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In silico characterization of structural and functional impact of the deleterious SNPs on FSHR gene

Dakshina Moorthy Janani, Gopalakrishnan Poornima & Balasundaram Usha*

Department of Genetic Engineering, School of Bioengineering, SRM Institute of Science and Technology, Kattankulathur- 603 203, Tamil Nadu, India

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FSHR is an important gene which plays a major role in the development of secondary sex characteristics and influences the female reproductive cycle by regulating the Follicle Stimulating Hormone. Though this gene and its protein are extensively studied, no attempts have been made yet to methodically analyze the variants in this gene. One of the chief objectives during the analysis of human genetic variation is to distinguish between the Single Nucleotide Polymorphisms (SNPs) that are functionally neutral from those that contribute to the disorder. To predict the possible impact of SNPs on the FSHR structure and function, data were obtained from NCBI (dbSNP and dbVar) and validated manually. Various bioinformatics tools were used to predict the alterations at transcriptional, post transcriptional stages and protein interaction. Around 38 variants reported by NCBI Variation Viewer were sorted by SIFT and 14 of them were reported damaging, 13 were reported to be either benign or damaging by PROVEAN and Panther. From these 13 SNPs, the most damaging (11 SNPs) were modeled using Pymol and the energy difference between the native and mutated structure was calculated by Swiss PDB – Viewer. Based on our analysis, we have reported potential candidate SNPs for the FSHR gene involved in the regulation of ovarian pathophysiology.

Keywords: Computational analysis, Follicle stimulating hormone receptor (FSHR), Ovarian regulation, Single nucleotide polymorphism (SNPs)

Follicle-Stimulating Hormone (FSH) is elemental for normal gametogenesis in humans and mammals[1-3]. In humans, FSH is vital for ovarian development and follicle maturation in females[2] whereas, FSH determines Sertoli cell number and is required for quantitatively and qualitatively normal spermatogenesis in males[3]. FSH action is mediated by a G-protein coupled receptor–FSHR, which is exclusively expressed on granulosa and Sertoli cells[4]. Follicle Stimulating Hormone Receptor (FSHR) is a single-copy gene that has been mapped to the region 2p21-2p16 and spans a region of 54 kb. It consists of ten exons; the first nine exons encode for the large extracellular domain and the trans membrane domain is comprised of the tenth exon[5]. Some structural changes occurring in the "sensitive" portions of the gene may give rise to variations in the amino acid sequence of the protein. This will affect the receptor's functional properties that may be amplifying (activating mutations) or diminishing (inactivating mutations) and have clinical consequences[6].

Single nucleotide polymorphisms (SNPs) are very significant and also a common form of genetic variation in the genome. Though many SNPs have no particular effect on the functionality of the cell and tend to be neutral, some of them are accountable for different reasons that lead to genetic variations affirmative of various diseases[7]. Therefore, they can be of use as biological markers for finding out the disease vulnerability[8]. SNPs tend to manifest approximately every 1000 to 2000 bases. A non-synonymous SNP (nsSNP) is a variation in a single base that occurs in the coding area which accounts for a change in an amino acid of the protein that it encodes. As a consequence of nsSNP protein, functionality alters and the changes in them can result in a phenotypic effect that turns out to be an apparent reason for the pathology of a disorder[9]. Variants which can possibly result in a premature stop were likely to be in association with diseases at about a probability of 2.77%[10].

*Correspondence:
Phone: 044-27452270
E-mail: sundaram.usha@gmail.com
Suppl. data available on respective page of NOPR

In silico prediction tools were used to efficiently filter out deleterious SNPs from the available data on the variants. It helps us in prioritizing SNPs of any functional significance thus narrowing the investigation of the disease-causing mutations which can contribute to disease susceptibility. Bioinformatics protein prediction tools can be used for finding out the structure and the structural variations that might occur due to the occurrence of SNPs. Here, we have attempted to predict the possible structural conformation of FSHR protein, identification of damaging variants and to find out possible changes that can occur in the protein.

Materials and Methods

Database of SNPs

The detailed information of the FSHR gene was obtained from Online Mendelian Inheritance in Man (OMIM) and Entrez Gene on National Centre for Biological Information (NCBI) websites. The database of NCBI for SNPs (dbSNP) found at https://www.ncbi.nlm.nih.gov/snp was used to retrieve SNP information such as rs ID, functional class, variation class, clinical significance and its allele frequency. Entrez Gene accessed at http://grch37.ensembl.org/index.html gives the information on chromosome location and coordinates in base pairs, consequence type and amino acid coordinates. The list of rs IDs for further downstream analysis was obtained from NCBI's Variation Viewer (https://www.ncbi.nlm.nih.gov/ variation/view/), since this table gives varied options for classification.

The particulars on the FSHR protein was obtained from Uniprot Knowledgebase accessed from http://www.uniprot.org/[11]. The mRNA accession number (NM_000145 and NM_181446) and the protein accession number (NP_000136 and NP_852111) of both the variants were retrieved. The native 3D structure and FASTA sequence for mutation modeling was obtained from Protein Data Bank (PDB) accessed at http://www.rcsb.org/pdb/ home/home.do[12].

Functional analysis prediction of non-synonymous SNPs

Functional effect of non-synonymous SNPs obtained from Entrez Gene was predicted using various *in silico* algorithms. The nsSNPs predicted to be damaging were characterized to be of high-risk nsSNPs and they were used for predictive studies. The tools used for characterizing the damaging effects are SIFT, PolyPhen-2, PROVEAN, SNP & GO and PANTHER.

Sorting Intolerant From Tolerant (SIFT) (http://sift.jcvi.org/) was used to observe the effect of amino acid (a.a.) substitution on protein function[13]. SIFT predicts the deleterious effect of nsSNPs by using algorithms that align closely related sequences using PSI-BLAST and predicts the damage using the degree of conservation of sequences. PolyPhen-2 (http://genetics.bwh.harward.edu/pp2) is the shortened form of polymorphism phenotyping version 2[14]. We used PolyPhen to understand the probable impact of an amino acid substitution on structural and functional properties of the protein by taking in to account physical and comparative approaches. Protein Variation Effect Analyzer (PROVEAN) (http://provean.jcvi.org/index.php) tool was used for predicting if an amino acid substitution has an effect on protein's biological function and on further filtering sequence variants to find out nonsynonymous variants[15]. PROVEAN tool helps us to gather pair-wise sequence alignment scores and enables us to generate pre-computed predictions. Protein Analysis Through Evolutionary Relationships (PANTHER) (http://www.pantherdb.org/tools/ csnpScoreForm.jsp) was used to predict the damaging effect of nsSNPs[16]. PANTHER calculates the substitution position-specific evolutionary preservation (sub-PSEP) score, to predict the a.a. substitution that will cause any functional effect using Hidden Markov Model (HMM)[17].

Predicting effect of mutation on protein stability

I-Mutant 2.0 (http://folding.uib.es/i-mutant/i-mutant2.0.html), a neural network based tool, predicts the variations in the stability of the protein upon mutation[18]. This tool automatically predicts protein stability changes upon single site mutations. Prediction can be done using a protein structure or sequence from databases. The FASTA sequence of the protein from PDB with the accession number 4AY9 was retrieved to predict the mutational effect on protein stability. The output obtained is in the form of the protein stability change upon mutation and Gibbs-free energy change ($\Delta\Delta G$).

Also, the energy changes caused by the deleterious SNPs on the stability of a protein were checked by using Pymol and Swiss PDB[11]. When the energy of a

mutated protein was lesser than that of the native protein the respective SNP was considered to have a deleterious effect on the function of the protein.

Prediction of Post-translational modification sites in FSHR

Glycation sites of ε amino groups of lysine residues were predicted using a NetGlycate 1.0 server (http://www.cbs.dtu.dk/services/NetGlycate/). In NetGlycate a score of >0.5 was considered glycated[19]. Phosphorylation sites were predicted using a NetPhos2.0 server (http://www.cbs.dtu.dk/services/NetPhos/). In NetPhos2.0, serine, threonine and tyrosine residues with a score of >0.5 were considered to be phosphorylated[20]. Ubiquitylation sites were predicted using UbPred (www.ubpred.org). In Ubpred, lysine residues with a score of ≥0.62 were considered ubiquitylated. Sumoylation sites were predicted using SUMO-plot (http://www.abgent.com/sumoplot). For SUMO-plot high probability motifs having a score of 0.5 were considered sumoylated[21].

Predictions of protein-protein interactions

STRING is a database that consists of detected and predicted protein-protein interactions[22]. The interactions include direct physical and indirect functional associations. The data develops from computationally predicted interactions, experimentally predicted interactions, knowledge transfer between organisms and information derived from other databases. This database was used to predict possible interactions of FSHR with other proteins.

Results

SNP dataset from dbSNP

The dbSNP database contains both validated and non-validated polymorphisms but it is the most extensive database. The allelic frequency of most of the nsSNPs of INSR has been recorded here and it was validated by manual curation. Totally 29117 SNPs were present in the FSHR gene for *Homo sapiens* as accessed on 24th August 2017. When these SNPs were classified according to their multiple allele frequencies and given a heterozygosity score of 40-50, only 795 SNPs were remaining. Then the list of rs IDs remaining was validated using dbSNP-Q and it reported 357 validated SNPs[23]. Also, the list of SNPs obtained from Entrez gene was merged with this list and the duplicates were removed. The remaining list of SNPs was used for input.

The 795 variants can be classified based on the variation type as 126 indels and 669 single nucleotide polymorphisms. Among the 669 SNPs, 26 belonged to the functional class 3′UTR, 7 SNPs were in 5′UTR, and 633 were in the intronic region. On the whole (SNPs and Indels), the mutations can be classified according to their function as 32-3′UTRs, 7- 5′UTRs, 736- introns, and 6-missense mutations.

Functional effects of nsSNPs on FSHR as predicted by different tools

SIFT characterizes the effect of amino acid substitution on protein function by using sequence homology. From SIFT results (Table 1), a total of 9 nsSNPs were predicted as damaging (score of

Table 1 — Functional Effects of nsSNPs as predicted by *in silico* tools

SNP	Amino acid change	SIFT	PROVEAN	POLYPHEN2	PANTHER
rs6166	S680N	TOLERATED		benign	
rs121909660	R573C	DAMAGING	Deleterious	probably damaging	probably damaging
rs28928871	D567N	DAMAGING	Deleterious	probably damaging	probably damaging
rs121909664	I545T	DAMAGING	Deleterious	probably damaging	probably damaging
rs121909662	P519T	DAMAGING	Deleterious	probably damaging	probably damaging
rs28928870	T449I	DAMAGING	Deleterious	probably damaging	probably damaging
rs121909663	T449A	DAMAGING	Deleterious	probably damaging	probably damaging
rs121909661	A419T	DAMAGING	Deleterious	probably damaging	probably damaging
rs6165	A307T	TOLERATED	Neutral	benign	probably begign
rs121909658	A189V	DAMAGING	Deleterious	probably damaging	probably damaging
rs111883853	R162K	TOLERATED	Neutral	benign	probably damaging
rs121909659	I160T	DAMAGING	Deleterious	benign	probably begign
rs121909665	S128Y	TOLERATED	Neutral	probably damaging	probably damaging
rs115030945	L8F	TOLERATED	Neutral	benign	probably damaging

0.00-0.04) by SIFT and 5 nsSNPs were simulated to be tolerated (score of 0.08-0.55). The rest had no records found.

PROVEAN calculates the effects of the variants on the respective biological function of the protein based on sequence homology. This algorithm functions mainly based on the primary sequence for prediction and other tools perform a similar task with the structure. PROVEAN has the advantage over other tools that it can predict a large number of substitutions and does not require structures. The scores of PROVEAN are classified as "deleterious" below a certain upper limit (here −2.5) and "neutral" above it. The list of SNPs was submitted manually to the "dbSNP rsIDs" page to calculate the PROVEAN score. The 9 SNPs claimed to be damaging by SIFT was also predicted as deleterious by PROVEAN and the 5 tolerated SNPs was predicted to be neutral (Table 1). Among the 9 deleterious nsSNPs mutations, R573C and P519T were predicted as highly deleterious with PROVEAN scores of −6.55 and −6.97, respectively.

PolyPhen estimates the difference between the variant scores using the algorithm where the homologues of the input sequences are identified *via* BLAST and PSIC scores are calculated for every variant and the difference of 0.339 is accepted as detrimental. The protein accession number of FSHR (NP_000136) and the amino acid change in each position, corresponding to each of the 14 nsSNPs (output of SIFT) were submitted as a batch query. Table 3 encapsulates the results obtained from the PolyPhen server. A PSIC score difference was allocated to categorize SNPs as benign and damaging. "PolyPhen-2: scores are assigned as 0.000 (most probably benign) to 0.999 (most probably damaging)." Nine of the 14 nsSNPs were predicted as "damaging". These nsSNPs were also predicted to be deleterious by the SIFT.

Protein ANalysis THrough Evolutionary Relationships (PANTHER) characterizes probable functional consequence of amino acid variation by means of HMM-based statistical modelling and also family multiple sequence alignments and phylogenetic trees. PANTHER analysis of FSHR nsSNPs was executed in order to add another layer of refinement in SNPs characterization. PSEP score was used to categorise the variants and it measures in million years (my). The longer an amino acid is preserved the higher the disturbance on its functional effect. This method of scoring is more sensitive than the previous version of subPSEP scoring. All the nsSNPs predicted as damaging by SIFT is also predicted to be "probably damaging" by PANTHER (Table 3). Also, two nsSNPs predicted as tolerant are also predicted to be probably damaging; rs111883853 and rs115030945.

Prediction of stability changes due to nsSNPs on FSHR

I-Mutant uses a neural network based algorithm for the analysis of protein stability alterations by taking into account the single-site mutations. I-Mutant also assigns the alterations with scores for free energy alterations which may be calculated with the FOLD-X energy based web server. A precision of about 93% is achieved by assimilating the FOLD-X estimations with those of I-Mutant and threshold of −1.5 Kcal/mol is considered to predict an SNP to be destabilized. Forty six nsSNPs were considered as destabilized with DDG values by I-Mutant. Finally, we selected 13 significant nsSNPs because they were predicted to be deleterious by PROVEAN, PolyPhen, and SIFT programs and showed decreased structural stability following analysis by I-Mutant (Table 2).

Effect of nsSNP variants on post translation modifications

Various *in silico* tools were used to study how nsSNP bring about changes in the protein structure and interaction due to post-translation modifications. NetGlycan predicted that 5 residues undergo

Table 2 — I Mutant table showing the stability changes due to polymorphisms

Pos	WT	NEW	Stability	RI	pH	T
680	S	N	Decrease	8	7.0	25
573	R	C	Decrease	4	7.0	25
567	D	N	Decrease	7	7.0	25
545	I	T	Decrease	5	7.0	25
519	P	T	Decrease	2	7.0	25
449	T	I	Decrease	7	7.0	25
419	A	T	Decrease	9	7.0	25
307	A	T	Decrease	2	7.0	25
189	A	V	Increase	6	7.0	25
162	R	K	Decrease	8	7.0	25
160	I	T	Decrease	7	7.0	25
128	S	Y	Decrease	7	7.0	25
8	L	F	Decrease	1	7.0	25

Table 3 — Post Translation modification sites

Glycation		Serine		Tyrosine		Threonine		Ubiquitation		Sumoylation	
Position	Score	Pos	Score	Pos	Score	Pos	Score	Residue	Score	Pos.	Score
191	0.6417	78	0.598	110	0.921	56	0.564	294	0.62	K598	0.8
199	0.6664	164	0.977	303	0.936	249	0.584	349	0.67	K349	0.61
293	0.5389	232	0.893	322	0.754	329	0.582			K74	0.5
318	0.5736	248	0.996	330	0.552	331	0.633				
680	0.4242	273	0.597	335	0.825	555	0.743				
		312	0.542	429	0.915	658	0.896				
		313	0.972	684	0.721						
		321	0.946								
		347	0.846								
		427	0.661								
		564	0.996								
		566	0.986								
		660	0.772								

glycation and the position 680 which is a site of glycation was predicted to be benign by Polyphen 2 software. According to NetPhos 13 serine, 6 threonine and 7 tyrosine residues undergo phosphorylation (Table 3). Protein sequence with the mutational position associated with nsSNPs was incorporated into the amino acid residue were submitted as input to UbPerd, 2 residue positions in the sequence had a score above 0, and these sites are predicted to have possible chances of ubiquitination in a mutated protein structure. Similarly, SUMOplot predicted 4 different positions with likely chances for sumoylation (Table 3). FTsite evaluated and gave 3 different ligand binding sites of FSHR gene (Table 3). None of the genomic variants predicted to be deleterious /damaging was found to be present on ligand binding sites; this may be due to the fact that the complete protein structure has not yet been elucidated.

Protein-Protein interactions

The protein interaction analysis carried out by STRING v10 showed that FSHR closely interacts with FSHB (Follicle stimulating hormone, beta polypeptide), PTH (Parathyroid hormone), BRD2 (Bromodomain containing 2), POMC (Proopiomelanocortin), MC2R (Melanocortin 2 receptor), TSHB (Thyroid stimulating hormone, beta), CGA (Glycoprotein hormones), INSL3 (Insulin-like 3 (Leydig cell)), ADRB2 (Adrenoceptor beta 2), LHB (Luteinizing hormone beta polypeptide). By exploring the KEGG database, it was known that the FSHR protein is involved in the cAMP signalling pathway, neuroactive ligand-receptor interaction pathway and ovarian steroidogenesis (Suppl. Fig. 1).

Discussion

Follicle Stimulating Hormone and its Receptor (FSHR) play very important roles in women's reproductive life[2]. FSHR inactivating mutations may cause infertility, primary or secondary amenorrhea and premature ovarian failure (POF), whereas activating mutations may induce ovarian hyperstimulation syndrome (OHSS) as an effect of exogenous FSH administration, or due to a spontaneous onset.

'Activating mutations' either make FSHR respond non-specifically to some tropic hormones (*e.g.*, TSH) or makes it highly responsive to FSH, making it dynamic even in the absence of the ligand. 'Inactivating mutations' relegate the receptor's function by either disrupting FSH signal transduction or altering the formation of the receptor-ligand complex.

The FSHR gene was mapped on the chromosome number 2p21 in human[24]. The *FSHR* gene is 54 kb in size and encompasses ten exons and nine introns[25].

The extracellular domain (ECD) is encoded by all the nine exons and a part of exon 10. Exon 10 is huge, encodes the transmembrane domain (TMD), and the intracellular domain (ICD) of the receptor apart from the ECD. This C-terminal part of the ECD is known as the hinge region and is responsible for the signal specificity[26]. The TMD is made up of seven α -helices which are spaced out and interconnected through three extracellular (ELs) and three intracellular loops (ILs). The ICD is predominantly coupled to a G protein that is responsible for initiating a cascade of intracellular events leading to specific biological effects of the ligand[21].

The ECD is a large hydrophilic domain with 349 amino acids and consists of the Leucine-rich repeat which is responsible for protein-protein interactions. Here, as predicted by the tools, the mutation at amino acid (a.a.) position 189 (Ala189Val, exon 7) completely abolished the FSH - stimulated cAMP production by granulosa cells[27]. The (a.a.) at position 191 is a highly conserved glycosylation site. The mutation at position 160 where isoleucine is changed to threonine leads to a condition where the primary follicles do not mature. The follicles developed normally up to the small antral stage and showed a disruption at the further stage this may be due to altered surface targeting[28].

The transmembrane domain, a highly conserved region of FSHR is composed of 264 (a.a). It is characterized by seven hydrophobic motifs of 20-25 a.a. forming transmembrane α helices connecting three extracellular and three intracytoplasmic loops of 10–23 (a.a). Many of the mutations in this region cause LH-independent, male-limited precocious puberty[29,30]. A heterozygous substitution Thr449Ile leads to Ovarian Hyper Stimulation Syndrome (OHSS) during pregnancy and can lead to complications[31]. A mutation at site 545 is predicted to be damaging by the *in silico* tools and it is to be noted that this site lies within a highly conserved stretch of amino acids (a.a. 535-a.a. 555) region responsible for the production of cAMP and estradiol[23]. Another heterozygous mutation Arg573Cys failed to show maturing follicles or corpus leuteum in the ovary[24,32].

Besides these mutations, FSHR gene polymorphisms at specific sites - 307 and 680 alter the ligand binding capacity to FSH and alter the response. The polymorphisms may persuade the FSHR protein responsiveness to exogenous FSH also, and finally, alter the effectiveness of *in vitro* fertilization (IVF) treatment in addition to the probability of developing a severe OHSS due to superovulation. Moreover, specific sites of the FSHR gene may show polymorphisms that influence FSHR protein responsiveness to exogenous FSH and have clinical relevance when FSH or HMG are administered.

Conclusion

The *in silico* prediction of a functional single nucleotide polymorphism is supportive to modern genetic analysis. Thus, here we have tried to analyse the FSHR gene and its products using computational biology since the resulting knowledge after an *in silico* analysis has assisted the genetic association study and reduced the cost of genotyping. This study demonstrates the first complete investigation that identifies the functional SNPs in FSHR gene using sequence- and structure-based homology algorithms. Of the 357 nsSNPs, 9 were predicted deleterious by SIFT, 9 by PolyPhen, 9 by PROVEAN and 11 by PANTHER. The structural consequences of S143P, G258V, and Y414D variants on FSHR are defined in the form of solvent accessibility, electrostatic interaction, energy calculation, and multiple alignment conservation. Altered FSHR function due to genetic variations and mRNA expression may possibly play an important role in ascertaining susceptibility to complex disorders. Furthermore, protein-protein interaction pathway helps us to understand its interaction with other proteins and thus its biological function. Finally, the thus predicted nsSNPs will not only help in deriving genotype-phenotype relations but to certain level give information about the molecular basis for diverse inter-individual response to certain drugs.

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