1% agarose gels with ethidium bromide staining. Concentrations of extracted DNAs were determined by Nanodrop. Two SNPs (A419T and T449I) were chosen for genotyping process. Designed regions of FSHR gene were amplified from genomic DNA by Amplification Refractory Mutation System PCR (ARMS-PCR) and Restriction Fragment Length Polymorphism PCR (RFLP-PCR). A419T genotyping was done by ARMS-PCR protocol (94 °C for 4 min, 94 °C for 30 s, annealing Time 60 °C for 30 s, 72 °C for 30 s, 72 °C for 5 min, and 4 °C for 30 min as holding time in 35 total cycles). ARMS primers (for A419T) were designed using oligo7 and Gene runner ver. 6.5 softwares as follows: G allele Forward: 5'-TGGAATCTACCTGCTGCTCACTG-3' and Reverse: 5'-TCACTAGAGGAGGACACGAT-3', A allele Forward: 5'-TGGAATCTA CCTGCTGCTCACTA-3', Reverse: 5'-ATCTTTCCAAGGTGATAGCTGT-3'.

Genotyping of rs28928870 was performed by RFLP-PCR. The PCR products were digested with 2 IU of restriction enzyme HPYCH4III for 2 h in 62 °C and analyzed by electrophoresis in 2% agarose gel respectively, and subsequently stained with ethidium bromide. Each PCR was carried out in a total volume 50 µL consisting of 2.5 µL extracted DNA, 50 pmol/µL each primer, 100 µM dNTP, 1 U/µL unit Taq DNA polymerase and 2 mM MgCl₂. We amplified exon 12 of FSHR gene with primers: (F: 5'-ATCTCTGCATTGGAATCTACCTG -3' and R: 5'-CAAGG AGGGACATGACATACAGC -3'). All reactions had an initial denaturation step of 3 min at 94 °C followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 62 °C for 30 s and 72 °C for 1 min, and a final elongation step at 72 °C for 10 min on a Gene. All reagents were purchased from Fermentas Company.

2.2. Statistical analysis

All statistical analyses were done by SNPalyze ver. 8.1.1., SPSS (ver.20, <http://www.spss.com/>), Medcalc ver. 12, and Web-Assotest online software (<http://www.ekstroem.com/assotest/assotest.html>). Pearson's chi-square test, Independent *t*-test and ANOVA were utilized to assess the associations of studied FSHR SNPs at both allelic and genotypic levels and all genotype-phenotype analyses. The corresponding odds ratios (ORs) and confidence intervals (95% CIs) were

calculated. The significance level was assumed lower than 0.05 ($P < 0.05$).

2.3. Homology modeling and validation of wild-type (A419), T419 and I449 structures

Given that FSHR protein was not previously modeled at A419T and T449I sites and other FSHR related structures in protein data bank (PDB) miss some residues in N- or C-terminal of the protein, the present study performed molecular modeling of human FSHR protein in wild-type and mutant alleles (T419 and I449). A 695 amino acid long sequence of FSHR was obtained from Uniprot (UniprotKB ID: P23945). A suitable template that had a high degree of sequence similarity with FSHR was identified by Blastp (PDB ID: 4AY9) (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The tertiary structures of human FSHR protein in wild-type and mutant alleles of A419T were modeled by the selected template with SWISS-MODEL, PS2, and Phyre2 [15] online servers. Then, best models from SWISS-MODEL were built after energy minimization by Swiss-PdbViewer DeepView ver. 4.1.0 software [16]. The structures of models were then validated through MolProbity [17], ProSA [18,19], and ERRAT [20]. PyMol v2.2 software was utilized for the superimposition and comparison of the wild-type and T419 structures [21]. To estimate the relative free energy of insertion into membrane the QMEAN server and QMEANBrane module from SWISS-MODEL server was utilized [22]. By using an implicit water model, the QMEANBrane specifically trained statistical potentials, were utilized on different regions of the membrane protein, to harvest the membrane insertion energy.

2.4. Pharmacophore modeling and virtual screening of T419 agonists

In the second part of *in silico* analyses, agonists and antagonists for A419 structure were gathered by investigation of Guo review article [23]. The structures of agonists and antagonists were imported into the Phase program [24,25]. 3D-structural features like hydrogen bond acceptor, hydrogen bond donor, hydrophobic group, positively and negatively charged moiety and aromatic ring were considered in the generation of pharmacophore hypotheses. The Phase hypo score was used to evaluate the results for the best alignment with most of our compounds. As the number of available agonists and antagonists for FSHR receptor were too short (to define a training and feature set and validate our results using Receiver Operating Characteristics (ROC) curve), virtual screening was used via Glide XP docking procedure and also molecular dynamics (MD) to confirm and validate the pharmacophore modeling results. The most potent and well-known small molecules in the literature as the FSHR agonist according to their EC₅₀ (7.2 nM and 14 nM) from previous studies were selected for virtual screening. The Zinc database was used for selection of similar compounds to these molecules. Tanimoto-60 method was utilized and a set of 3041 molecules were identified. The structures of these compounds were prepared with Ligprep (in pH = 7.4 ± 1) and the prepared molecules were screened for having the previously defined pharmacophore framework. Among these molecules, a series of 2672 compounds embodied the pharmacophore structure and were subjected to Glide XP docking with T419 to filter weak agonists and cultivate the results.

2.5. Molecular docking and MD of A419

Using Glide XP precision [26–28], molecular docking was used for screening of 2672 compounds. Docking was employed by OPLS-2005 method through sampling flexible ligand structures as well as ring conformations and nitrogen inversions. Epik state penalties were added to the final scores and a post-docking minimization was performed to further improve the accuracy of the results. A set of 5000 poses were set for initial step of docking and 800 best poses for each ligand were submitted for energy minimization. The binding region (extra-cellular

domain of FSHR and near the transmembrane helices which is known as hinge region or HinR) for generation of the grid file was inferred from previous study by Jiang et al. and based on that study, Tyr335 seems to play one of the most important role in ligand recognition for FSHR (in the hinge region) [29]. In this study, a grid box with $20 \text{ \AA} \times 20 \text{ \AA} \times 20 \text{ \AA}$ coordinates by centering Tyr335 residue was applied for molecular docking.

To investigate the MD simulation of FSHR with the best compounds determined in the previous steps, Desmond v5.3 (Schrodinger suite 2018–1 [30]) was utilized. OPLS-2005 force-field was applied for the simulation, in a drenched box with SPC water model and a concentration of 0.15 M of sodium chloride. The system was loaded with 74020 atoms. Steepest descent minimization was carried out following the Limited-memory Broyden-Fletcher-Goldfarb-Shanno (LBFGS) method of energy minimization to converge the system to a gradient of 1 kcal/mol/Å with a maximum iteration of 2000. MD simulation was settled in NPT ensemble (constant number of atoms, constant pressure i.e. 1.01325 bar and constant temperature i.e. 310 K) and before running the MD simulation the temperature of system was raised to 410 K for 0.5 ns to remove the non-selective interactions and returned to the normal 310 K after that period (simulated annealing). The Nose-Hoover chain and Martyna-Tobias-Klein approach were used as the default thermostat and barostat with 1.0 ps and 2.0 ps interval by isotropic coupling style respectively. For computation of near and far range forces, a 2 fs and 6 fs Reversible Reference System Propagator Algorithm (RESPA) integrator time-step, was exploited. Summation of long-range electrostatic forces was performed by Particle-mesh-based Ewald (PME) method. A cut-off radius of 9.0 Å was set for Coulombic forces [31].

The Root Mean Square Deviation (RMSD) and Root Mean Square Fluctuation (RMSF) of the protein and ligand were monitored throughout simulation (20 ns) in reference to the first frame. Interactions lasting more than 30% of the time of simulation were documented in final results.

3. Results

3.1. Association analysis of A419T and T449I in study groups

Tetra-ARMS technique was used for genotyping of samples. PCR products included the fragments with the lengths of 468 base pairs (G as wild-type Allele) and 170 base pairs (A as mutant allele). Allele frequency of A419T was represented in Table 1. The statistical analysis for the A419T allele frequency indicated that A allele increases the risk of infertility ($P = 0.02$). Data showed that out of 77 case subjects, 17, 48, and 12 individuals had GG, GA, and AA genotypes, respectively. Also, out of 74 control subjects, 34, 32, and 8 individuals had GG, GA, and AA genotypes, respectively. Results indicated that A419T was statistically significant under dominant model of inheritance ($P = 0.005$). Based on this, A Allele was considered as a risk factor in case group and it might be involved in disease onset.

The PCR-RFLP technique was performed to determine samples' genotypes. The replicated fragment included T449I with a restricting site in C nucleotide for cutting by *HPYCH4III* enzyme. The bands caused

by enzymatic restriction were seen in CC and CT genotypes. 125 and 259 base pair bands were seen in CC genotype and 384, 259, and 125 base pair bands were obtained in CT genotype. No enzymatic restriction occurred in TT genotype and a 384-base-pair-long fragment was obtained. Allele and genotype frequencies were assessed and represented in Table 2. Results revealed that C allele had the highest frequency between the case and control groups. In case group, 32, 7, and 4 individuals were with CC, CT, and TT genotypes, respectively. Also, in control group, 42, 5, and no individuals had CC, CT, and TT genotypes, respectively. Based on genotype analyses, it was observed that there is a weak association between T449I genotypes and susceptibility to infertility among study groups in recessive model of inheritance ($P = 0.03$) (Table 2).

3.2. Genotype-phenotype sub-analyses of infertile women based on A419T genotypes

Genotype-phenotype analyses were done through independent t-test and one-way ANOVA based on allelic and genotypic levels of A419T and T449I with clinical characteristics of infertile women including age, abortion status, and LH and FSH levels in blood (Table 3). According to Table 3, no significant difference was observed among genotypes of A419T in association with age, and abortion status. However, FSH and LH levels represented significant difference among three genotypes ($P = 0.01$ and $P < 0.0001$, respectively). Women with GG genotype had a higher mean level of FSH (6.36 ± 1.97 mIU/ml) while women with AA genotype showed the lowest level of FSH (4.43 ± 2.19 mIU/ml). Also, subjects with GG compared with GA and AA genotype had the highest level of LH (7.40 ± 1.11 compared with 5.63 ± 1.53 and 4.7 ± 1.92 mIU/ml, respectively). Thus, G allele carriers had higher FSH and LH levels compared with A allele carriers. Genotype-phenotype analysis of T449I showed no association in both allelic and genotypic levels with studied characteristics (Not shown in table).

3.3. Results of wild-type (A419), T419 and I449 modeling

After performing Blastp of FSHR protein sequence, follicle-stimulating hormone in complex with the entire ectodomain of its receptor (PDB structure 4AY9) was selected as the template with Query coverage of 50% and an identity of 99% with a low E value of 0. Target human protein FSHR in wild-type and mutant alignments modeled using SWISS-MODEL, then energy minimization was performed for the two structures using Swiss-PDB viewer version 4.1.0. Wild-type, T419, and I449 structures of FSHR were analyzed by ProSA (z-score: 4.82, -5.66 , and -5.9 respectively). Ramachandran plots were used for validation analysis of FSHR models with MoProbit which revealed 99.1%, 99.6%, and 95.6% of amino acids were in the favored and allowed regions for wild-type, T419, and I449 models. Moreover, Errat values of wild-type, T419, and I449 structures were 87.94, 88.50, and 83.28, respectively (Figures and pdb files for A419 (wild) and T419 are incorporated in supplementary data).

Table 1

The association analysis of FSHR gene polymorphism Ala419Thr (rs121909661) among patients and control subjects.

SNP	Subjects	Genotype frequency			Allele frequency		P-value	Dominant model		Co-dominant model		Recessive model	
		MM	Mm	mm	Major	minor		Allele	P-value	OR (95% CI)	P-value	OR (95% CI)	P-value
Ala419Thr (G > A)	Case	0.22 (17)	0.62 (48)	0.16 (12)	0.53	0.47	0.02	5.85E-3	0.38	0.7E-2	2.00	0.39	0.66
	Control	0.46 (34)	0.43 (32)	0.11 (8)	0.68	0.32							

Number of subjects with each genotype and number of alleles (frequency in %). OR; Odds ratio, CI; confidence interval. ORs for different modes of inheritance were assessed by the SNPalyze ver. 8.1.1 and Web-Assotest program. MM, Mm, and mm are Major homozygote, Heterozygote, and minor homozygote respectively. The significance level of P is less than 0.05.

Table 2

The association analysis of FSHR gene polymorphism Thr449Ile (rs121909661) among patients and control subjects.

SNP	Subjects	Genotype frequency			Allele frequency		P-value	Dominant model		Co-dominant model		Recessive model	
		MM	Mm	mm	Major	minor		Allele	P-value	OR (95% CI)	P-value	OR (95% CI)	P-value
Thr449Ile (C > T)	Case	0.75 (32)	0.16 (7)	0.9 (4)	0.83	0.17	0.64	0.14	0.51 (0.21–1.25)	0.32	1.84 (0.53–6.33)	0.03	10.82 (0.56–207.21)
	Control	0.89 (42)	0.11 (5)	0.00 (0)	0.95	0.05							

Number of subjects with each genotype and number of alleles (frequency in %). OR; Odds ratio, CI; confidence interval. ORs for different modes of inheritance were assessed by the SNPalyze ver. 8.1.1. and Medcalc ver. 12. MM, Mm, and mm are Major homozygote, Heterozygote, and minor homozygote respectively. The significance level of P is less than 0.05. NA means not applicable.

Table 3

Clinical characteristics of infertile patients according to Ala419Thr (G > A) genotypes.

Clinical Characteristics	Individuals with infertility				
	GG	GA	AA	P-value	
Age (years)	31.11 ± 5.19	31.53 ± 4.70	31.92 ± 4.30	0.90	
FSH (mIU/ml)	6.36 ± 1.97	5.45 ± 1.51	4.43 ± 2.19	0.01	
LH (mIU/ml)	7.40 ± 1.11	5.63 ± 1.53	4.7 ± 1.92	1E-4	
Abortion statuses	No-Abortion	10 (25.0)	26 (65.0)	4 (10.0)	0.67
	One-Abortion	4 (18.2)	13 (59.1)	5 (22.7)	
	Continuous Abortion	4 (26.7)	8 (53.3)	3 (20.0)	

Data are presented as means ± SD for Age, FSH, and LH; also, frequency (%) for Abortion statuses, P-values indicate independent ANOVA and Pearson Chi-Square test, FSH: Follicle-Stimulating Hormone, LH: Luteinizing Hormone.

3.4. Comparison of wild-type A419 and mutated T419 structures

According to the strong association of A419T (P = 0.005) with female infertility among Iranians compared to the T449I (P = 0.03) and significant associations of A419T with clinical characteristics (FSH and LH levels) of studied infertile individuals, further *in silico* investigations (secondary docking, pharmacophore modeling, and MD) were carried out on A419T site. The membrane insertion energies of the two modeled FSHR structures reveal that the wild-type FSHR protein (A419) had slightly more tendency toward being inserted into membrane and thus A419 structure is adequately more stable in the membrane but the significance level of this observation (without the other findings of present study) in the real cellular environment is not completely distinctive and might be negligible in practice (Fig. 1). The superimposition of A419 and T419 structures demonstrated a considerable amount of divergence between the two structures (RMSD = 2.887 Å). The folding of some α -helices was distorted in the membrane or altered in a way that transform the normal FSHR activity (Fig. 2). Based on the

current findings in the experimental part on genotyping of A419T, this conformational transformation of the transmembrane segment of A419T is pertinent to its diminished activity.

3.5. Pharmacophore modeling and virtual screening

The results of Pharmacophore modeling revealed that 3 major pharmacophore features were necessary for binding of the agonists to the FSHR protein. The first one was the presence of an aromatic ring. Next one was the presence of an acceptor hydrogen bond adjacent to the aromatic ring and the last one was a hydrophobic fragment located at the other side of the molecule (Fig. 3). The results of virtual screening revealed the top agonistic compounds against FSHR T419 protein. The top 5 compounds, all indicated more negative XP scores than the previously identified agonists, and their structures are depicted (Fig. 4). From the results of the virtual screening, it can be postulated that at least the existence of one amide group near to the aromatic ring contributes to more negative docking values. Attachment of methoxy

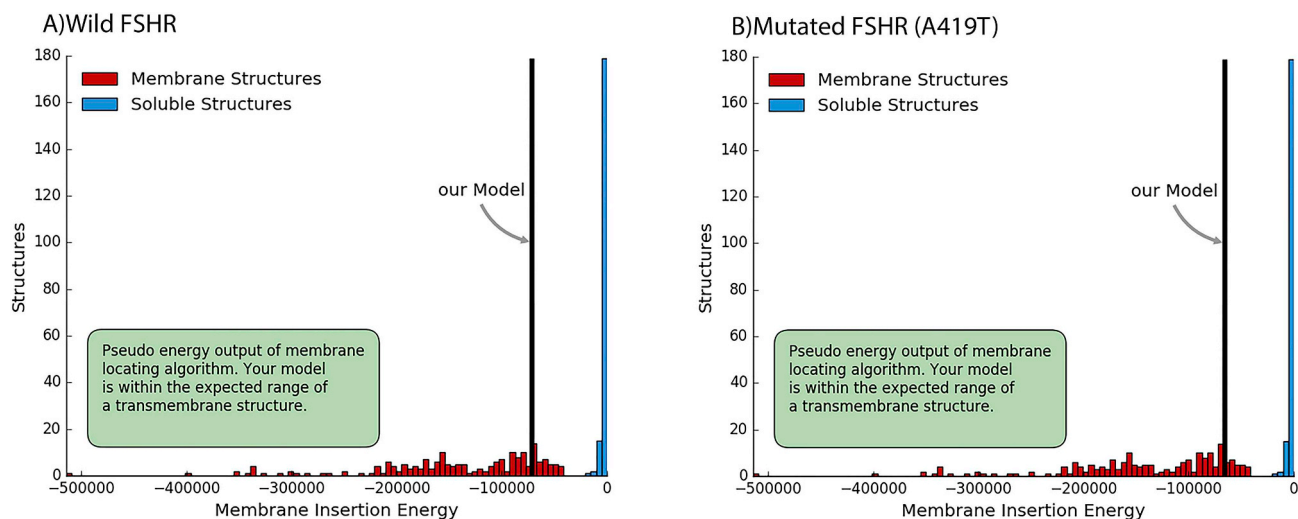


Fig. 1. Membrane insertion energy comparison of wild-type FSHR and A419T FSHR.

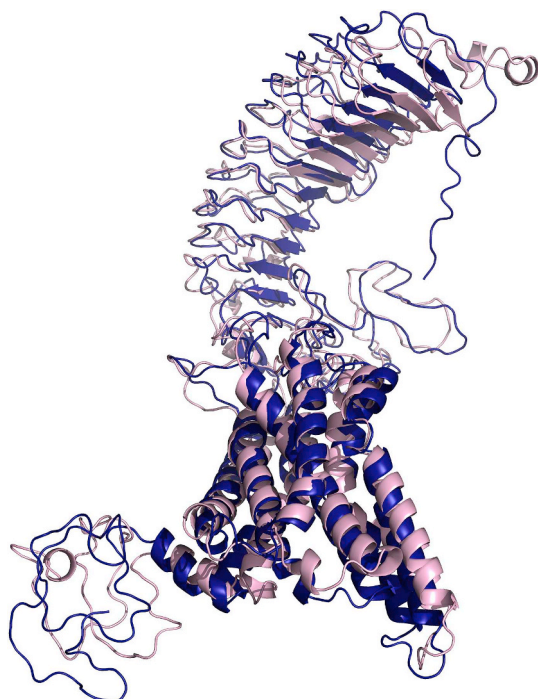


Fig. 2. The superimposed structures of A419 (dark blue) and T419 (pink) of FSHR exposed the subtle differences of the two structures which was mostly evident in the intra-membrane region (serpentine domain, i.e. transmembrane α -helices) of FSHR structure. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

groups near to the meta- or ortho-substitution of the aliphatic hydrocarbon chain yields also in more favorable binding interactions with the receptor. N1-(3-carbamoylbenzyl)-N2-(4-hydroxy-3-methoxyphenethyl) oxalamide is the IUPAC name of our best compound according to chemdraw software.

3.6. Molecular dynamics (MD)

To examine whether the binding of the screened compounds provide a potent interaction with FSHR A419T, we set up a MD study with compound A as the most potent agonist (Fig. 5) and recorded the chemical binding interactions along with other parameters related to

the potency of interactions. The RMSD of ligand and the receptor should be compared after the point of initial semi-equilibrium phase in which the temperature is maintained in 300 K thereafter. The system displayed significant stability with an RMSD fluctuation beginning from 6.4 Å and ending with 6.6 Å. Therefore a total oscillation of 0.2 Å was observed in total 20ns simulation which demonstrate that the system has reached the equilibrium throughout the simulation process and no major conformational changes were disclosed. For a large protein like FSHR with 695 amino acid residues, this amount of fluctuation is very satisfactory and confirms that the *in silico* MD simulation is set up with a valid FSHR structure and resulted authentic outcomes. Correspondingly, the behavior of the ligand was stable with a RMSD of 1.2 Å at the beginning and 1.6 Å at the end of simulation (about 0.4 Å fluctuation indicating that the ligand is tightly bound to the receptor). This low scale of RMSD implies that the pharmacophore modeling and virtual screening were successful in identifying novel and potent agonists for FSHR T419 structure (Fig. 5). The other interesting finding which confirms that the system has reached equilibrium and the FSHR structure is stable, is the RMSF diagram which indicates no fluctuation of more than 4 Å for every protein residues and the basal fluctuation for most part of the protein residues is less than 2 Å (Fig. 7).

Surprisingly, the analysis of the 2D-binding interactions revealed that there were four strong interactions between the ligand and the receptor which last more than 90% of the time of simulation. All of these interactions were Hydrogen bonds which are among the most stable inter-molecular forces (compared with hydrophobic, π - π stacking and other van der Waals forces) and this signifies that the formation of ligand-protein complex is remarkably favorable in terms of enthalpy. Hydrogen bonds between Lys608 and the phenolic -OH, Ser605 and the carbonyl group of one of the amides (the oxamide), Phe353 and the other carbonyl group (of the oxamide) and finally between the amine group of the benzamide ring and Lys513, collaborate to produce these four Hydrogen bonds. Some other strong interactions were also uncovered by the MD simulation. A water bridge was reported 61% of the time of simulation between the phenolic -OH and Val514 and another Hydrogen bond was recorded in 59% of the time (Fig. 6).

The fluctuations of each atom of the ligand was very small (for most atoms of the ligand was less than 1 Å) as proposed by the RMSF diagram of the ligand. A considerable increase in the fluctuation was addressed to the carbonyl group of the benzamide which failed to entail a remarkable interaction with receptor residues. Protein RMSF was analyzed and revealed three major fluctuation surges. First, the initial high variation in the position of atoms which is linked to a natural tendency of the unfolded structure of the protein (the part which is not classified

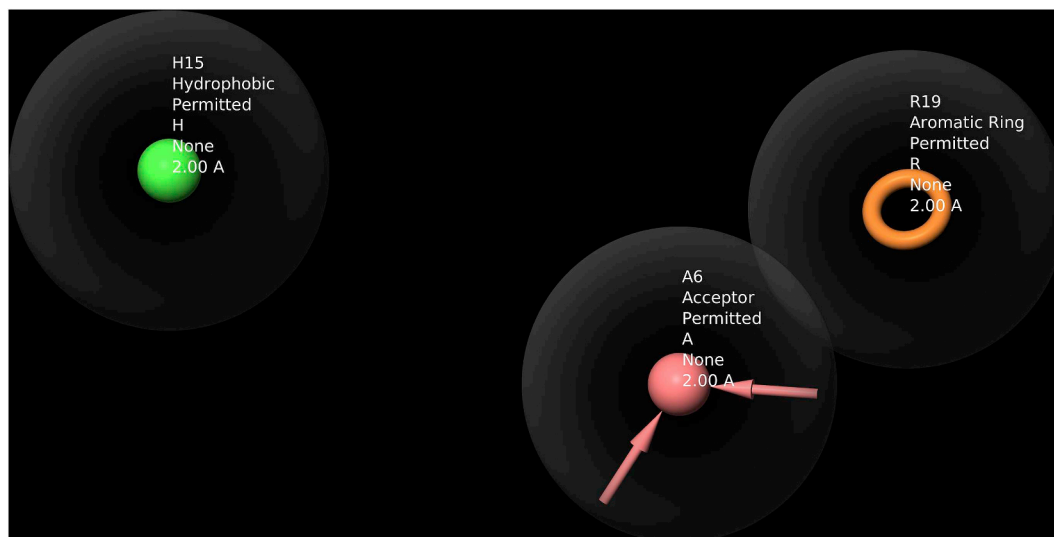


Fig. 3. Three main features of our pharmacophore hypothesis used in virtual screening for identification of novel and potent agonists of FSHR T419.

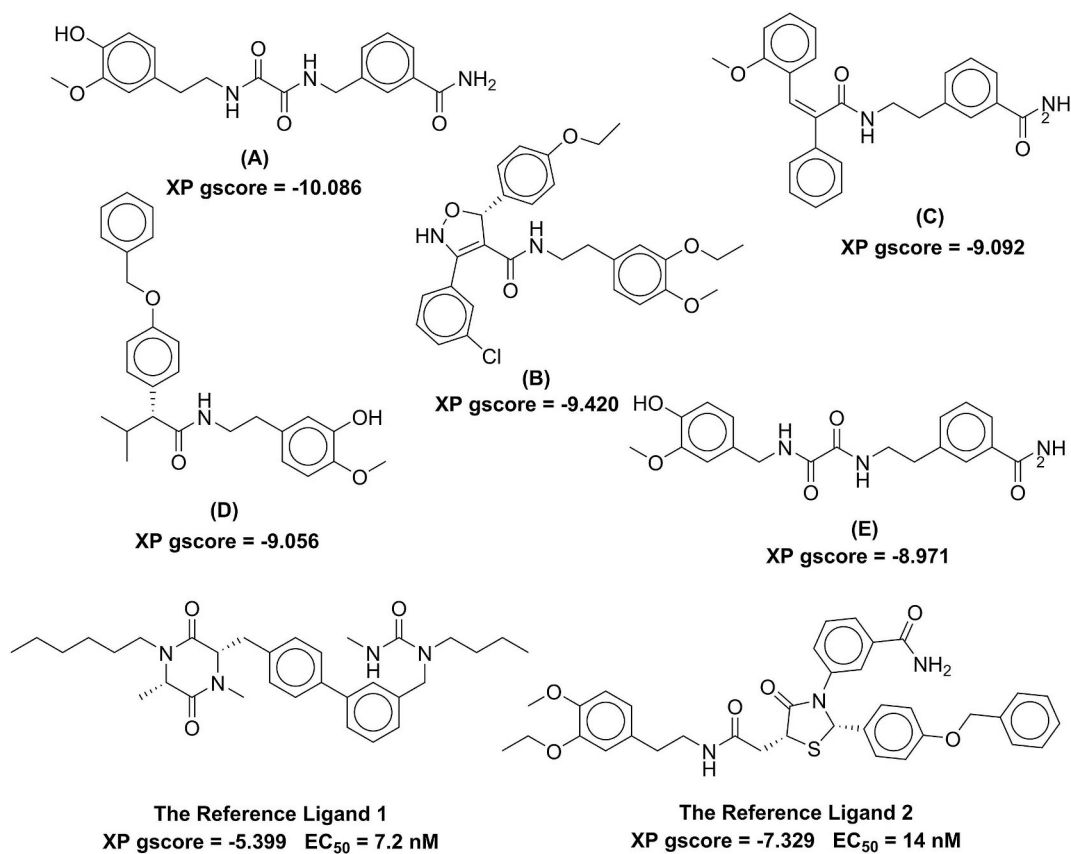


Fig. 4. The pharmacophore features derived from previous pharmacophore hypothesis can be easily seen in the structures of our best virtually screened compounds. Our hit compounds all displayed more negative binding energies in reference to reference ligand structures obtained from previous works (28).

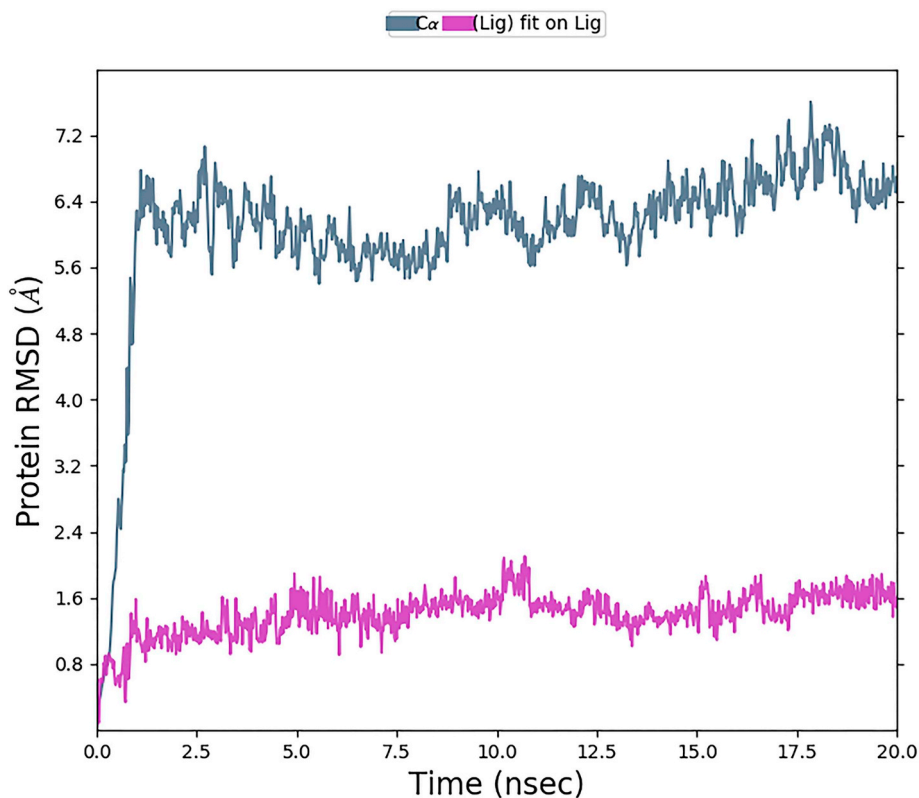


Fig. 5. RMSD of ligand and $C\alpha$ of the protein suggest the overall stability of the system and is consistent with potent interactions between the ligand and receptor.

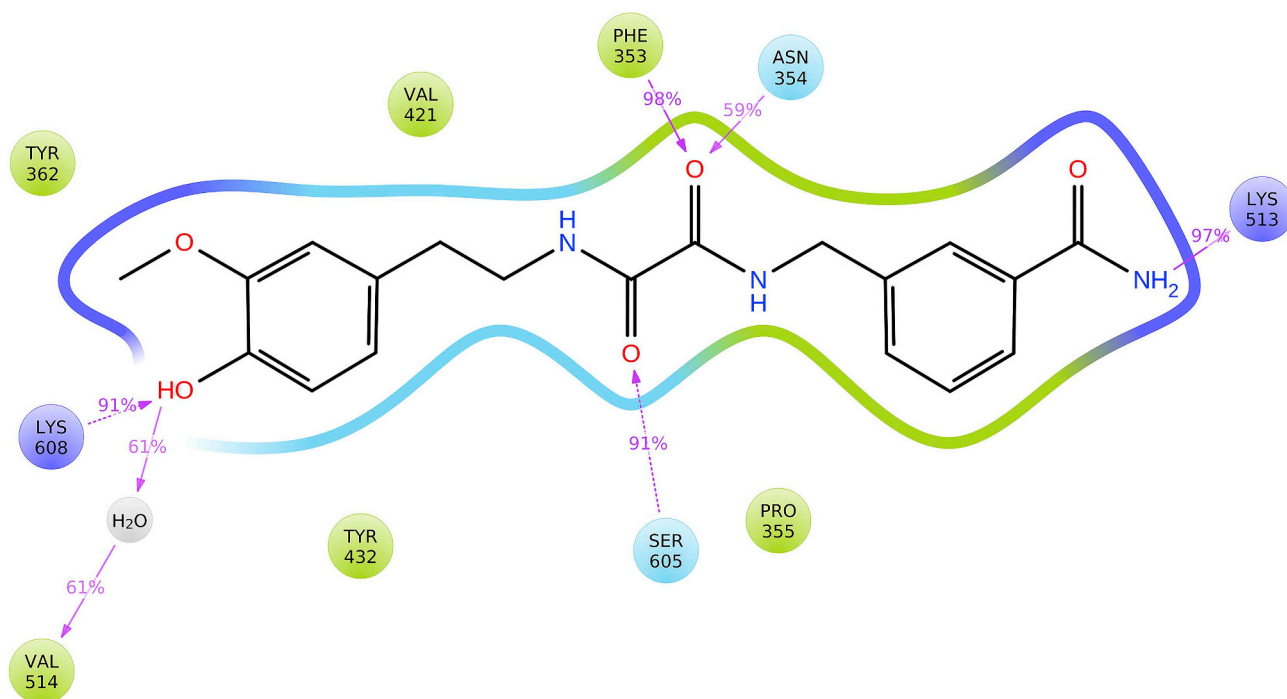


Fig. 6. Ligand-Protein interactions which last more than 30% of the time of simulation are illustrated (Positively charged groups (dark blue), hydrophobic groups (green), polar groups (bright blue), and water (gray)). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

as the common secondary structures like either an α -helix or a β -sheet). The next important fluctuation is located around the middle of protein structure (residues around 300) which is the linker part between the outer β -sheet structure of FSHR and the transmembrane α -helices. This linker part was previously indicated as the hinge region (HinR) of Glycoprotein Hormone Receptors (GPHR) and is responsible for the hormone binding and intramolecular signal transduction [32]. The last major fluctuation corresponds to a turn structure connecting the two terminal α -helices (Fig. 7).

4. Discussion

The current study aimed to investigate the association of missense mutations of *FSHR* gene (A419T and T449I) with female infertility. The results revealed a significant association of A419T with susceptibility to infertility among Iranian women in a dominant hereditary model ($P = 0.005$) but T449I represented a weak association in recessive model of inheritance ($P = 0.03$). According to the genotype-phenotype sub-analysis, significant differences observed among three genotypes of A419T with FSH ($P = 0.01$) and LH ($P < 0.0001$). G allele carriers of A419T had augmented FSH and LH levels. Thus, there may be a remarkable association between G allele carriers of A419T and both FSH and LH levels. Previously it was confirmed that *FSHR* gene polymorphism, is linked with serum LH levels [33]. Our homology modeling and structure comparison indicate a possible diminished FSHR activation in T419 structure which may explain the interesting serum LH levels in patients with A419T SNP. Based on lower activation of FSHR pathway, hypothalamus probably tries to compensate the neuroendocrine balance, by overproduction of GnRH which ultimately increases the serum LH levels. Consistent with our results, high LH levels (> 10 mIU/ml) were considered to increase the susceptibility of individuals for miscarriage and infertility in earlier studies [34].

Moreover, the molecular implications of A419T were investigated. To combat the molecular dysfunctions originated from this mutation, the agonistic structures in Guo paper [23] were surveyed and three pharmacophore features necessary for FSHR agonistic activation were

mapped. Related structures for agonistic activation of T419 FSHR were screened by utilizing this pharmacophore hypothesis and docking-based virtual screening and ultimately the best compound was subjected to a 20 ns MD simulation; the results were gratifying owing to potent interactions of the best compound for binding to T419 structure and low RMSD and RMSF values. The structures identified as possible agonists of FSHR, could be used further in synthesizing new chemical compounds in drug discovery. It should be noted that these agonists might have agonistic effect on A419 and other missense SNPs of *FSHR* gene but their characterization and *in vitro* as well as *in vivo* investigation are necessary to test this hypothesis. Nevertheless, the structures of the virtual screening results of this study, are among the few compounds with predicted agonistic activity on FSHR protein. Hence, they can be used as template for comparison of FSHR agonists and antagonists and further research.

Recombinant human FSH, has remarkably contributed to the treatment of infertility since its first production in 1989 [35]. As recombinant technology is a costly and time-consuming procedure [36], small chemical molecules are drawing attention in medicinal and pharmaceutical chemistry. Additionally, FSH medication requires extensive monitoring by specialists and it is not orally available for patients [37]. Hence, small-molecules are being investigated as oral medications instead of FSH [38]. Our contemporary drug development techniques fundamentally rest upon small-molecule based new chemical entities (NCEs) [39] and in this study we tried to broaden our knowledge about these molecules for FSHR by virtual screening and molecular dynamics.

Since a diminution in cAMP signaling pathway is observed for T419 variant, then why should we quest for a small-molecule agonist structure for reverting to the normal endocrine balance? There are only a handful number of studies which employed small-molecule agonists for bringing back the normal FSHR function by activating the receptor for treatment of infertility in animals or human. This study proposes new agents as potential candidates for future studies to be tested. Although the cAMP levels are lower in patients with A419T SNP, there is not a "lack" of FSH response and there is a continuum of phenotypes by these

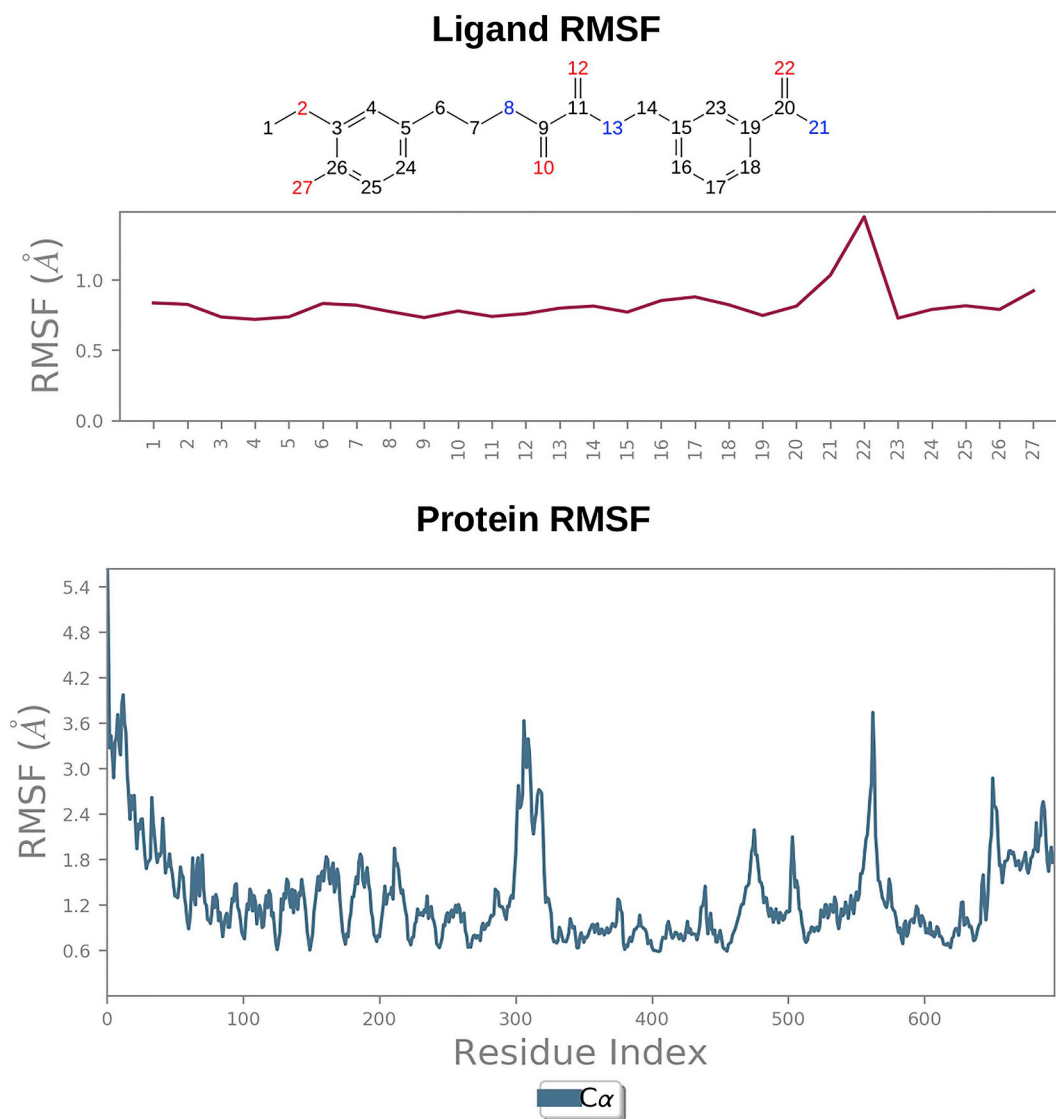


Fig. 7. RMSF analysis of ligand (up) and protein (down) display regions with highest and lowest fluctuation throughout the simulation.

FSHR mutations [9]. As a result, higher activation of the receptor with more potent agonists (than FSH), which is possible for a GPCR structure like FSHR, would be a possible strategy to overcome infertility in these patients.

To the best of our knowledge, this study is the first virtual screening for identification of new potent agonists of FSHR. Although, T419 FSHR structure was used for virtual screening, the established ligand structures obtained in this study, might be potent activator of wild type FSHR and future studies should be introduced to broaden our knowledge about the agonistic and antagonistic structural dependencies of this receptor. MD simulation used in this study substantially supported the agonistic activity of one of the top compound found and due to the remarkable structural similarity with other top ranked virtually screened compounds, it might be assumed that other structures could also be considered as potential FSHR agonists.

How does A419T mutation affect the cellular response to FSH? To explain the possible underlying mechanisms, a closer look at the biochemical structure of FSHR seems necessary. FSH receptor is a member of GPCRs, a group of family of G Protein-Coupled Receptors (GPCRs) [40,41]. GPCRs display three important regions; first an extracellular Leucine-Rich-Repeats (LRR), a hinge region (HinR) and a transmembrane serpentine domain (SD) [32]. The residue 419 in FSHR protein is part of SD and SD is involved in the proper folding of 7TM α -helices in

the membrane and signal transduction; in which activation of this domain provokes the production of cAMP by enhancing the stimulatory action of G_s ; but the details of the interplay between the three functional domains were not fully elaborated [42]. The residue site 419, is in the relaying part of GPCR and consequently a change in the conformation of this part could wreak havoc on the communicating part between FSHR SD and G_s . The current study verified this assumption by two interesting observations. First, a significant conformational change was observed between the T419 and A419 based on RMSD value from validated homology modeling structures. Second, the membrane insertion energy of two structures as assessed with QMEANBrane were slightly different and this instability which is observed in T419 structure might be due to the partially unfavorable structure induced by this mutation.

The activation of GPCRs is a complex process and involves the transition between two or more structures and it is believed that the endogenous ligand, selectively stabilize the active structure and thus allows an augmentation in cellular response to the stimuli [43]. The findings of the present research might shed a leading light on the understanding of agonistic activity on this receptor. For this complexity, four main types of ligand interactions with GPCR were characterized; inverse agonists, antagonists, partial agonists and full agonists. The aim of this paper was to represent new potent full agonists of FSHR

according to T419 structure site based on pharmacophore modeling and selection of the potent agonists as the reference structures for virtual screening (to exclude other types of interactions except full agonistic activity) for treatment of female infertility; however, to be more specific, we only identified the most potent ligands which can strongly interact with A419T missense site. Hopefully, the current findings would be helpful and inspiring enough for future studies to untangle the structural and functional complexity of this receptor and its ligands.

T449I (rs28928870) SNP, which is located in the upper section of the third transmembrane domain of FSHR protein, was also studied in this research. Similar to the A419T, this zone is highly preserved among other GPHR. T449I alters threonine at codon 449 into isoleucine. Data of present study showed a weak difference between the case and control groups in recessive model of inheritance. No study has been conducted extensively on association of T449I with female fertility. Vasseur et al. reported a correlation of T449I missense mutation on OHSS in a Moroccan family [11]. Binder et al., suggested that T449I is a rare mutation which was not observed in patients with OHSS and nor with patients with reduced response to gonadotropin stimulation (which “seems” to contradict Vasseur et al. findings) [44,45]. Indeed, the fact that all individuals in Binder et al. studies in German population, were homozygous for wild-type Thr⁴⁴⁹/Th⁴⁴⁹, does not necessarily mean that there is no relationship with this allele and infertility or OHSS and thus does not belie our findings. On the other hand, polymorphism of some other genes like *CYP19A1* [46] in premature ovarian failure and *IGF2R* [47] in age of menarche and age at natural menopause, have been shown to impact the *FSHR* gene response via an epistatic behavior. As long as T449I mutation was thought to be an activating mutation (with increased FSHR response) [45] and A419T was considered inactivating [9] and the fact that only a weak association is observed for T449I missense mutation in this study (in only recessive model of inheritance), a large epistatic propensity for T449I seems very likely.

Some studies have focused on mutations of FSHR at exon 10 [4]. The most frequent association study of exon 10 was related to the replacement of alanine by threonine at codon 307 and replacement of serine by asparagine at codon 680 [48]. Studies have reported that these two missense SNPs are the risk factors of developing OHSS and reducing ovarian reserve in the infertile females treated by different doses of FSH [49,50]. A419T is situated on the second intracellular loop of FSHR protein. Vasseur et al. have shown that A419T affects the specificity of ligand-receptor binding [11]. As A419T is the substitution of a non-polar amino acid into uncharged polar amino acid in the second intracellular loop of FSHR protein. This zone is highly protected among the families of GPHRs and it plays a major role in performance of receptor and identification of ligand [9]. Doherty et al.'s study demonstrated the association of A419T with infertility and reported that heterozygote individuals were at a higher risk of primary amenorrhea [9]. Our structural results for T419 FSHR structure conforms with Doherty et al.'s findings which supposed that this mutation is inactivating [9]. This is probably due to the structural changes of A419T in FSHR protein folding which decreases the relaying activity of FSHR structure.

By this study, Phe353, Asn354, Ser605, Lys608 and finally Lys513 were identified as the most important residues in the FSHR binding pocket for small molecules. This region is at the intersection of extracellular region with 7TM segment. Unexpectedly, our results clearly indicate that the binding pocket for small molecules is essentially different from those involved with FSH binding which is placed in extracellular region and was studied by Jiang et al., and Fan et al. [4,29]. Other studies involved in small molecule agonists and antagonists for FSHR also lack molecular modeling or X-ray crystallography investigations [51–57]. Nonetheless, prior radiolabeling studies by Straten et al. had confirmed that the binding site of small molecules is different from FSH binding site and they presumed that this binding site should be located in 7TM region [58]. The observation is in accordance with our findings that extracellular region of FSH binding site is not the

same binding site for small molecules; but to be more precise the binding region is at the edge of 7TM region and extracellular domain. Therefore this is the first study which describes the exact potential binding site of small-molecules acting on FSHR.

Pharmacophore modeling indicates three main feature for a FSHR agonist: 1. a hydrogen bond acceptor (which is an oxamide group for our best compound) interacts mainly with Thr603 and Ser605 residues; 2. an aromatic group attached to this hydrogen bond acceptor by one or two carbon spacer and is mainly involved in removing solvent exposure and confinement of the ligand; 3. and finally a hydrophobic phenyl alkyl group in the opposite side of the molecule which provides a π - π stacking bond (presumably with Tyr432 or Tyr362) to stabilize the ligand-protein complex structure. The high percentage of stability of oxamide group's interactions (three interactions which two of them last more than 90% of the time of simulation) with FSHR residues confirms and validates the methodology of pharmacophore modeling and virtual screening.

Pharmacogenetics, the concept of introducing a drug according to gene polymorphisms, has gained much attention in recent years. Patients are categorized into responders and non-responders to a certain drug in pharmacogenetics discipline. Genotyping of individuals with A419T SNP in different populations (especially Iranian population which this study was designed) and applying personalized-medication based on FSHR agonists on this missense site is very promising according to the current MD findings. Considering that T419 structure is inclined to have a lower relaying intracellular signaling activity, agonists providing potent binding to this receptor are of great interest in treatment of these patients.

As infertility is a multifactorial disease, determination of other genetic and environmental factors and their effects on each other requires other studies in other populations. The interactions of other genes on the path of puberty and reproduction might have effects on SNPs investigated in the current study. Recognition of the precise mechanism of SNPs which effect *FSHR* on infertility development might be useful in clinical trials and drug designing. Investigating the association of rs121909661 (A419T) and other key SNPs of *FSHR* with female and male infertility among larger sample sizes in the other populations is highly recommended.

5. Conclusion

A thorough knowledge of which SNPs are involved in the misbehavior of proteins and why these SNPs obstruct the normal function of the proteins is prerequisite for drug discovery and treatment of human illnesses. FSHR was identified as a potential protein having several SNPs demonstrating significant associations with infertility. In the current study, it is disclosed how A419T missense SNP contributes to the disruption of FSHR signalling and demolition of HinR activity, the relaying part of FSHR. The clinical study presented alongside the molecular modeling studies substantially supports this abnormal activity.

Agonistic structures for FSHR might be a potential treatment strategy for infertile women, especially those who demonstrate some kind of dysfunction in the FSHR activity. A virtual screening based on pharmacophore modeling of previously derived potent agonistic structures of FSHR was set up and new compounds were identified with higher potency for this receptor. Molecular dynamics displayed very satisfactory results and confirmed the previous searches for finding new potent agonists.

GPCRs are one of the favorable druggable targets in the field of drug design and many drugs act by manipulating the function of these proteins. Histaminergic, dopaminergic, serotonergic, adrenergic, muscarinic, opioidergic and some prostaglandin, cytokine and hormonal receptors are among the pathways or receptors which GPCR signaling plays a critical role and there are a battery of FDA-approved drugs which selectively target them. Designing various types of receptor

modulation (e.g. partial agonistic or inverse agonistic) is also a matter of consideration in pharmaceutical researches and again these various types of modulations are affirmed for these FDA-approved GPCR drugs [59].

The power of GPCR in treatment of human disorders will be more discernible by the fact that more than a quarter of FDA-approved drugs target directly or indirectly these receptors and together with Ligand-Gated Ion-Channels (LGICs) and Voltage-Gated Ion-Channels (VGICs) they account for approximately 40% of all known drug targets [60,61]. Yet little studies have accomplished related to FSHR small molecule ligands and extraction of agonistic features necessary for its activation. This study can lay the foundation for more studies governing the role of small molecule ligands of FSHR in manipulation of endocrine system and treatment of infertility.

At a glance, in this study, a significant association was found between A419T missense mutation and infertility but the significance of T449I missense mutation in the infertility was weak in the Iranian population. The relationship between the lowered FSHR pathway activation by A419T missense mutation and increased LH and FSH serum levels was discussed (based on hypothalamic GnRH response). Besides, for the first time a virtual screening on FSHR T419 was performed based on previous agonists to investigate promising future drug candidates. In this study, the potential binding site of small-molecules to FSHR, was also explained for the first time. Identification of these novel agonists push our knowledge a little further in the edge of FSHR activity.

Conflicts of interest

The authors have not any conflict of interest to disclose.

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