Original article:

Presence of the ASN680SER Polymorphism in women with Primary Amenorrhea from South India

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ABSTRACT:

Introduction: The hormones that play a role in the menstrual cycle are FSH, LH, estrogen and progesterone. For a hormone to function normally, the structural and functional integrity of its receptor is a pre-requisite. The Asn680Ser polymorphism in the FSHR gene has been the more studied polymorphism as it seems to play a role in the receptor function. The prevalence of the FSHR gene polymorphism in patients with primary amenorrhea and its possible significance has not yet been explored in the South Indian population

Methods: 139 subjects were taken for this study of which 92 of them had primary amenorrhea and 47 [controls] of them had regular menstrual cycles. Cytogenetic analysis was done by routine karyotyping methods to rule out the presence of a chromosomal abnormality. This was followed by DNA extraction using organic solvents phenol and chloroform. The extracted DNA was quantified and then amplified using primers designed for this study. The amplified PCR product was then purified and sequenced.

Observation & Results: 26.1% of the patients were found to have a homozygous mutant status while 12.8% of the controls were also found to have a homozygous mutant status.

Conclusion: This is the first study to report the prevalence of the FSHR gene polymorphism in women with primary amenorrhea from the South Indian population. 26.1% of the patient group and 12.8% of the control group were found to have the polymorphism. While the FSHR gene polymorphism cannot be attributed as the sole cause of primary amenorrhea; other genes of the menstrual cycle need to be screened to get an answer for the same.

Keywords: Asn680Ser, FSHR gene, Primary Amenorrhea

Introduction:

The failure of spontaneous onset of menstrual cycles by the age of 16 is called Primary amenorrhea [1]. Normal menstruation requires integration of the hypothalamic-pituitary-ovarian [HPO] axis with a functional uterus and a normal 46,XX karyotype. The main hormones that play a role in the menstrual cycle are the Gonadotropin releasing hormone, secreted from the hypothalamus, the Follicle Stimulating hormone [FSH] & Luteinizing Hormone [LH] secreted from the anterior lobe of the pituitary gland, estrogen secreted from the cells of the ovary and progesterone secreted by the corpus leuteum [2].One of the foremost factors necessary for the menstrual cycle to occur in a cyclic manner, as it should, is the adequate action of these hormones and for the hormones to act accurately, it is very essential that the receptors of these hormones are structurally and functionally sound.

The follicle stimulating hormone [FSH], a glycoprotein, secreted from the anterior lobe of the pituitary, under the action of the gonadotropin releasing hormone, binds on to specific receptor cells seen on the granulosa cells of the ovaries and as the name suggests, it stimulates the developing

follicles in the ovaries. It also induces the secretion of estrogen and then progesterone, at a later stage. For the FSH to function properly it is very essential that the receptor of the FSH, the FSH-receptor [FSHR] is structurally and functionally sound. This is because chemical signals are the mode of communication between cells. The signalling molecules bind on to specific receptor cells on the target organ, and this in turn sets of a series of chain reactions, which is amplified at each level resulting in a highly specific response. Any alteration in the structure of the receptors can disrupt this chain of reactions, leading to a change or even in the absence of the final output [3].

As the knowledge about the human genome and its sequence increased over the years, it became evident that genetic variations due to single nucleotide polymorphisms [SNPs] are frequent in hormones and receptors of the hypothalamic pituitary gonadal axis. It is probable that such genetic changes, in the form of single SNPs or in various combinations, are able to modify the endocrine feedback systems and hormone action, which could result in variable reproductive performance ranging from fully functional gametogenesis to infertility [4].

The FSHR located on the granulosa cells of the ovary in females and sertoli cells of the testis in males. The gene has been localized to chromosome 2p21 and belongs to the family of G protein coupled receptors. The gene coding for the receptor is 54kbp in size. Structurally the FSHR gene can be divided into three parts: an extracellular domain, a transmembrane region and an intracellular domain. The gene has 10 exons and 9 introns. The first nine exons code for the extracellular region while the exon 10, which is the largest, codes for the C terminal part of the extracellular domain, the transmembrane region and the intracellular domain. [4].The proband had primary amenorrhea, poorly developed secondary sexual characteristics and recessively inherited hypergondatropic ovarian failure. [5] Once the first description of a FSHR mutation/polymorphism was described, the search for mutations in several conditions of male and female infertility began. Scientists, over the years, discovered other naturally inactivation mutations in the FSHR gene in women with primary amenorrhea namely Asp224Val (A671T) and Leu601Val (T479C), Pro348Arg (C1043G), Ala419Thr (G1255A), Pro519Thr (A1556C), and Val221Gly (C1801G) [6-10]

The analysis of a large number of both patients and controls all over the world has lead to the discovery of 2 common polymorphisms in the FSHR gene and their role and possible effect in the various aspects of gonadal function are being studied. [11]

A total of eight polymorphisms have been detected in the coding region of the FSHR gene, of which six are non-symptomatic. The other two polymorphisms are present at position 307 [Thr307Ala] and position 680 [Asn680Ser]. The two SNPs in the coding region of the FSHR gene occur at nucleotide 919 & 2039 in exon 10, where nucleotide 1 is the A of the ATG start codon, which corresponds to amino acid positions 307 & 680 respectively of the mature protein. The polymorphism at position 307 is located in the hinge region of the extracellular domain whereas the polymorphism at position 680 is present in the intracellular domain of the receptor. Since exon 10 contains both the polymorphisms, they are believed to be in strong linkage with each other. The polymorphism at 680 alone was focused in most of the association studies. The Asn680 contains a sequence for glycosylation which may play a role in receptor trafficking and Ser680 contributes as a phosphorylation site which may be involved in receptor turnover. Hence these isoforms are believed to play a role as potential glycosy-

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lation/phosphorylation sites of *FSHR*. [12] Our aim was to estimate the prevalence of polymorphisms of the FSH Receptor gene at position 680 in the subjects referred with primary amenorrhea.

MATERIALS & METHODS:

Patient Selection: 139 females were selected for this study. Of that 92 of them had primary amenorrhea and 47 of them had regular menstrual cycles and were considered as controls. All subjects recruited were from the South Indian states of Karnataka, Tamil Nadu, Kerala and Andhra Pradesh

Study setting: Division of Human Genetics, St. John's Medical College, Bangalore. Ethical clearance was obtained from the Institutional Ethics Committee [IEC Ref No: 83/2010]

Methodology: The methodology involved can be broadly divided into 2 parts: Cytogenetic analysis to rule out a chromosomal abnormality followed by molecular analysis to detect the presence of a polymorphism in the exon 10 of the FSHR gene.

1. **Cytogenetic Analysis**:

Chromosomal abnormality was ruled out by karyotyping by routine GTG banding technique [13]. [Software & Microscope used: BX53 Olympus Trinocular Microscope with Applied Spectral Imaging Ltd (Israel) Automated Karyotying System Software version 7.2.6]

2. **Molecular Analysis**:

2a. **DNA Extraction**: DNA was extracted from peripheral venous blood by using the phenol chloroform method of extraction [14].

Principle: The red blood cells in the blood are first lysed and discarded as they do not contain a nucleus and in turn DNA. The pellet obtained is then treated with a detergent, salt and an organic solvent. The detergent [10% SDS] disrupts the cell membrane and dissolves the lipids so that the contents of the cell spill out. The salt used, 5M NaCl, stabilizes the DNA and helps it remain in the double helix form. The organic solvents phenol and chloroform have two effects: 1) they dissolve the hydrophobic molecules and 2) they denature proteins (which makes them insoluble in water). As a result, cell membranes and cellular proteins are either dissolved in the phenol (which is then discarded) or trapped in the interface between the two phases. DNA which remains in the aqueous phase is easily separated. The extraction was confirmed by running a 0.8% agarose gel and then quantified using a Qubit flurometer and stored at -20°C for further use. [Equipment used: Eppendorf microcentrifuge- model no 5468. Fluorometer: Qubit 2.0 Fluorometer, Invitrogen.]

2b. **Polymerase Chain Reaction [PCR]**:

A 473bp region of the exon 10 of the FSHR gene which included the position of interest was amplified from the extracted DNA using primers designed for this study. [Table1]. The cycle conditions were standardized [Table 2] and the amplification was confirmed by running a 2% agarose gel [Image 1]

Fwd/Rev primers	Length	Tm °C	GC content	Product size [bp]
3.Fwd: 5'ATCGTGTCCTCCTCTAGTGACAC 3'	23	57.06	52.17%	473
Rev: 5'GACATACCCTTCAAAGGCAAGACTG 3'	25	57.68	48%	

Table 1: Primer sequences and features

The PCR cycle conditions for the Primer pair is as follows

Pre-Denaturation		Denaturation		Annealing		Extension		Post Extension	
Temp	Time	Temp	Time	Temp	Time	Temp	Time	Temp	Time
94°C	3min	94°C	30sec	60.4°C	30sec	72°C	30sec	72°C	3min

Table 2: PCR cycle conditions

Once the amplification was confirmed, the PCR products were purified using EXO-SAP IT [An enzyme] to remove impurities such as excess dNTP's etc.

[Thermal cycler: Long gene thermal cycler; gradient thermal cycle. Vendor: Helini biomolecules, Chennai; Gel documentation system: Chemidoc XRS + imaging system, Bio Rad.]

2c. **Sequencing and Analysis**: The purified PCR products were then sequenced using Sanger sequencing. The samples were outsourced for the same to the National Centre for Biological Sciences, Bangalore. The raw sequences were obtained and the chromatograms were analysed using software available online, such as NCBI align & Finch TV, and the data obtained was studied [Image 2]

3. Statistical Analysis: The frequency of the presence of the Asn680Ser polymorphism in the patients with primary amenorrhea and in the controls was analysed [Table 3 & 4]. In order to evaluate the difference in the prevalence in both the groups, the odds ratio was calculated [Table 5] and the test for significance used was the Chi square test. Since the subjects with heterozygous status have one normal allele and a significant percent of heterozygous subjects were seen in both the groups, patients & controls with the heterozygous status were considered as normal for the calculation of the odds ratio and chi square test. SPSS version16 was used for statistical analysis.

OBSERVATION & RESULTS:

The sequences obtained for each patient were analyzed and were divided into 3 categories for the polymorphism as follows:

1. Homozygous Wild: normal status [A]
2. Homozygous Mutant: The Allele changed from A to G
3. Heterozygous: Both A & G present.

The homozygous/heterozygous status is confirmed by studying the chromatogram as shown in Fig 2.

26.1% of the patients with primary amenorrhea were found to have a homozygous mutant status while 42.4% of them had a heterozygous status. 12.8% of the controls had a homozygous mutant status while 25.5% of them had a heterozygous status.

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The odds ratio calculated was 2.4 at 95% confidence interval and the p value was 0.127; chi square test

Genotype	Frequency	Percentage
Homozygous Wild	29	31.5%
Homozygous Mutant	**24**	**26.1%**
Heterozygous	39	42.4%

Table 3: Frequency of the FSHR gene polymorphism in patients with primary amenorrhea

Genotype	Frequency	Percentage
Homozygous Wild	29	61.7%
Homozygous Mutant	**6**	**12.8%**
Heterozygous	12	25.5%

Table 4: Frequency of the FSHR gene polymorphism in controls

		Primary Amenorrhea		
		Present	Absent	
Polymorphism Asn680Ser	Present	24	6	31
	Absent	68	40	108
		92	47	139

Table 5: Data for calculation of odds ratio

DISCUSSION:

26.1% of the patients [24/92] with Primary Amenorrhea [PA] that were studied were found to have a change in their sequence. All the 24 patients were homozygous mutants. The change from A to G at position 2039 causes a change from Asparagine to Serine at position 680 in the intracellular domain of the receptor which in turn introduces a potential phosphorylation site [15]. This change, though involving a change in only one base pair each, could affect the downstream mechanisms of the FSH receptor which could in turn affect the menstrual cycle resulting with the patient presenting with primary amenorrhea.

42.4% of the patients with PA were found to have a heterozygous condition. This means that while one allele had the normal "A", the other allele had changed to "G". Since these patients have one allele that is normal and functioning, this heterozygosity cannot be attributed as the cause for PA in them and other causes should be explored. Similarly in the 31.5% of the patients with PA who had a homozygous wild status, the exon 10 of the FSHR gene is not the cause for the presence of PA and as in the case of the heterozygotes, other possible causes should be explored.

12.8% [6/47] of the controls i.e. women with normal female karyotype and regular menstrual cycles, were found to have a homozygous mutant status for the Asn680Ser polymorphism. Review of literature showed that this polymorphisms is seen in up to 30% of the normal population [16].While these individuals do not have a phenotypic effect of the SNP [hence called *polymorphisms*], these changes especially the Asn680Ser change, is said to play a role in ovarian reserves and also in case IVF treatment is sort for later in life. The dosage of exogenous FSH given has to be increased in the case of a ser680ser condition compared to the Asn680ser condition [17]. In a study done by Simoni et al in 2002 it was found that while on the whole, the frequency distribution of the polymorphisms in

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the probands [i.e. ovulatory women undergoing IVF treatment] was similar to reported data but the volume of FSH introduced to achieve the required effect was significantly different among the three groups. The number of ampoules of FSH required in the ser680ser group was 46.8 ±5 compared to the 31.8 ± 2.4 in the Asn680Asn group [$p<0.01$][4]. Looking into this aspect is beyond the scope of this study.

The polymorphisms in the FSHR gene have been extensively studied and it has been found to have a strong ethnic influence1 [16].Various studies have been done in the South Asian population including Japan [17],Indonesia[18], China[19] & India [Chennai & Western India][20,21]. While the study done in Chennai focused only on patients with polycystic ovarian syndrome, the Indonesian & Chinese study focused mainly on patients undergoing IVF treatment. These studies found a significant relation between the FSHR polymorphism and their patient population of interest.

Sudo et al studied included a large cohort of 522 Japanese women with gynaecological issues and also those with spontaneous ovulation. 33 of them had primary amenorrhea and in these 33 patients 4 of them [12.1%] had a homozygous mutant status, 9 [27.3%] had a homozygous wild status and 20 [60.6%] had a heterozygous status. The Study done by Acherekar et al focused only on patients with Primary and secondary amenorrhea but included subjects only from Western India. In this study, 48 of the subjects had primary amenorrhea out of which 08 (16.67%) had a homozygous mutant status, 15 (31.25%) had a homozygous wild status and 25 (52.08%) had a heterozygous status. This study also found significant difference in the frequency distribution in the different FSHR polymorphism haplotypes between patients with primary amenorrhea and controls.

Among the controls studied by Achrekar et al which included females with regular menstrual cycles & proven fertility, 13% of the women were found to have a homozygous mutant status. Kambalachenu et al screened, 101 healthy women without history of infertility and any signs of hyperandrogenism for the FSHR polymorphism in addition to those with polycystic ovarian syndrome and reported that 18 (17.8%) had a homozygous mutant status.

There is no reported data on the prevalence of the FSHR gene polymorphism in women with primary amenorrhea from the South Indian population. The present study exclusively screened women with primary amenorrhea as opposed to the mixed patient population of other studies. The subjects recruited for the present study were all from the South Indian states of Karnataka, Kerala, Tamil Nadu and Andhra Pradesh. Out of the 92 patients with Primary amenorrhea, 24 of them were found to have a homozygous mutant status indicating that this polymorphism is seen in more than 25% of the patients presenting with primary amenorrhea as compared to the 16% seen in the study done in the western Indian population by Achrekar et al. In the case of controls, i.e. normally menstruating women with regular cycles, the results of the present study are similar to the results reported from Chennai by Kambalachenu et al, as 12% of the controls were found to have a homozygous mutant status. This indicates that in the South Indian population, the FSHR gene polymorphism is seen in the affected as well as normal population similar to the findings reported by Acherekar et al. Here the ethnic difference does not seem to play a significant role.

Conclusion:

The result of these changes varies from individual to individual and can range from fully functional gametogenesis to sub fertility. [22]

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In this study, the gene of interest was the Follicle Stimulating Hormone Receptor gene which has been mapped to chromosome 2p21-16.

While 26.1% of the patients with primary amenorrhea did have a homozygous mutant status, it can be concluded that these polymorphic changes may not be the sole cause for their presenting problems, as these changes are also seen in the controls with regular menstrual cycles. Other causes which include polymorphisms or mutations in the various genes of the menstrual cycle including the FSH gene itself, could give an answer to this. It has been found that the combination of the FSHß gene and FSHR gene polymorphisms have a much stronger impact as compared to either of them alone[15].

The present study is the first to report the prevalence of the FSHR gene polymorphism in patients with primary amenorrhea from the South Indian population. The data obtained from the present study, along with the other studies published from India indicates that in the Indian population, the FSHR gene polymorphisms can be ruled out as the sole cause for primary amenorrhea. The various possible causes for primary amenorrhea have been explored over the years and with the increase in the knowledge of the human genome, it has been found that genes have a significant contribution to the regularity of menstrual cycles. The association of mutations in these genes and primary amenorrhea has to be explored in more detail involving a larger cohort of patients in order to arrive at a significant conclusion.

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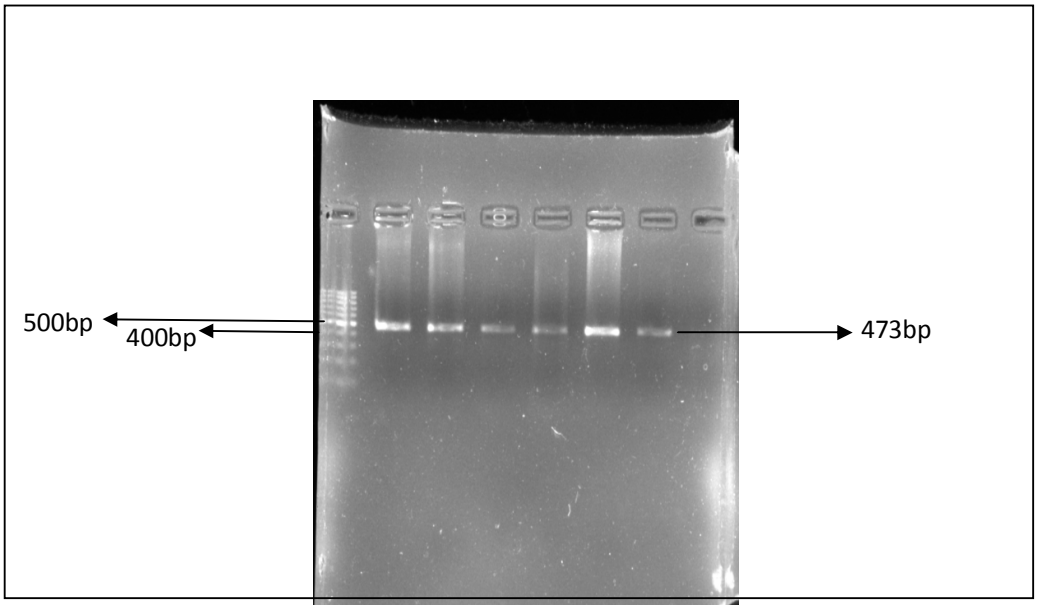

Image 1: Gel image showing the 473bp fragment of the FSHR gene

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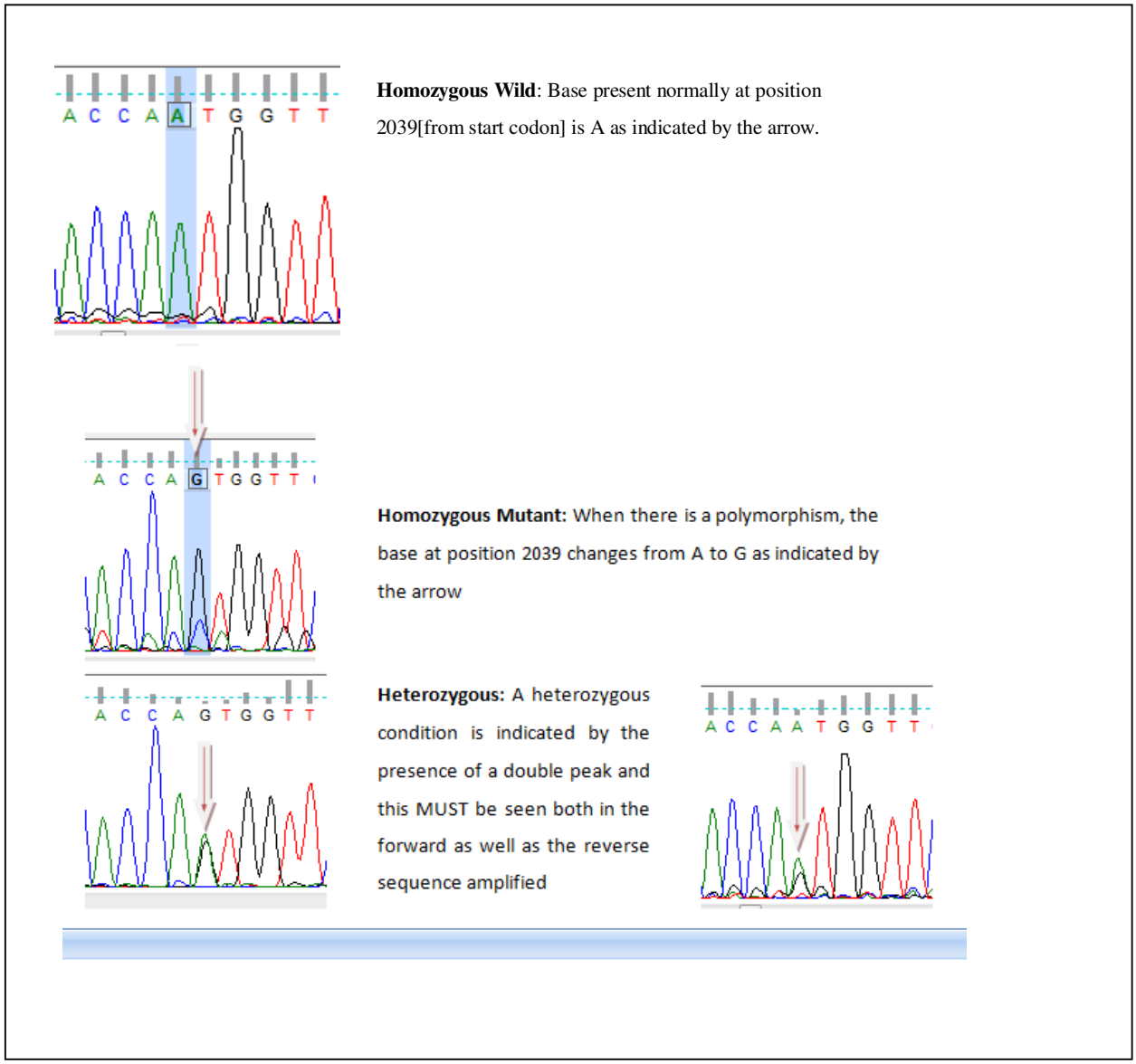

Image 2: Chromatogram showing Homozygous Normal, Homozygous Mutant & Heterozygous condition

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