Mutations and polymorphisms in FSH receptor: functional implications in human reproduction

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Abstract

FSH brings about its physiological actions by activating a specific receptor located on target cells. Normal functioning of the FSH receptor (FSHR) is crucial for follicular development and estradiol production in females and for the regulation of Sertoli cell function and spermatogenesis in males. In the last two decades, the number of inactivating and activating mutations, single nucleotide polymorphisms, and spliced variants of *FSHR* gene has been identified in selected infertile cases. Information on genotype–phenotype correlation and *in vitro* functional characterization of the mutants has helped in understanding the possible genetic cause for female infertility in affected individuals. The information is also being used to dissect various extracellular and intracellular events involved in hormone–receptor interaction by studying the differences in the properties of the mutant receptor when compared with WT receptor. Studies on polymorphisms in the *FSHR* gene have shown variability in clinical outcome among women treated with FSH. These observations are being explored to develop molecular markers to predict the optimum dose of FSH required for controlled ovarian hyperstimulation. Pharmacogenetics is an emerging field in this area that aims at designing individual treatment protocols for reproductive abnormalities based on *FSHR* gene polymorphisms. The present review discusses the current knowledge of various genetic alterations in *FSHR* and their impact on receptor function in the female reproductive system.

Reproduction (2013) **146** R235–R248

Introduction

Follicle-stimulating hormone (FSH), luteinizing hormone (LH), and thyroid-stimulating hormone (TSH) are glycoproteins secreted by the anterior pituitary. They are heterodimers comprising a common α -subunit and a hormone-specific β -subunit that contributes to receptor-binding specificity (Pierce & Parsons 1981). FSH and LH are under the regulation of gonadotropin-releasing hormone (GNRH) and in turn regulate gonadal functions in males and females by activating their cognate receptors. FSH receptors (FSHR) along with other glycoprotein hormone receptors (LH/CGR and TSHR) form a subgroup of G-protein-coupled receptors (GPCR). FSH binds to its receptors expressed on the membrane of granulosa cells (GCs) in the ovary and Sertoli cells in the testis to bring about folliculogenesis (Richards & Midgley 1976) and spermatogenesis (Nieschlag *et al.* 1999) respectively.

Sprengel *et al.* (1990) cloned the cDNA of FSHR from rat testicular cells. Later, the mapping of *FSHR* gene on the chromosome number 2p21 in human was reported (Rousseau-Merck *et al.* 1993). The *FSHR* gene is 192 kb in size and comprises ten exons and nine introns (Gromoll *et al.* 1994). The first nine exons encode the

N-terminal part of the extracellular domain (ECD). Exon 10 is large and encodes the C-terminal part of the ECD (hinge region), the transmembrane domain (TMD), and the intracellular domain (ICD) of the receptor. The TMD comprises seven α -helices, interconnected through three extracellular (ELs) and three intracellular loops (ILs). The ICD is predominantly coupled to a Gs protein that is responsible for initiating a cascade of intracellular events leading to specific biological effects of the ligand. The X-ray crystal structures of FSH complexed with the ECD of FSHR (Fan & Hendrickson 2005, Jiang *et al.* 2012) have immensely contributed to understanding the interaction of FSH with its receptor at the molecular level. However, it does not provide information on ligand-induced activation of the receptor leading to steroidogenesis.

Fsh β gene (Kumar *et al.* 1997) and *Fshr* gene (Dierich *et al.* 1998) knockout female mice were found to be infertile with thin uteri and streak ovaries. But in both cases the male mice were fertile, although there was a decrease in testicular size and partial spermatogenic failure. These observations clearly indicate that FSH is absolutely essential for female fertility. In recent years, naturally occurring mutations in the *FSH* and *FSHR* genes have been reported. *In vitro* functional characterization

of these mutants has provided valuable information with respect to genotype–phenotype correlation in the case of both FSH and FSHR. Studies of FSHR mutants also provide an opportunity to understand the extracellular and intracellular events, such as hormone binding, signal transduction, and receptor trafficking, in response to FSH action. The *FSHR* gene is highly polymorphic in nature and three single nucleotide polymorphisms (SNPs) at positions –29, 307, and 680 have been extensively studied by various investigators using different ethnic populations both in males and females. Currently, FSH is used as a therapeutic in reproductive medicine, and published data on SNPs of *FSHR* has provided a new dimension with an emphasis on ‘pharmacogenetics of FSH action’ (Laan *et al.* 2012). Alternatively spliced variants of the *FSHR* gene have been reported and some of them affect fertility, mainly in females (Song *et al.* 2002, Gerasimova *et al.* 2010).

Our group is working on understanding the structure–function relationship of FSHR and one of the approaches employed toward this is studying the naturally occurring mutations, SNPs, and spliced variants of *FSHR* in women enrolled in IVF and infertility clinics. In the present review, we have made an attempt to provide an update on *FSHR* gene mutations and their effect on hormone–receptor interaction and signal transduction. We have also reviewed the usefulness of studying *FSHR* polymorphisms in developing a molecular marker for predicting ovarian response in women who are treated with FSH. Toward the end, the available information on the spliced variants of *FSHR* is also included.

Naturally occurring mutations of the *FSHR* gene

The presence of naturally occurring inactivating/activating mutations in the *FSHR* gene has been found to be a rare phenomenon when compared with the occurrence of mutations in the *LHR* and *TSHR* genes (Dufau *et al.* 1995). Studies suggest that the probable reason for the rare occurrence of the *FSHR* gene mutations could be due to the virtual absence of disease characterized by the deficiency or hypersecretion of FSH. Moreover, the FSHR mutations leading to severe forms of phenotype causing infertility are not inherited by the next generation. Therefore, screening of subjects with the phenotypes related to lack or hypersecretion of the FSH might remain unnoticed. The loss-of-function mutations in FSHR have been found in women with ovarian dysgenesis (ODG), primary amenorrhea, and secondary amenorrhea. Such mutations are also reported in men with small testes and impaired spermatogenesis (Table 1). On the other hand, gain-of-function mutations have been identified in women with ovarian hyperstimulation syndrome (OHSS) and in a single case of a hypophysectomized man (Table 2). The positions of the mutations reported so far in the ECD

and TMD of FSHR have been shown in Fig. 1. For depicting the mutations in the ECD, the X-ray crystal structure of FSH complexed with ECD-FSHR (Jiang *et al.* 2012) is used. Whereas, for depicting the mutations in the TMD, the homology model generated for TMD-FSHR based on the rhodopsin receptor structure is used (Okada *et al.* 2004).

FSH action on its target cells involves initial binding of hormone to the receptor followed by signal transduction. These two events of hormone binding and signal transduction are generally studied by *in vitro* experiments while characterizing the naturally occurring mutations of *FSHR*.

Inactivating mutations

Identification of an inactivating mutation in the *FSHR* gene was first reported by Aittomäki *et al.* (1995) in women with ODG. In Finland, a large genetic survey of women with ODG was carried out. ODG is characterized by elevated gonadotropin levels and primary amenorrhea with normal karyotype. By genetic analysis, the locus segregating the disease was mapped on chromosome number 2p, which corresponds to the locus for both FSHR and LHR. As the males in these families were unaffected and their androgen levels were found to be normal, the mutation screening for only the *FSHR* gene was carried out. The observed mutation was at position 189 substituting Ala to Val in the ECD of FSHR. This mutation was found to be homozygous in affected women. *In vitro* functional studies with the mutant receptor expressed in MSC-1 cells showed reduced ligand-binding ability probably due to reduced expression on the cell membrane. However, the ligand-binding affinity was not affected. FSH-induced cAMP was abolished in the mutant receptor when compared with the WT receptor. Amino acids from 189 to 193 (AFNGT) in FSHR are conserved across all the glycoprotein hormone receptors and also across all the species. N¹⁹¹ is the proposed glycosylation site and the mutations at position 189 may have an effect on glycosylation and probably on trafficking of mature receptor onto the membrane (Davis *et al.* 1995).

Although this mutation in a homozygous condition has impaired fertility in females, the affected male siblings homozygous for this mutation showed a variable degree of spermatogenic failure. None of the five men investigated was found to be azoospermic. Only one was infertile, while two of them had fathered children (Tapanainen *et al.* 1997). The phenotype observed in women and men was similar to the one shown by *Fshr* knockout female and male mice respectively. Another mutation in the ‘AFNGT’ motif (Asn¹⁹¹Ile) was reported in a healthy woman in a heterozygous condition (Gromoll *et al.* 1996). *In vitro* studies with this mutation showed impairment in FSH-induced cAMP production and this defect could also be due to lack of glycosylation,

Table 1 Inactivating mutations of *FSHR* in men and women: phenotype of the affected individual and findings from *in vitro* functional studies with mutants when compared with WT receptor.

S. no.	Nucleotide change (exon number)	Amino acid change (region)	Phenotype of subjects	Effect of mutation/s on (<i>in vitro</i> analysis)			References
				Cell surface expression of FSHR	FSH binding	FSH-induced cAMP	
1	c.566C>T (exon 7) Homozygous mutation	p.Ala ¹⁸⁹ Val (ECD)	Hypergonadotropic ovarian dysgenesis Variable degrees of spermatogenic failure	Affected	Decreased	Decreased	Aittomäki <i>et al.</i> (1995) Tapanainen <i>et al.</i> (1997)
2	c.573A>T (exon 7) Heterozygous mutation	p.Asn ¹⁹¹ Ile (ECD)	Fertile woman with normal ovarian function	ND	ND	Abolished	Gromoll <i>et al.</i> (1996) Gromoll <i>et al.</i> (2002)
3	c.662T>G (exon 8) Heterozygous mutation	p.Val ²²¹ Gly (ECD)	Primary amenorrhea	ND	ND	ND	Nakamura <i>et al.</i> (2008)
4	c.1043C>G (exon 10) Hemizygous mutation with inherited/ <i>de novo</i> microdeletion or <i>de novo</i> gene conversion	p.Pro ³⁴⁸ Arg (ECD hinge region)	Primary amenorrhea with increased serum FSH levels	ND	Abolished	Abolished	Allen <i>et al.</i> (2003)
5	c.479C>T (exon 6) c.1717C>T (exon 10) Compound hetero- zygous carrier of both mutations	p.Ile ¹⁶⁰ Thr (ECD) p.Arg ⁵⁷³ Cys (IL3)	Secondary amenorrhea with increased serum FSH levels	Affected ND	Decreased Similar	Decreased Decreased	Beau <i>et al.</i> (1998)
6	c.671A>T (exon 7) c.1801C>G (exon 10) Compound hetero- zygous carrier of both mutations	p.Asp ²²⁴ Val (ECD) p.Leu ⁶⁰¹ Val (EL3)	Primary amenorrhea with increased serum FSH levels	Affected ND	Decreased Similar	Abolished Decreased	Touraine <i>et al.</i> (1999)
7	c.566C>T (exon 7) c.1255G>A (exon 10) Compound hetero- zygous carrier of both mutations	p.Ala ¹⁸⁹ Val (ECD) p.Ala ⁴¹⁹ Thr (TMD helix 2)	Primary amenorrhea with increased serum FSH levels	Affected ND	Decreased Similar	Abolished Abolished	Doherty <i>et al.</i> (2002)
8	c.1555C>A (exon 10) Homozygous mutation	p.Pro ⁵¹⁹ Thr (EL2)	Primary ovarian failure with FSH levels	Affected	Minimal	Abolished	Meduri <i>et al.</i> (2003)
9	c.1231A>T (exon 10) Heterozygous mutation	p.Ile ⁴¹¹ Asn (TMD helix 2)	PCOS	ND	ND	Similar	Orio <i>et al.</i> (2006)
10	c.1760C>A (exon 10) Mutation on single allele with unbalanced translocation	p.Pro ⁵⁸⁷ His (TMD helix 6)	Primary amenorrhea	ND	ND	Abolished	Kuechler <i>et al.</i> (2010)
11	c.1723C>T (exon 10) Homozygous mutation	p.Ala ⁵⁷⁵ Val (TMD helix 6)	Primary amenorrhea with hypergonadotropic hypogonadism	ND	ND	ND	Achrekar <i>et al.</i> (2010)

ECD, extracellular domain; EL, extracellular loops; IL, intracellular loops; ND, not determined; TMD, transmembrane domain.

Table 2 Activating mutations of *FSHR* in men and women: phenotypes of the affected individuals and findings from *in vitro* functional studies with mutants when compared with WT receptor.

S. no.	Nucleotide change (exon number)	Amino acid change (region)	Phenotype of subjects	In vitro functional analysis				References
				Cell surface expression of FSHR	FSH binding	Basal (constitutive activity)	cAMP levels	
1	c.1700A>G (exon 10) Heterozygous mutation	p.Asp ⁵⁶⁷ Gly (IL3)	Hypophysectomized man with undetectable amount of serum FSH	ND	ND	1.5-fold higher	Similar upon FSH stimulation	Gromoll <i>et al.</i> (1996)
2	c.1699G>A (exon 10) Heterozygous mutation	p.Asp ⁵⁶⁷ Asn (IL3)	Women with sOHS	Decreased cell surface expression	Similar	Threefold higher	Similar upon FSH stimulation, increase upon hCG and TSH stimulation	Smits <i>et al.</i> (2003)
3	c.1292A>T (exon 10) Heterozygous mutation	p.Asn ⁴³¹ Ile (EL1)	Normal spermatogenesis, suppressed serum FSH	Reduction cell surface expression	Decreased	Lower	Higher upon FSH stimulated when receptor expression is normalized	Casas-González <i>et al.</i> (2012)
4	c.1346C>T (exon 10) Heterozygous mutation	p.Thr ⁴⁴⁹ Ile (TMD helix 3)	Women with sOHS	Similar	Similar	Similar	Similar upon FSH stimulation, increase in hCG stimulation, no effect by TSH	Vasseur <i>et al.</i> (2003)
5	c.1345A>G (exon 10) Heterozygous mutation	p.Thr ⁴⁴⁹ Ala (TMD helix 3)	Women with sOHS	Twofold increase receptor expression	ND	2.5-fold higher	Similar upon FSH stimulation, increase in response to hCG and TSH	Montanelli <i>et al.</i> (2004)
6	c.1634T>C (exon 10) Heterozygous mutation	p.Ile ⁵⁴⁵ Thr (TMD helix 5)	Women with sOHS	77% cell surface expression	Similar	130% increase	Increase upon FSH, hCG, and TSH stimulation	De-Leener <i>et al.</i> (2006)
7	c.383C>A (exon 5) Heterozygous mutation	p.Ser ¹²⁸ Tyr (ECD)	Women with sOHS	36% cell surface expression	ND	Similar	Increased sensitivity to hCG, no response upon TSH stimulation	De-Leener <i>et al.</i> (2008)

ECD, extracellular domain; EL, extracellular loops; IL, intracellular loops; ND, not determined; sOHS, spontaneous ovarian hyperstimulation syndrome; TMD, transmembrane domain.

Figure 1 Positions of naturally occurring activating and inactivating mutations in FSHR (A) X-ray crystal structure of human FSH complexed with ECD of human FSHR (PDB ID: 4ay9; Jiang *et al.* (2012)). α - and β -subunits of FSH are shown in yellow and green color respectively. ECD of FSHR (in slate blue) shows the presence of leucine-rich repeats. Inset shows hydrophobic interaction of V²²¹ of FSHR with P⁴² and A⁴³ of β -subunit of FSH. (B) Homology model of TMD of FSHR constructed based on the crystal structure of bovine rhodopsin receptor (PDB ID: 1U19; Okada *et al.* 2004). H1–H7 are the helices that are interconnected by extracellular and intracellular loops. In both A and B, amino acids shown in orange and red correspond to inactivating and activating mutations of FSHR respectively.

resulting in a poor surface expression of the receptor (Gromoll *et al.* 2002). Recently, it has been reported that the 'AFNGT' motif plays an important role in maintaining the structural integrity of the receptor (Casas-González *et al.* 2012). In spite of the presence of mutation at position 191, this woman became pregnant, suggesting that the heterozygous mutations of *FSHR* do not affect functioning of the receptor (Gromoll *et al.* 1996).

Recently, a heterozygous mutation at Val²²¹Gly position in the ECD of FSHR has been identified in women with primary amenorrhea (Nakamura *et al.* 2008). Interestingly, this woman responded to human menopausal gonadotropin (hMG) treatment. *In vitro* functional characterization of this mutant has not been reported so far. X-ray crystal structural of FSH complexed with the ECD of FSHR suggests that Val²²¹ of FSHR is present at the hormone binding site and is in close proximity (<5 Å) with P⁴² and A⁴³ of β -subunit of FSH (Fig. 1). This mutation may be affecting the hormone binding affinity, and the increased ligand concentration (hMG used in this case) is possibly required for receptor activation. On the other hand, the mutation at Pro³⁴⁸Arg from the hinge region of the ECD was detected in a woman with primary amenorrhea. Functional analysis revealed that this mutation abolished both binding and signaling of the receptor (Allen *et al.* 2003). As in both the above-mentioned studies the level of mature receptor expression on plasma membrane was not analyzed, the effect of the mutations on receptor conformation and trafficking cannot be assessed.

There are three different cases of the presence of compound heterozygous mutations reported in different

women. In a woman with secondary amenorrhea, mutations at Ile¹⁶⁰Thr and Arg⁵⁷³Cys positions were detected. The other two cases were of primary amenorrhea where the mutation reported was at Asp²²⁴Val, Leu⁶⁰¹Val, Ala¹⁸⁹Val and Ala⁴¹⁹Thr. Both these women developed high plasma gonadotropin levels, contrasting with normal-sized ovaries and the presence of antral follicles. Functional studies revealed that mutations located in the ECD affect receptor trafficking, thereby affecting ligand binding and subsequent signaling, whereas mutations at Arg⁵⁷³Cys, Leu⁶⁰¹Val, and Ala⁴¹⁹Thr did not impact greatly on the ligand-binding ability of the receptor but did, however, show reduced cAMP production (Beau *et al.* 1998, Touraine *et al.* 1999, Doherty *et al.* 2002). But, when Arg⁵⁷³ was replaced by Ala, the plasma membrane expression was observed to be severely affected, suggesting that the nature of amino acid substitution and location of the mutation are important and are observed to be independent determinants of FSHR function (Zarinan *et al.* 2010).

Meduri *et al.* (2003) reported a novel mutation in the EL2 of FSHR at the position Pro⁵¹⁹Thr. This mutation has been observed in a homozygous condition in women with primary amenorrhea. Histological studies revealed a markedly enhanced number of (24.2 ± 4.6) primary follicles in the small ovaries. This mutation completely abolished the ligand binding and cAMP production *in vitro*, due to the defect in the trafficking of the receptor. Therefore, it can be concluded that Pro⁵¹⁹ is probably a prerequisite for the FSHR trafficking and receptor function. Thus, along with ECD, ELs also seem to play an important role in ligand binding and receptor trafficking.

Apart from inactivating mutations identified in the ECD, a number of mutations in the helices of the TMD have also been reported so far. A mutation at Ile⁴¹¹Asn position was detected in women with polycystic ovary syndrome (PCOS; Orio *et al.* 2006). On the other hand, the mutations at Pro⁵⁸⁷His and Ala⁵⁷⁵Val were observed to lead to more adverse phenotypes such as primary amenorrhea (Achrekar *et al.* 2010, Kuechler *et al.* 2010). However, *in vitro* studies on the effect of the mutations in TMD on receptor trafficking have not yet been reported so far.

Genotype–phenotype association studies and *in vitro* functional characterization of these inactivating mutations of FSHR have provided valuable information about the contribution of the mutated residues and the corresponding region in hormone receptor interaction and signal transduction. In case of certain mutations, the defect is at the level of maturation of the receptor which is responsible for its trafficking onto the cell surface. Recently, efforts have been made to rescue the trafficking defective mutation to the cell surface using a pharmacopone (Org41841) by Janovick *et al.* (2009). This drug was observed to bind to the FSHR at a site other than the FSH binding site and it increased the membrane

expression of mutant FSHR (A¹⁸⁹V) associated with misrouting and endoplasmic reticulum retention. Such studies need to be undertaken in future, which will form the basis for developing treatment modalities in some cases of reproductive abnormalities due to non-functioning of the receptor.

Activating mutations

Activating or gain-of-function mutations in the *FSHR* gene have been reported in both men and women. However, the number of cases reported is few when compared with the incidences and severity of the phenotype reported with activating mutations of the *LHR* gene (Meehan & Narayan 2007).

The first activating mutation was reported in a hypophysectomized, hypogonadotropic man (Gromoll *et al.* 1996). Although the serum gonadotropin levels were undetectable, he had normal testis volume and semen parameters. He had also fathered three children with testosterone treatment. The mutation was identified at Asp⁵⁶⁷Gly position in the IL3 of FSHR. This mutant exhibited ligand-independent constitutive activity when tested *in vitro*. It was reported that Asp⁵⁶⁷ is conserved across other glycoprotein receptors. The mutation in the corresponding residues in TSHR and LHR has also been reported. A mutation at Asp⁶¹⁹Gly in TSHR was reported in cases with thyroid hyperfunctioning adenoma (Russo *et al.* 1996) and Asp⁵⁶⁴Gly in LHR was reported in cases of pseudoprecocious puberty (Laue *et al.* 1995). Interestingly, the first activating mutation in a female was identified at the same residue (567) of FSHR. The Asp⁵⁶⁷ was found to be mutated to Asn in a woman with spontaneous OHSS (sOHSS). The mutant receptor displayed threefold higher constitutive activity when studied *in vitro* and also responded to hCG and TSH treatment (Smits *et al.* 2003). Apart from Asp⁵⁶⁷Gly mutation, there is another activating mutation at Asn⁴³¹Ile reported in an asymptomatic man (Casas-González *et al.* 2012). *In vitro* experiments demonstrated decreased cell surface expression of the mutant receptor. Further, the agonist-induced desensitization and internalization were found to be markedly altered.

All the activating mutations reported in women till date are associated with sOHSS. There are two cases of sOHSS where the Thr⁴⁴⁹ in the TMD was substituted. In one case, the mutation was at Thr⁴⁴⁹Ile (Vasseur *et al.* 2003) and in the second case it was Thr⁴⁴⁹Ala (Montanelli *et al.* 2004). Both the mutations were heterozygous in condition and displayed decreased hormone binding specificity of the receptor as these mutant receptors also responded to hCG. Substitution of Thr to Ala was observed to give constitutive activity to the receptor and was also observed to be nonspecifically responding to both hCG and TSH, whereas in the case of mutation at Thr⁴⁴⁹Ile, no constitutive activity was observed and the mutant receptor was not responding to TSH.

The mutation at Ile⁵⁴⁵Thr, also from the TMD, was detected in a woman with sOHSS. When tested, this mutation was found to cause cross-sensitivity of the receptor to both hCG and TSH together. The mutant receptor was also observed to be constitutively active. It was interesting to note that the symptoms of sOHSS were observed in this patient in the first trimester of pregnancy with normal hCG levels. The examination of an other seven women with sOHSS and high hCG or TSH levels indicated the presence of normal *FSHR* genotype. This suggests that, for these seven patients, sOHSS results from the natural promiscuous stimulation of WT FSHR by very high levels of hCG or TSH (De-Leener *et al.* 2006). It is worth noting that most of the germ line mutations in *FSHR* causing sOHSS were identified in the TMD (remote to the hormone binding site) of the FSHR. These mutations were observed to cause a non-specific response to hCG and TSH while displaying constitutive activity.

So far, there is only one report on an activating mutation (Ser¹²⁸Tyr) from the ECD observed in a case of sOHSS (De-Leener *et al.* 2008). In contrast to the other mutations, this mutation in the ECD did not show any constitutive activity but exhibited higher affinity and sensitivity toward hCG but not to TSH. Subsequently, the extensive site-directed mutagenesis study at position 128 demonstrated that the increase in the sensitivity to hCG selectively is not a consequence of the loss of serine residue, as some substitutions were found to be neutral (Ser to Ala/His), whereas some substitutions were observed to be sensitive to both hCG and TSH (e.g. Ser to Ile/Val).

In general, it is observed that the gain-of-function mutations in the *FSHR* gene can cause a phenotype even when present in the heterozygous condition with dominant inheritance whereas the loss-of-function mutations are mostly transmitted recessively and give a phenotype only when present in the homozygous or compound heterozygous condition. Although studies were carried out to detect mutation in diseases such as ovarian tumors, megalotestes, precocious puberty, and twin pregnancies, no change in the *FSHR* gene was observed. This suggests that the phenotypes associated with the activating mutations of *FSHR* are still unclear in both men and women.

SNPs of the *FSHR* gene

Various SNPs in the coding and non-coding region (> 1300 SNPs) of the *FSHR* gene have been identified in diverse populations. Of the total of eight polymorphisms present in the coding region, six are non-symptomatic and the other two polymorphisms present at position p.Thr³⁰⁷Ala (rs6165) and p.Asn⁶⁸⁰Ser (rs6166) are most extensively studied to assess the response of the receptor to FSH stimulation. The polymorphism at position 307 is located in the hinge region of the ECD whereas the polymorphism at position 680 is present in the ICD of the

receptor. As both the polymorphisms are present in the same exon 10, they are observed to be in strong linkage disequilibrium. As the frequency distribution in various populations revealed that the Thr³⁰⁷-Asn⁶⁸⁰ and Ala³⁰⁷-Ser⁶⁸⁰ isoforms are predominant, most of the association studies focused on the polymorphism at position 680 alone. These two isoforms were believed to be present as potential glycosylation/phosphorylation sites of FSHR as Asn⁶⁸⁰ introduces a consensus sequence for glycosylation which in turn may play a role in receptor trafficking and Ser³⁰⁷ may contribute as a phosphorylation site, which might be involved in receptor turnover (Davis *et al.* 1995). Other than the polymorphisms in the coding region, Wunsch *et al.* identified the two polymorphisms at positions -29 and -114 in the 5' UTR of the *FSHR* gene. These two polymorphisms are also linked due to their close proximity with each other. SNP in the -29 position (rs1394205) has been observed to be present in the viral E26 transformation-specific sequence (c-ETS-1) transcription factor binding site (Wunsch *et al.* 2005).

Association of FSHR gene polymorphisms with ovarian response

As FSH plays a central role in stimulating follicular growth, it is used for controlled ovarian stimulation during IVF protocol and for various other infertility treatments in both females and males. Although similar stimulation protocols are used in IVF, the ovarian response to exogenous FSH varies widely ranging from poor to hyper-responsive. Many parameters, such as age and diminished ovarian reserve (Klingman & Rosenwaks 2001) and serum AMH levels (Nardo *et al.* 2009), have been used as a marker to predict the ovarian response. However, determining the dose of FSH to attain optimum response is one of the ongoing challenges in the field of infertility management in IVF clinics. By far, polymorphisms in the *FSHR* gene are most studied in relation to ovarian response in various populations and findings are summarized in Table 3.

Perez-Mayorga *et al.* (2000) demonstrated for the first time the association of higher serum FSH levels with the Ser⁶⁸⁰ allele in women undergoing IVF. This paper served as a real breakthrough in signifying the role of *FSHR* gene polymorphisms as a genetic marker to predict ovarian response. Studies on the functional characteristics of these two isoforms were reported by various investigators. The *in vitro* functional analysis of the Thr³⁰⁷-Asn⁶⁸⁰ and Ala³⁰⁷-Ser⁶⁸⁰ isoforms suggested that the FSH binding and cAMP production are similar for both these isoforms (Tilly *et al.* 1992, Minegishi *et al.* 1994, Simoni *et al.* 1999, Sudo *et al.* 2002). Response of these isoforms to TSH stimulation was also characterized in HEK-293 cells and was observed to be alike (Ryan *et al.* 2007). Therefore, the molecular mechanism by which both these polymorphisms might be altering the ovarian response is still unclear. Significantly higher

basal FSH levels and lower estradiol production upon hCG stimulation were associated with Ser⁶⁸⁰ allele in studies conducted in different ethnic groups (Sudo *et al.* 2002, de-Castro *et al.* 2004, Behre *et al.* 2005, Greb *et al.* 2005). Recently, a meta-analysis carried out by Morón & Ruiz (2010), Altmäe *et al.* (2011), and Yao *et al.* (2011) suggested that the Ser⁶⁸⁰ allele is the only promising marker available to be used in clinical setup to predict the ovarian response to FSH stimulation.

Nevertheless, in the above studies, the significant associations of Ser/Ser genotype with total amount of FSH administered, number of follicles, and mature oocytes were not observed when compared with Asn/Asn genotype alone or in combination with Asn/Ser genotype at position 680. In a study reported by de-Castro *et al.* (2004), although the percent of poor responders were significantly higher in women with Ser/Ser genotype, the other clinical parameters, such as peak E₂ levels, number of follicles and oocytes, and amount of exogenous FSH, were observed to be similar in all the three genotypes at position 680 of FSHR. Additionally, it was noted that in a systematic randomized control study, women with either Ser/Ser or Asn/Asn genotype with 150 or 225 IU/day of FSH dose resulted in a similar number of follicles and retrieved oocytes, fertilization rate, and clinical pregnancy (Behre *et al.* 2005). Furthermore, there are reports from different ethnic origins from The Netherlands (Laven *et al.* 2003, Klinkert *et al.* 2006) and UK (Mohiyiddeen *et al.* 2012), which did not report any association of the polymorphism at position 680 with poor ovarian response.

On the other hand, in the study by Greb *et al.* (2005), the number of antral follicles was observed to be significantly greater in women with Ser/Ser genotype (22.6 ± 1.3) when compared with Asn/Asn genotype (17.8 ± 1.1). Similarly, the systematic study of subjects with ODG, poor responders, and good responders demonstrated that subjects with Ser/Ser genotype generate a significantly higher amount of estradiol and higher number of follicles and oocytes when compared with subjects with Asn/Asn genotype (Loutradis *et al.* 2006). Daelemans *et al.* (2004) also reported that women with the Ser⁶⁸⁰ allele were at risk of showing hyper-response when compared with the Asn⁶⁸⁰ allele. Similarly, our group has observed that although not significant, 50% of the subjects (three of six subjects) with p.Ser⁶⁸⁰Ser genotype developed OHSS (Achrekar *et al.* 2009a).

The association of polymorphism at position 307 with ovarian hyper-response was reported in an Indian population. Almost 85% of the subjects (six of seven subjects) with Ala³⁰⁷Ala genotype were found to be significantly associated with iatrogenic OHSS. These subjects developed OHSS when stimulated with significantly lower amount of exogenous FSH and demonstrated significantly higher levels of estradiol before and on the day of hCG stimulation (Achrekar *et al.* 2009a). As mentioned earlier, Ala at 307 is linked with the Ser⁶⁸⁰

Table 3 Studies on association of the polymorphism in the *FSHR* gene with ovarian response in women undergoing the IVF program.

Polymorphism	Subjects screened (n)	Outcome of the studies	Reference
p.Asn ⁶⁸⁰ Ser	161	Women with Ser/Ser and Asn/Ser genotype showed significantly higher basal FSH levels and required significantly higher FSH dose for ovarian stimulation when compared with Asn/Asn genotype	Perez-Mayorga <i>et al.</i> (2000)
		Peak estradiol levels, number of follicles, and oocytes were similar in women with all three genotypes	
	58	Women with Ser/Ser genotype were significantly associated with increased exogenous FSH and decreased estradiol levels on the day of hCG administration	Sudo <i>et al.</i> (2002)
		The number of oocytes retrieved was similar in all three genotypes	
	102	Ser/Ser genotype displayed significantly higher basal FSH levels and higher number of IVF cycles	de-Castro <i>et al.</i> (2004)
		The peak estradiol levels, number of follicles, and oocytes were similar in all three genotypes	
	93	Women with Ser/Ser genotype required higher total FSH dose (225 U/day) for ovarian stimulation when compared with women with Asn/Asn genotype (who received 150 U/day) to attain similar ovarian response	Behre <i>et al.</i> (2005)
		Basal FSH, number of follicles, oocytes, and pregnancy rate was not different among Ser/Ser (150 U/day), Ser/Ser (250 U/day), and Asn/Asn (150 U/day) groups	
	148	Women with Ser/Ser genotype displayed significantly higher basal FSH levels, but the peak estradiol levels, exogenous FSH levels, number of follicles, and oocytes were similar when compared with women with Asn/Asn and Asn/Ser genotype	Laven <i>et al.</i> (2003)
	105	Women with Ser/Ser genotype had implantation and pregnancy rate significantly higher than women with Asn/Asn genotype	Klinkert <i>et al.</i> (2006)
	421	The total amount of FSH stimulated and the number of oocytes retrieved were similar in all three genotypes	Mohiyiddeen <i>et al.</i> (2012)
	64	Women with Ser/Ser genotype were associated with higher ovarian threshold to FSH, decreased negative feedback of luteal secretion, longer menstrual cycles but displayed significantly higher number of antral follicles	Greb <i>et al.</i> (2005)
p.Thr ³⁰⁷ Ala		Peak estradiol levels, inhibin B, and growth velocities of dominant follicles were not significantly different among the three genotypes	
	79	Women with Asn/Ser genotype generate a significantly higher amount of estradiol and higher number of follicles and oocytes when compared with women with Ser/Ser and Asn/Asn genotype	Loutradis <i>et al.</i> (2006)
	37	Ser ⁶⁸⁰ allele present more risk of giving iatrogenic OHSS when compared with Asn ⁶⁸⁰ allele, but the Asn ⁶⁸⁰ allele was significantly associated with severity of iatrogenic OHSS	Daelemans <i>et al.</i> (2004)
	427	Women with Ser/Ser genotype were more likely to have hyper-response than women with Asn/Asn genotype	Boudjenah <i>et al.</i> (2012)
	50	Women with Ala ³⁰⁷ Ala genotype were significantly associated with lower dose of exogenous FSH, higher estradiol levels before and on the day of hCG stimulation	Achrekar <i>et al.</i> (2009a)
g.G>A -29		85% of the women with Ala/Ala genotype developed OHSS	
	202	Basal FSH and estradiol levels were not significantly different among the three genotypes at position -29	Wunsch <i>et al.</i> (2005)
	50	Women with AA genotype displayed lower estradiol levels, number of follicles and oocytes, and higher total amount of exogenous FSH administered when compared with women with GG genotype	Achrekar <i>et al.</i> (2009b)
	100	The level of FSHR expression at transcript and protein levels was significantly reduced in women with AA genotype when compared with GG genotype	Desai <i>et al.</i> (2011)

allele. Thus, it is more likely that the Ser⁶⁸⁰ allele along with the Ala³⁰⁷ allele may be associated with hyper-response to FSH stimulation.

The rate of pregnancy in an IVF cycle is very important as it is considered as a measure to determine the IVF outcome. Klinkert *et al.* (2006) reported that the Ser⁶⁸⁰ allele is more likely to give a higher pregnancy rate when compared with the Asn⁶⁸⁰ allele. Recently, a genome-wide analysis carried out by Boudjenah *et al.* (2012) reported that women with Ser/Ser genotype were more likely to have a high response than women with Asn/Asn genotype at position 680 (24 vs 12% respectively, $P=0.013$). These observations suggest that there is a

lack of consistency in the outcome of these association studies and there is a need for clarity in defining the poor ovarian response. Various attempts have been made to study the impact of the polymorphism at position 680 on FSHR activity using GCs. GCs obtained from women with three different genotypes at position 680 were cultured for a short time (~6–7 days) and were used to study the response of FSH stimulation. Results revealed that the amount of cAMP production and estrogen and progesterone synthesis was similar in GCs obtained from all three genotypes at position 680. Although the basal estradiol levels in GCs with Ser/Ser genotype were significantly lower, the estrogen production upon FSH

stimulation did not differ significantly between the three genotypes, suggesting that the probable contribution of polymorphism at position 680 needs further investigation in long-term GC culture system (Nordhoff *et al.* 2011, Zalewski *et al.* 2013).

The level of FSHR expression also has an impact on the FSH action. Moreover, the reduced expression of FSHR on GCs has been shown to be associated with poor ovarian response (Cai *et al.* 2007). The polymorphism at position -29 of the 5' UTR in the *FSHR* gene has been studied with respect to its effect on receptor expression as this polymorphism may modulate the cETS-1 transcription factor binding site. *In vitro* analysis carried out in CHO cells demonstrated that the A⁻²⁹ allele expressed a significantly lower level of luciferase activity when compared with the G⁻²⁹ allele of the *FSHR* gene (Nakayama *et al.* 2006). Despite the fact that Wunsch *et al.* (2005) did not observe any association of the polymorphism at position -29 with ovarian response, the clinical parameters considered in this study were limited (basal FSH levels and E₂ levels). Recently, we observed that subjects with AA genotype were significantly associated with a reduced number of follicles and retrieved oocytes when compared with subjects with GG genotype. The amount of FSH required for optimum response was also significantly higher in subjects with AA genotype when compared with GG genotype. Almost 66.67% (odds ratio 8.154; 95% CI 2.79 to 23.77; $P < 0.0001$) subjects with AA genotype were poor ovarian responders (Fig. 2; Achrekar *et al.* 2009b, Desai *et al.* 2011, 2013). Further, using GCs obtained from women undergoing IVF, we observed that subjects with AA genotype expressed significantly reduced FSHR expression both at transcript and protein level when compared with GG genotype (Desai *et al.* 2011). This study provides the experimental evidence to suggest that reduced receptor expression observed in subjects with AA genotype could be the probable reason for the poor ovarian response to FSH stimulation observed in them. However, a greater number of subjects from various ethnic populations needs to be analyzed to assess the usefulness of screening this polymorphism, which could be used as a biomarker to predict poor ovarian response.

Association of FSHR gene polymorphisms with PCOS, premature ovarian failure, and amenorrhea

PCOS represents a clinical condition distinguished by impaired menstrual cycle and formation of multiple cysts in the ovary. In these subjects, LH:FSH ratio is generally increased and there is excessive production of androgen (Goodarzi *et al.* 2011). So far, a number of polymorphisms has been studied in various candidate genes to predict the risk of developing PCOS. A meta-analysis across the eight studies suggested that the Asp⁶⁸⁰ allele was associated with lower risk and the Ala³⁰⁷-Ser⁶⁸⁰

Figure 2 Association of AA genotype at position -29 of the *FSHR* gene with poor ovarian response (A, B, C and D). Amount of (recombinant or urinary) exogenous FSH administered during controlled ovarian hyperstimulation, number of preovulatory follicles, number of retrieved oocytes, and percent poor ovarian responders respectively in women ($n = 150$) with different genotypes who are undergoing an IVF protocol (Achrekar *et al.* 2009b, Desai *et al.* 2011, 2013). The same letter (a or b) indicates statistically significant difference at $P \leq 0.05$ among different genotypes.

genotype was associated with an increased risk of developing PCOS (Du *et al.* 2010).

Generally, treatment of infertility in cases with PCOS is based on increasing the level of FSH either by antagonizing the effect of estrogen by clomiphene citrate (CC) or by administering recombinant FSH (rFSH). Recently, it was observed that subjects with Ser/Ser genotype at position 680 exhibited high resistance to CC treatment but showed the most favorable response to rFSH stimulation (Overbeek *et al.* 2009). Likewise, in a study in PCOS women from Italy, Asn/Thr genotype at position 307 displayed higher response to FSH stimulation when compared with both the homozygous genotypes (Dolfin *et al.* 2011).

Premature ovarian failure (POF) is also referred to as primary ovarian insufficiency (POI) and is characterized by low levels of gonadal hormones (estrogen, inhibins) and elevated levels of FSH with impaired ovarian folliculogenesis. The phenotype of the *FSHR* knockout mice was observed to be similar as observed in POF (Dierich *et al.* 1998). Thus, FSHR could serve as a strong candidate gene responsible for the phenotype observed in the females with POF. Although there are no clear associations observed in *FSHR* gene polymorphism with POF, recently Kim *et al.* (2011) reported that epistasis between *FSHR* and *CYP19A1* polymorphisms was significantly associated with POF.

In the case of subjects with amenorrhea, an association with the Ser⁶⁸⁰ allele has been observed (Sudo *et al.* 2002). Recently, our group has investigated the association of polymorphisms at position -29, 307, and 680 in women with primary and secondary amenorrhea. We observed that in the subjects with primary amenorrhea, AA genotype at position -29 was significantly associated with increased basal serum FSH levels when compared with GG and GA genotype (Achrekar *et al.* 2010).

Association of FSHR gene polymorphisms with male infertility

In recent years, several studies on the association of the *FSHR* gene polymorphisms on sperm motility, sperm counts, and testicular volume were carried out. Most of the reports showed no significant association of the polymorphisms at position -29, 307, and 680 with these parameters (Ahda *et al.* 2005, Pengo *et al.* 2006, Li *et al.* 2011, Lindgren *et al.* 2012). The recent three meta-analyses that summarized the seven original papers also suggest no association of these polymorphisms with reproductive abnormalities in men (Tüttelmann *et al.* 2007, Lend *et al.* 2010, Wu *et al.* 2012). However, it was interesting to note that subjects with AA genotype at position -29 showed significantly smaller mean testicular volumes when compared with subjects with GG genotype (Lend *et al.* 2010). Taken together, these studies from different populations suggest that *FSHR* genotype does not seem to have a significant impact on male reproductive physiology.

Association of FSHR gene polymorphisms with gonadal cancer

There are limited studies on *FSHR* gene polymorphisms in cases with ovarian or testicular cancer. Contradicting observations with respect to the association of *FSHR* polymorphisms with low or high risk of forming gonadal cancers have been reported. Ala³⁰⁷/Ser⁶⁸⁰ allele alone or in combination with G⁻²⁹/T⁻¹¹⁴ allele was observed to be associated with lower risk of developing testicular cancer (Ferlin *et al.* 2008). On the other hand, homozygous Ala³⁰⁷/Ser⁶⁸⁰ alleles have been associated with higher risk and recurrence of developing ovarian cancer (Ludwig *et al.* 2009). However, the mechanism by which these alleles could modulate the *FSHR* function in ovarian or testicular cancer cells is still unknown and holds a great research interest.

Alternatively spliced variants of the FSHR gene

Alternatively spliced variants of the *FSHR* gene have been identified in various species such as cattle, rat, and sheep. Alternatively spliced variants of the *FSHR* gene in rat and sheep have been observed to result in the soluble

form of the protein or modification of the C-terminal domain of the receptor (Sairam *et al.* 1996, Yarney *et al.* 1997). In humans, alternate splicing of exon 9 in normal testicular tissue has been reported, which suggests that this spliced variant does not affect the FSHR function in testis (Gromoll *et al.* 1992). Deletion of exons 6 and 9 and insertion of part of intron 8 has also been identified in infertile men (Song *et al.* 2002). Additionally, a number of spliced variants such as del exon 2, 6, and 9 and insertion of 102 bp of intron 8 along with the WT *FSHR* in the GCs obtained from women undergoing IVF has also been reported. The clinical parameters of these women suggested that deletion of exon 2 was associated with poor ovarian response whereas deletion exon 6 was associated with a high response (Gerasimova *et al.* 2010). In our study, in subjects undergoing IVF, we observed that out of a total of 20 subjects screened, only one subject was observed to possess insertion of a part of intron 8 (102 bps) along with the WT-*FSHR* (S S Desai and S D Mahale 2011, unpublished data). However, this spliced variant did not seem to affect the various clinical parameters. Therefore, screening of spliced variants in a greater number of subjects is essential to corroborate their role in ovarian response and female infertility.

Summary and future perspective

Studies reported so far clearly indicate that mutations, polymorphisms, and alternatively spliced variants in *FSHR* have varied effects on receptor function. The overview of the phenotypes associated with genetic alterations in the *FSHR* and their functional characterization carried out so far has been summarized in Fig. 3. Identification of different inactivating mutations in the *FSHR* gene and correlating them with respective phenotypes clearly suggest that receptor function is impaired in women with such mutations, resulting in infertility. However, the affected men had no effect on their fertility. *In vitro* functional studies undertaken to characterize these mutants have provided valuable information in understanding FSH–FSHR interaction at the molecular level. These mutations are believed to bring about structural change in the receptor, thereby reducing the hormone binding ability or hormone-induced signaling ability. Few of the mutations have shown any defect at the level of trafficking to the cell membrane, probably due to a defect in the maturation of the receptor (Casas-González *et al.* 2013). Further studies with these mutants will possibly help in understanding the events involved in post-translational modifications of *FSHR*, its interaction with intracellular adaptor molecules, internalization, recycling, degradation of the activated receptor, etc. In addition, generation of the corresponding mutant animal model will help in developing modalities to rescue the receptor function. Toward this, efforts have been made to use small molecules capable of restoring the receptor function

Figure 3 Schematic representation of an overview of the genetic alterations in *FSHR* and associated phenotypes reported so far. *In vitro* functional studies employed for characterizing the mutant receptor are also included.

using COS cells (Janovick *et al.* 2009). In the case of activating mutations of *FSHR* gene, the receptor activity was found to be enhanced mainly due to loss of hormone binding specificity. It was observed that in most of the cases, the receptor was activated by hCG or TSH or by both. In women, such mutations resulted in sOHSS.

The polymorphic nature of the *FSHR* gene is being explored to develop molecular markers mainly in the case of women undergoing controlled ovarian hyperstimulation protocol during IVF procedure. SNPs at –29, 307, and 680 have been studied in detail by various investigators in different ethnic populations. Polymorphism at –29 (AA) has been linked to poor ovarian response, which is found to be due to reduced expression of the receptor both at transcript and protein levels. On the other hand, mechanisms involved in the association of the altered ovarian response with SNP at 307 and 680 position is not well understood and efforts need to be made toward this. It would be interesting to study these SNPs in combination with each other with an aim to increase the predictive value of the marker. Preliminary findings from the analysis of data in women ($n=150$) suggest that A^{–29}A/Asn⁶⁸⁰Asn genotype is associated with poor ovarian response with an odds ratio of 7.92 ($P=0.009$) (Desai *et al.* 2013). Thus, studying SNPs of the *FSHR* gene is emerging as an important arm of pharmacogenomics and personalized medicine and

needs to be explored further to optimize the dose of FSH required for superovulation and also to predict the ovarian response in women undergoing FSH treatment.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

Funding

The work carried out in authors' laboratory is supported by Indian Council of Medical Research, New Delhi, India (NIRRH/MS/103/2013) and (BIC/12(10)/2013). S S Desai is a recipient of Senior Research Fellowship of Indian Council of Medical Research, New Delhi, India.

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Received 31 July 2013

First decision 4 September 2013

Revised manuscript received 16 September 2013

Accepted 19 September 2013