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Functional characterization of two naturally occurring mutations $V^{221}G$ and $T^{449}N$ in the follicle stimulating hormone receptor

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ABSTRACT

Naturally occurring mutations in follicle stimulating hormone receptor (FSHR) affect the receptor function. Here, we characterized two such previously reported mutations, $V^{221}G$ and $T^{449}N$, in the extracellular domain and transmembrane helix 3, of FSHR, respectively. Functional studies with the $V^{221}G$ mutant demonstrated an impairment in FSH binding and signaling. Validation of X-ray crystallography data indicating the contribution of FSHR specific residues in the vicinity of V^{221} to contribute to FSH-FSHR interaction was carried out. *In vitro* mutational studies showed that these residues are determinants of both FSH binding and FSH induced signaling. Analysis of the $T^{449}N$ mutation revealed that it results in an increase in FSH binding and high cAMP response at lower doses of FSH. A marginal hCG induced and no TSH induced cAMP production was also observed. These findings corroborated with the clinical manifestations of primary amenorrhea ($V^{221}G$) and spontaneous ovarian hyperstimulation syndrome ($T^{449}N$) in women harbouring these mutations.

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1. Introduction

Follicle stimulating hormone (FSH), binds to and activates its cognate G-protein coupled receptor, the follicle stimulating hormone receptor (FSHR). FSHR is expressed on the granulosa cells of the ovary in females (Sanford and Batten, 1989) and Sertoli cells of the testes in males (Fletcher and Reichert, 1984). The signaling pathways that are triggered by the FSH-FSHR interaction are essential for folliculogenesis in females and maintenance of spermatogenesis in males (Dias et al., 2002). FSHR belongs to the subfamily of glycoprotein hormone receptors (GPHRs) along with the common receptor for luteinizing hormone/choriogonadotropin (LH/CG receptor) and the receptor for the non-gonadotropin thyroid-stimulating hormone (TSH receptor) (Salesse et al., 1991). These receptors are characterized by the presence of a large extracellular domain (ECD) made up of N-terminal leucine rich repeats followed by a hinge region which connects the ECD to the serpentine transmembrane domain (TMD). The leucine rich repeats mediate ligand binding with high affinity and specificity (Vassart et al., 2004). Following FSH binding to the leucine rich repeats of FSHR, a sulfotyrosine (sTyr) binding pocket is created in FSH into which the sulfated Tyr^{335} residue from the hinge region is inserted, resulting in FSHR activation (Jiang et al., 2012). The TMD of FSHR mediates signal transduction. The seven α-helices of the transmembrane domain are connected to each other by means of three extracellular and three intracellular loops. A short C-terminal tail is present in the cytoplasm.

Several naturally occurring point mutations in FSHR manifesting into pathophysiological conditions have been reported (reviewed by Tao and Segaloff, 2009; Desai et al., 2013; Siegel et al., 2013). The site of the receptor mutation, that is, the domain in which the mutation is located, can be attributed to the function that gets affected, as a result, in its life cycle. For example, mutations in the ECD, $I^{160}T$ (Beau et al., 1998), $A^{189}V$ (Aittomäki et al., 1995), $D^{224}V$ (Touraine et al., 1999), $P^{348}R$ (Allen et al., 2003), result in impairment in hormone binding ability and subsequent diminished

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receptor function. One such heterozygous mutation, $V^{221}G$, has been reported in a patient with primary amenorrhea (Nakamura et al., 2008). The residue affected here, V^{221}, is also implicated in forming a hydrophobic pocket for FSH binding as reported by Jiang et al., in 2012. The FSH-$FSHR_{ED}$ crystal structure reported by Jiang et al. (2012) indicates a wide-ranging hormone-receptor interface leading to a more compact hormone-receptor interaction. Other than the primary site of hormone binding (Fan and Hendrickson, 2005), several FSHR specific residues especially V^{221} (non conserved across the GPHRs) contribute to this. The presence of a naturally occurring mutation at this site along with its involvement in FSH binding as shown by crystallography studies signifies that Valine at 221 position is a crucial residue for FSH-FSHR interaction. Since the crystal structure is a partial one and provides only a molecular glimpse of the FSH-FSHR interacting sites, experimental validation of the residues predicted to be involved is necessary.

On the other hand, the interhelical ionic locks between the helices of the transmembrane domain keep the receptor in a constrained, and thus, inactive state. Several reports have shown that mutation in the TMD not only can result in weakening of these ionic interactions (Montanelli et al., 2004b) but also promiscus activation of the receptor by the non-cognate ligands hCG and TSH (Vasseur et al., 2003; Montanelli et al., 2004b; De Leener et al., 2006). This can result in a life threatening complication known as ovarian hyperstimulation syndrome (OHSS) due to exogenous FSH administered in the setting of assisted reproduction program (iatrogenic OHSS). OHSS can also occur due to overproduction of hCG in the first trimester of pregnancy or high TSH levels as a result of hypothyroidism during pregnancy (spontaneous OHSS). One such novel heterozygous mutation $T^{449}N$ in the transmembrane helix 3 (TMH3) has been identified by our group in a patient exhibiting symptoms of sOHSS (Chauhan et al., 2015). Functional characterization of this naturally occurring mutation is necessary in order to establish genotype-phenotype association.

Structure-function relationship studies by site directed mutagenesis approach is useful in characterizing the functional consequences of mutations in FSHR. In the present study, the effect of the naturally occurring mutations $V^{221}G$ and $T^{449}N$ on FSH receptor function was determined.

We also identified the FSHR residues in close proximity of V^{221} shown to be involved in interaction with FSH from the FSH-FSHR complex structure. Hence, we proposed to check for the contribution of the residues K^{146}, E^{197}, I^{222} and K^{243} in conferring functional specificity to FSHR. This was done by swapping with the corresponding residues from the closely related LH/CGR as these closely related GPHRs can coexist in mature ovarian granulosa cells. The effect of the naturally occurring and induced mutations on FSH receptor expression, FSH binding ability and the signaling response generated thereafter was investigated. This provided insights into the contribution of the above mentioned residues in FSH receptor function.

2. Materials and methods

2.1. Residues of FSHR selected for site directed mutagenesis

The naturally occurring and induced mutations chosen for *in vitro* functional studies are listed in Table 1.

2.1.1. Naturally occurring mutations

Mutant FSHR $V^{221}G$ was selected to identify the cause of impairment in the mutant receptor in the patient with primary amenorrhea. The woman with primary amenorrhea harbouring the $V^{221}G$ mutation in FSHR (Nakamura et al., 2008) had moderately developed secondary sexual characteristics, normal sized ovaries but small antral follicles. Her serum FSH levels were not high and serum estradiol levels were low but detectable. Only administration of high doses of human menopausal gonadotropin (hMG) triggered follicular growth. This indicated that the follicles had the potential to respond to high doses of gonadotropin and develop to the antral stage. The ovarian response to high dose hMG stimulation suggested an FSHR dysfunction of her ovaries.

The $T^{449}N$ substitution was selected for delineation of the effect of the mutation in the receptor in the patient presenting symptoms of sOHSS during her first pregnancy (Chauhan et al., 2015). She had a normal thyroid profile and normal levels of hCG β but elevated estradiol levels of 12,927 pg/dl (normal range 188–2497 pg/dl). Multiloculated cystic lesion was revealed by ultrasonography. Ascites and bilateral pleural effusion were also observed. Magnetic resonance imaging confirmed the features of OHSS.

2.1.2. Induced mutations

The 3-D model of FSH-$FSHR_{ED}$ complex as reported by Jiang et al., 2012 (PDB ID: 4AY9) was visualized using Discovery studio 3.5 (Accelrys, Inc., San Diego, CA, USA). Residues in close proximity to V^{221} of FSHR which interacted with FSH were selected for further studies. The ECD mutants $K^{146}N$, $E^{197}S$, $V^{221}G$, $V^{221}K$, $I^{222}T$ and $K^{243}R$ were subjected to Protein interactions calculator (PIC) server analysis (Tina et al., 2007). Mainly the hydrophobic, ionic, main chain-main chain and main chain-side chain interactions that differed between the WT receptor and the mutants were studied. All the mutants including $T^{449}N$ were also subjected to SIFT (Sorting Intolerant from Tolerant) analysis which predicts the propensity of amino acid substitutions to affect protein structure, and hence function, prior to carrying out functional validation (Sim et al., 2012).

2.2. Construction of FSHR mutants

The substitution mutants were generated by site-directed mutagenesis as mentioned in Dupakuntla et al. (2012). hFSHR cloned into pSG5 vector was used as the template for mutagenesis (Dias et al., 2010). DNA sequencing was carried out to confirm the mutations. The plasmid DNA extraction of mutant constructs for transfection was then carried out using a midi-prep kit (Sigma, St. Louis, MO, USA).

2.3. Transient transfections in CHO cell line

Chinese hamster ovary (CHO) cells were used as the transfection host. CHO cells were obtained from the Cell Repository at the National Centre for Cell Science, Pune, India, and were maintained in DMEM-F12 (Gibco; Invitrogen, Carlsbad, CA, USA) supplemented with 5% fetal bovine serum (Gibco; Invitrogen, Carlsbad, CA, USA) and antibiotics. Transient transfection of wild type FSHR and the substitution mutants was performed using Lipofectamine and Plus reagents (Invitrogen, San Diego, CA, USA) as per manufacturer's protocol. All the assays were performed 48 h post transfection. Cells transiently transfected with empty vector pcDNA 3.1 + which did not encode FSHR served as the negative control.

2.4. Determination of FSHR expression by western blotting

Western blotting was performed to determine total FSHR expression in transfected cells. 1.5×10^5 of CHO cells were seeded per well in a 24 well plate. Twenty four hours post seeding, cells were transfected with 500 ng of WT or mutant FSHR plasmid constructs diluted in Opti-MEM (Invitrogen, San Diego, CA, USA) using Lipofectamine and Plus reagents. The two naturally occurring mutations, $V^{221}G$ and $T^{449}N$ were heterogyzous in nature. Hence,

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