Inactivating Mutation screening of Exon 6 and Exon 10E of *FSHR* gene in women with Polycystic Ovarian Syndrome in Vellore population

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Inactivating Mutation screening of Exon 6 and Exon 10E of *FSHR* gene in women with Polycystic Ovarian Syndrome in Vellore population

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Abstract. Polycystic Ovarian syndrome (PCOS) is a major cause of infertility in females of reproducing age and is typified by oligo-anovulation, hyperandrogenism, hirsutism and polycystic ovaries. FSHR gene located on chromosome 2 p21 is responsible for the normal follicular development and any deletion or mutation in the gene affects the interaction of FSH with its receptor. Thus, it becomes the candidate gene for PCOS study**.** Inactivating mutation in FSHR gene limits the receptor's function by creating a complete block, changing the receptor-ligand complex or the basic hormone signal transduction.To screen the inactivating mutations in Exon 6 and Exon 10E of FSHR gene in women diagnosed with PCOS.PCR-RFLP analysis indicated that there were no inactivating mutations found in Exon 6 and Exon 10E. Variations in hormone levels were seen amongst the PCOS patients. There were no inactivating mutations found in FSHR gene of the women diagnosed with PCOS according to the Rotterdam criteria in Vellore population.

1. Introduction

According to National Institute Health (1990), 6-10% of females of reproductive age are affected by Polycystic Ovarian Syndrome (PCOS) across the globe. PCOS can be shortly described as an endocrine system disorder in which 10-12 small cysts of diameter ranging from 2-9 mm are formed inside either one or both the ovaries [1].Diagnostic features of PCOS are oligo-anovulation, hyperandrogenism, hirsutism, polycystic ovaries [2].

Based on these features, three diagnostic strategies have been established by the Androgen Excess PCOS Society, Rotterdam Criteria, and National Institutes of Health [1].The 1990 NIH and Androgen Excess-PCOS Society criteria for PCOS accentuates hyperandrogenism, which is related to hyperinsulinism, which makes these criteria's important for understanding metabolic dysfunction in PCOS whereas the Rotterdam criteria are useful for diagnosis in ethnic groups (for example, Asian subjects) who do not show clinical hyperandrogenism. Overproduction of insulin is normally observed in women with PCOS. This causes insulin resistance that initiates the production of androgens, like testosterone, in the ovaries. Obesity is seen due to this insulin resistance. Because of excess androgens, there is a disruption in the cyclical hormone balance in women that leads to male pattern hair growth and its conversion to estrogen represses the flow of follicle-stimulating hormone and results in anovulation. Small cysts are formed due to these unreleased ova [3].FSH is a glycoprotein polypeptide hormone responsible for the stimulation and incorporation immature ovarian follicles in the ovary [4]. The receptor for this hormone, known as Follicle Stimulating Hormone

Receptor/FSHR (G protein coupled receptor), is expressed on granulosa cells of ovaries in females and Sertoli cells of the testis in males. In humans, the location of FSHR gene is chromosome 2 p21 and traverses a region of about 54kb consisting of 10 exons and 9 introns [4].There are numerous genetic variants in FSHR which show phenotypic effects including PCOS, amenorrhea, serum levels of FSH, alteration in the development secondary sex characteristics [5].Hindrance in follicular development can be caused by mutations or deletions in FSHR gene which in turn disturbs the interaction of FSH with its receptor making it a candidate gene for PCOS study [14].There are associations found between inactivating mutations of the FSHR gene and women with ovarian failure with primary and secondary amenorrhea [10].The aim of our study to screen the inactivating mutations in Exon 6 and Exon 10E of FSHR gene in women diagnosed with PCOS.

2. Materials and Method

2.1 Study population

This study includes PCOS patients (n=10) and controls group (n=5) randomly chosen from Sandhya Hospital**,** Vellore and was approved by the Institutional Ethical Committee, VIT University, Vellore. The PCOS diagnosis was established in the patients using the 2004 Rotterdam criteria for the diagnosis of PCOS. All women in the control group were healthy without hirsutism according to the Rotterdam criteria.

2.2 Clinical measurements

Information considered such as age, height, weight, menstrual history, acne, Acanthosis nirgricans, ultrasound assessment, and other features are summarized in Table 1.

2.3 Biochemical measurements

Hormone levels-Follicle Stimulating Hormone (FSH), Prolactin (PRL), Thyroid Hormones (T3, T4), Thyroid Stimulating Hormone(TSH), Luteinizing Hormone (LH), Total Testosterone & Free Testosterone.

2.4 Molecular analysis

2.4.1 DNA isolation and polymerase chain reaction DNA was isolated from 2mL venous blood sample according to the lab protocol and stored at -20°C. Using this isolated genomic DNA, Exon 10E and Exon 6 of the FSHR gene were amplified by PCR using specific oligonucleotide primers shown in Table 2. PCR conditions were decided for each fragment. DNA sample (3µl) was amplified with initial denaturation at 95°C for 5 minutes which was followed by denaturation for 45 seconds at same temperature, annealing step was achieved at 52°C and 56°C for Exon 6 and Exon 10E respectively for 50 seconds, initial extension was carried out at 72°C for 1 minute and final extension at 72°C for 10 minutes. First, the PCR products were separated using gel electrophoresis (2% agarose) and then visualized using UV trans-illuminator. *2.4.2 Restriction Fragment Length Polymorphism (RFLP)* Using the restriction enzymes Msc1 and Mun1 respectively for Exon 10E and Exon 6, the PCR products were digested for 5 minutes at 37°C. The digested PCR products were then run on 3% agarose and visualized by using UV trans-illuminator and photographed by Gel Doc software.

3. Results and Discussion

During the data collection, it was observed that BMI indicates obesity in some cases as shown in Figure 1. By comparing the hormone levels in patients with the reference level it can be deduced that in few patients FSH, T3, and T4 were below normal level whereas levels of total testosterone, free testosterone and thyroid stimulating hormone were also found to be elevated in few patients which can be seen in Figure 2.

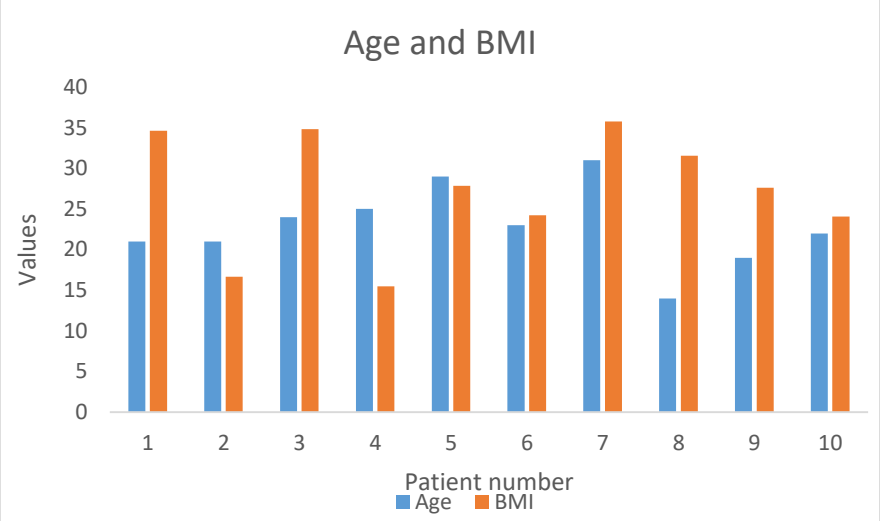

Figure 1. Age and BMI

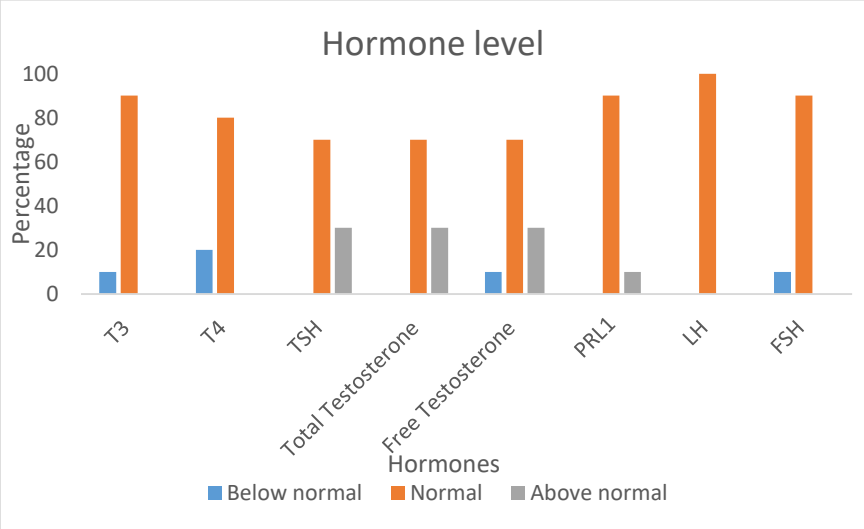

Figure 2. Hormone levels

FSH (Follicle Stimulating Hormone), LH (Luteinizing Hormone), PRL1 (Prolactin), T3 (Serum Triiodothyronine), T4 (Serum thyroxine), TSH (Thyroid Stimulating Hormone

The number and size of cysts found in the patients varied from 4-16 and 3-5 mm respectively.
The PCR amplicons of the expected size, which is 219bp and 101bp in case of Exon 10E and Exon 6 respectively, were obtained using genomic DNA of the patients and control. RFLP analysis of Exon 10E using MscI restriction enzyme showed that the PCR product size in lane1 and lane2, whereas the patient sample in lane3-7 showed 2 fragments of length approximately 119bp and 100bp represented in Figure 3. RFLP analysis of Exon 6 using Mun I restriction enzyme showed that the PCR product size in lane1 and lane2, whereas the patient sample in lane3-7 showed two fragments each of length85bp and 16bp indicating no mutation in the patient sample represented in Figure 4.
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Figure 3.RFLP analysis exon 10E of *FSHR* gene in PCOS Patients. Lane1&2- undigested PCR product of approx. 219bp; Lane3-7: show two fragments each of length 119bp and 100bp approx. indicating no mutation in the patient sample.

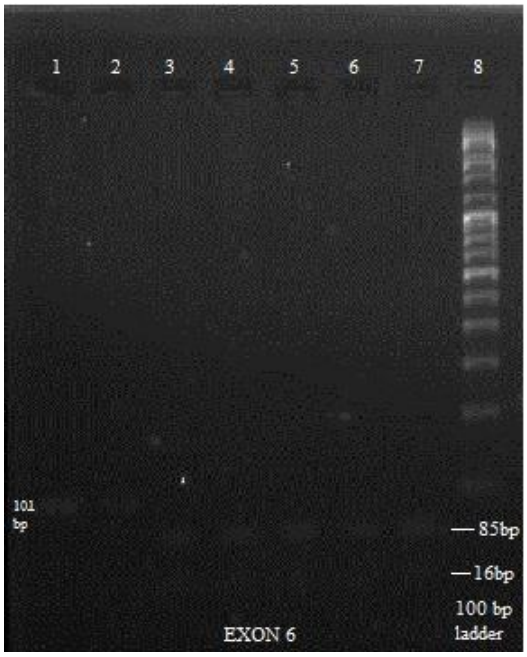

Figure 4.RFLP analysis exon 6 of *FSHR* gene in PCOS Patients: Lane 1& 2-undigested PCR product of approx.101bp; Lane 3-7: show two fragments each of length85bp and 16bp indicating no mutation in the patient sample.

Table 1. Clinical Measurements

Patient code	Age (years)	Height	Weight	BMI	Irregular periods	Acne	Acanthosis Nigricans	Insulin problem	Cardio problem
PCOS1	21	158	86.5	34.64	Yes	During periods	No	No	No
PCOS2	21	154	39.5	16.65	Yes	During periods	No	No	No
PCOS3	24	153	81.6	34.85	Yes	No	No	No	No
PCOS4	25	155	37.2	15.48	Yes	Medium	No	No	No
PCOS5	29	163	74	27.85	Yes	No	No	No	No
PCOS6	23	145	51	24.25	Yes	Medium	No	No	No
PCOS7	31	153	74	35.79	Yes	During periods	No	No	No
PCOS8	14	145	66.4	31.58	Yes	No	No	No	No
PCOS9	19	162	72.54	27.64	Yes	No	Yes	No	No
PCOS10	22	158	60.1	24.07	Yes	During periods			

Table 2. Primer Sequence

Region of FSHR gene	Primer sequence	Product size in bp	Exon location
Exon 6	5′ACATTCAAGATAACATAAAC3′ 5′GCGGATCCTTACAGAATCACACTTTC3′	101	Intronic
Exon 10E	5′ CCTTGTGCTCAATGTCCTGG 3′ 5′ GCTTTGGACACAGTGATGAG 3′	219	1601-1820

Recently it has been seen that somatic or germline mutations affect proteins involved in signaling pathways and establish key mechanisms for diseases of the endocrine system. In our study, we have tried finding inactivating mutations in Exon 6 and Exon 10E of follicle stimulating hormone receptor gene in Vellore patients diagnosed with PCOS. However, more studies are required to infer a polymorphism as a biomarker since the results are insignificant due to lesser sample size. When the personal traits in the patient groups were evaluated, it was found that BMI values were higher.

Russell et al.1998 demonstrated similar results in Exon 7 and Exon 10 of FSHR gene in Brazilian patients with PCOS [8]. Another study by Takakura et al. 2001 done on 50 women with idiopathic POF, 38 women with PCOS showed that the absence of point mutations in Japanese patients [9].

However, a novel homozygous mutation in FSHR gene of women diagnosed with primary amenorrhea in western Indian population was demonstrated by Achrekar et al. 2010 [10]. The general mutations in association with FSHR gene including the inactivating mutation and their physical consequences are summarized by Lussiana et al.2008 [11]. Our results along with combining the data of the previous studies it may be deduced that using the restriction enzymes Msc1 and Mun1 may show mutations/polymorphisms of $Ala^{575}Val$ and $Ile^{160}Thr$. $Ala^{575}Val$ mutation causes the decrease in cell surface expression and high internal hormone receptor complex [12]. $Ile^{160}Thr$ mutation shows altered FSHR expression on the cell surface found in heterozygous secondary amenorrhea [11]. Therefore, there is a positive association found between inactivating mutations of the FSHR gene and women with primary and secondary amenorrhea but due to small sample size, the results are insufficient to conclude that the inactivating mutations in Exon 6 and Exon 10E indicates the women with resistant ovarian syndrome, premature ovarian failure as well as PCOS [10].

4. Conclusion

Although we cannot exclude the possibility of the presence of inactivating mutations in other regions of FSHR gene, the inactivating mutations in Exon 6 and Exon 10E may be considered uncommon in Vellore patients with PCOS. Polymorphisms/mutation studies with the larger population will give more significant results and can also provide insights into various risks factors associated with these mutations in PCOS.

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