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To cite this article: Nishu Sekar *et al* 2017 *IOP Conf. Ser.: Mater. Sci. Eng.* **263** 022036

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Association of Exon 10A and 10B inactivating mutation of follicle stimulating hormone receptor gene (FSHR) and Polycystic Ovarian Syndrome in Vellore cohort

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Abstract. Polycystic ovarian syndrome is the most common heterogenous endocrine disorder in women. Follicle stimulating hormone receptor is associated with normal development as well as maturation of follicles and triggers estrogen production in granulosa cells of the ovary. Inactivating mutation in FSHR gene correlated with reduction of ovarian function in women is due to damage to receptor function. This study aims to investigate whether inactivating mutations, in follicle stimulating hormone receptor gene is related to polycystic ovarian morphology in women with PCOS. Genomic DNA isolated from 15 subjects from Sandhya Hospital, Vellore (10 patients with PCOS and 5 healthy controls) was taken for this study. Patient data included a clinical report, hormonal levels, and ovarian morphological details. DNA isolation was followed by DNA amplification by polymerase chain reaction using Exon 10 A and Exon 10 B primers. The PCR-RFLP analysis was performed using DdeI restriction enzyme. Here we discuss inactivating mutation found in Exon 10 of FSHR gene in patients with PCOS. The absence of inactivating mutation was observed through PCR-RFLP study on Exon 10A and Exon 10B.

1. Introduction

Polycystic ovarian syndrome (PCOS) is a frequently observed endocrine disorder in women of conceptive age. Polycystic ovary disorder is a complicatedly inherited condition, an exceptionally common heterogeneous disorder of clinical and additionally biochemical androgen abundance, ovulatory dysfunction and PCO. The clinical symptoms differ from person to person [3]. The 2003 Rotterdam criteria consist of amenorrhea, hyperandrogenism and polycystic ovary morphology. Women with PCOS are at higher risk of developing metabolic syndromes such as diabetes, dyslipidemia, obesity and high blood pressure which cause heart-related diseases [16]. Although the development of the disease is not clear, factors such as environment and influence of heredity also play a major role in the cause of the disease. Studies show that there are various genes related to this syndrome. These include growth hormone receptors such as exon 3, CYP11A1 and GnRH. The FSH is important in women for the normality of follicles, for steroid genesis and oocyte maturation. It triggers FSH receptor by its function [3].

FSHR is present on chromosome 2p21 and is of G-protein family. It consists of 10 exons and 9 introns at the promoter area of the chromosome. It is a heterodimeric glycoprotein synthesized by the pituitary gland. FSH is responsible for the normal functioning of follicular growth and maturation in females [4].



Studies show that FSHR is relevant for studying the regulation of ovaries with respect to the hormone action, determining the hyper stimulation of ovaries, causing pre-mature ovarian failure and PCOS. The FSH acts together with a particular receptor (FSHR) which is expressed by the granulosa cells present in the ovaries [2]. When the hormone binds to the receptor it triggers the activation of Nucleotide-binding G proteins that cause an intracellular effect of releasing the enzyme adenylyl cyclase [2]. The G-protein family is associated with this receptor. These receptors have an exceptionally normal structure of seven trans membrane domains, containing a big extracellular domain in N-terminal part of a polypeptide which is necessary for communications [5]. In chromosome 2 the gene coding for FSHR often displays variations caused by point mutations in the amino acid assembly of the receptor protein. Due to the structural alteration the functional properties of the receptor are affected that may be either upgraded (activating) or impeded (inactivating). Total block function of the receptor is reduced due to inactivating mutations. This causes damage to the formation of receptor-ligand interaction or FSH signal pathway [7]. Determination of the inactivating mutation in the FSHR gene is a crucial step in knowing the role played by FSHR in polycystic ovarian syndrome (PCOS). We, therefore, took 15 samples from the Vellore population and did DNA isolation and PCR-RFLP to identify the possible polymorphisms or inactivating mutations in the A and B regions of exon 10.



Figure 1.Location of FSHR on chromosome 2 [17]

2. Materials and Method

2.1 Study population

All the subjects incorporated in this study were from Sandhya Hospital, Vellore. All the women present in this study gave their well-briefed consent, and collection of peripheral blood samples for molecular studies, approved by Institute of Ethical Community, VIT Vellore, India. A total sample size of 15 (10 patient of PCOS and 5 control) was used for the study. These women were aged between 18-28 years old [8]. The data collection sheet consisted of clinical factors such as acne, Acanthosis Nigricans, obesity problems, cardio problems and irregular periods. The patients were also tested for hormonal levels and the test included were that of FSH, LH, PRL1, T3, T4, TSH, Total testosterone and free Testosterone. The ovarian Morphology was studied using Ultrasonography (USG) [10].

2.2 Inclusion criteria

The age group of subjects ranged from 18-35 and were recognized using the 2006-Androgen Excess (AES) criteria: one hyperandrogenism, clinical or biochemical and either; two oligo-anovulation or third polycystic ovarian morphology [9,10].

2.3 Exclusion criteria

Women with inherited disorders like congenital adrenal hyperplasia, androgen secreting neoplasms, androgenic/anabolic Drug use or abuse, Cushing's syndrome, syndromes of severe insulin resistance, thyroid dysfunction and hyperprolactinemia were excluded from the study. The age of subject ranged from 18-35 years and hirsutism, acne or male-type alopecia were absent. Each one of the controls had normal menstrual cycles and none of them were suitable for any of the AES-2006 criteria. Every healthy subject also was examined by ultrasonography and found with the absence of cysts in the ovary [9,10].

Table 1. Baseline characteristics of all patients

Patient Code	Age	Height	Weight	BMI	Age of problem Recognised	Marital Status	Problem Recognised Since
PCOS 01	24	143	38.8	18.97	23	NO	1 year
PCOS 02	19	150	60	26.66	18	YES	1 year
PCOS 03	25	160	68.8	26.88	19	YES	6 year
PCOS 04	27	156	80	32.87	26	YES	1 year
PCOS 05	24	150	63	28	17	YES	3 months
PCOS 06	18	146	52.5	24.63	18	NO	6 months
PCOS 07	26	148	55	25.1	24	YES	2 year
PCOS 08	25	163	105	39.52	20	YES	5 year
PCOS 09	23	157	72.1	29.25	23	YES	6 months
PCOS 10	28	154	87.2	36.76	13	YES	15 years

2.4 Genotype determination of FSHR gene EXON 10 A

DNA was isolated from peripheral blood using DNA isolation technique. Amplification of DNA was done by using 5'GCT ATA CTG GAT CTG AGA TG 3' as forward primer and 5'ACC ACT TCA TTG CAT AAG TC 3' as reverse primer by PCR in a thermal cycler (Eppendorf master cycler) using 20µl PCR mixture containing 1µl of each primer, 9µl of milli-Q water and 6µl master mix (Amplicon). DNA sample (3 µl) was multiplied for 35 cycles with initial denaturation for 5 minutes at 95°C followed by denaturation at 95°C for 1 minute, annealing at 52°C for 30 seconds, extension for 50 seconds at 72°C and final extension for 10 minutes at 72°C. The PCR products were separated by electrophoresis on 2% agarose gel containing 5 µl ethidium bromide (50 µg/µl) and were observed using a UV Transilluminator (Medox). PCR method resulted in a 221 bp product. These products were digested using fast digestion restriction enzyme *DdeI* for 5 minutes at 37°C. Digested PCR products were subjected to electrophoresis on 3% agarose gel, visualized under UV Transilluminator (Medox) and photographed using Gel Doc software.

Table 2.RFLP analysis carried out for detection mutations of FSHR gene giving information about primers and enzymes used along with other conditions

Mutations/ polymorphism	PCR primer used	Enzyme used	Enzyme per reaction of 20 μ l	Digestion at 37°C (min)
C1043G(Pro348Arg)	11F,12R	Dde I	5	5

2.5. Genotype determination of FSHR gene EXON 10B

DNA was extracted from circumferential blood using DNA isolation technique. Amplification of DNA was done by using 5'TTGACATGACGTACACTGAG3' as forward primer and 5'CAACTGATGCAATGAGCAG3' as a reverse primer by PCR in a thermal cycler (Eppendorf master cycler) using 20 μ l PCR mixture containing 1 μ l of each primer, 9 μ l of milli-Q water and 6 μ l master mix (Amplicon). DNA sample (3 μ l) was multiplied for 35 cycles with initial denaturation for 5 minutes at 95°C followed by denaturation at 95°C for 1 minute, annealing at 52°C for 30 seconds, extension for 50 seconds at 72°C and final extension for 10 minutes at 72°C. The PCR products were separated by electrophoresis on 2% agarose gel containing 5 μ l ethidium bromide (50 μ g/ μ l) and were visualized using a UV Transilluminator (Medox). PCR method resulted in a 221 bp product. These products were digested using fast digestion restriction enzyme *DdeI* for 5 minutes at 37°C. Digested PCR products were subjected to electrophoresis on 3% agarose gel, visualized under UV Transilluminator (Medox) and photographed using Gel Dock software.

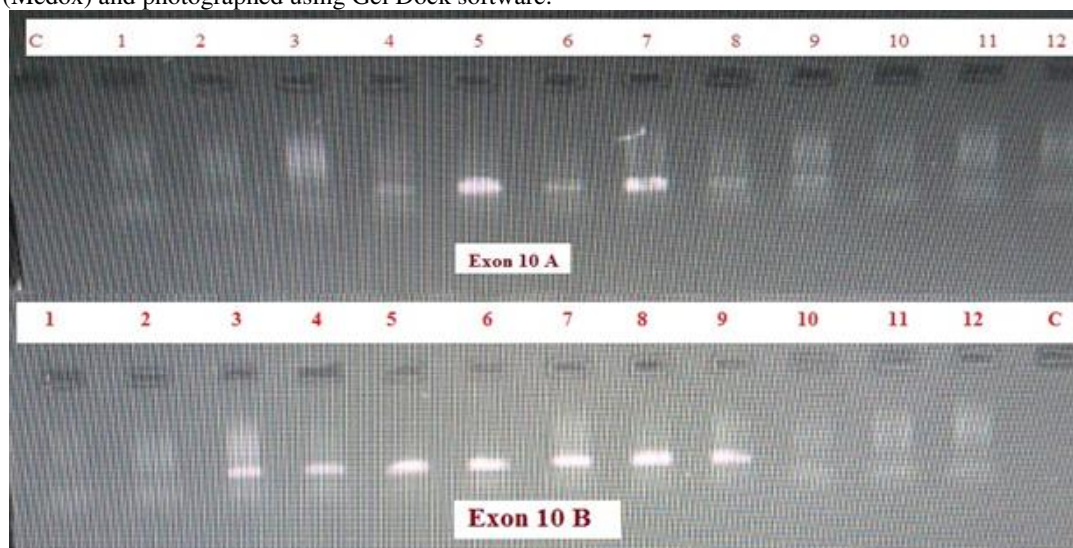


Figure 2. Gel image of PCR product

3. Results

In all the subjects BMI and the hormonal profile was studied as seen in Table 1 and Figure 3. All of the subjects showed irregular menstruation cycles. 60% had obesity problems with reference to BMI calculated and while some showed acne problem. The morphology of right and left ovary and number and size of cysts was studied using ultrasonography which was satisfying the Rotterdam criteria as seen in Table 4. Most of the subjects showed increased ovarian volume (>10 ml) [10]. Restriction enzyme *DdeI* was used for screening C¹⁰⁴³G transition in the subjects with help of RFLP analysis. The recognition site for *DdeI* is C^ATNAG, where the enzyme cuts between C and T.

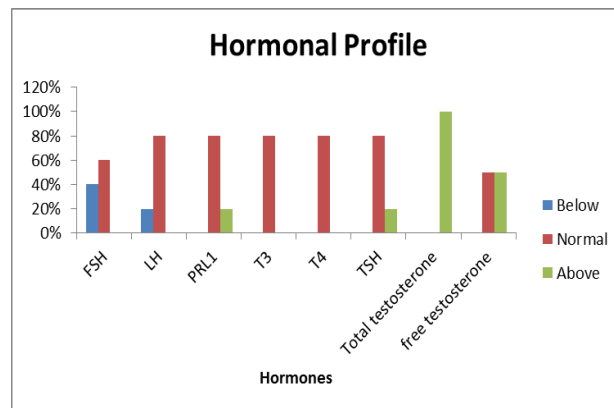


Figure 3.Hormonal Profile

The hormonal profile concluded that various hormonal levels such as FSH, LH, PR1 (Prolactin), T3 (triiodothyronine), T4 (thyroxine), TSH (thyroid stimulating hormone) were found to be within normal range whereas free testosterone and total testosterone were elevated than normal.

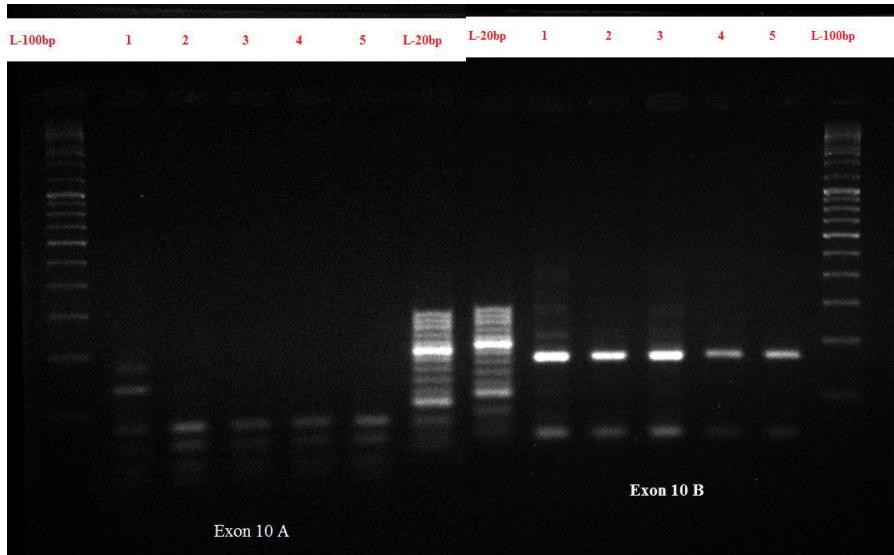


Figure 4.RFLP analysis of Exon 10A

Figure 5.RFLP analysis of Exon 10B

In figure 4 before 1st lane a 100bp ladder is added and after the 5th lane, 20bp ladder is added. From 2nd lane onwards 3 bands are observed. In figure 5 before 1st lane 20bp ladder is added and after 5th lane 100bp ladder is added. From 1st lane onwards 2 bands are observed.

Table 3.Physiological characteristics of all patients

Patients Code	Irregular periods	Insulin problem	Obesity problem	Cardio problem	Acne	Acanthosis Nigricans
PCOS 1	Yes	NO	NO	NO	NO	NO
PCOS 2	Yes	NO	YES	NO	MEDIUM	NO
PCOS 3	Yes	NO	YES	NO	MEDIUM	NO
PCOS 4	Yes	NO	YES	NO	DURING PERIODS	NO
PCOS 5	Yes	NO	YES	NO	NO	NO
PCOS 6	Yes	NO	YES	NO	LESS	NO
PCOS 7	Yes	NO	NO	NO	MEDIUM	NO
PCOS 8	Yes	NO	YES	NO	MEDIUM	NO
PCOS 9	Yes	NO	NO	NO	DURING PERIODS	NO
PCOS 10	Yes	NO	NO	NO	NO	NO

Table 4.Ovarian report of all PCOS subjects

Patients code	ovary measurement(cente meter)		No ofFollicles cyst		Size of Follicles(milli meter)	
	RIGHT SIDE	LEFT SIDE	RIGHT SIDE	LEFT SIDE	RIGHT SIDE	LEFT SIDE
PCOS 01	3.6 X 2 X 1	3.5 X 2 X 1	10	11	3	5
PCOS 02	3.1 X 1.6 X 1	3.5 X 1.5 X 1	10	10	4	4
PCOS 03	4.3 X 3.2 X 2.7	3.7 X 3.4 X 2.9	9	12	5	4
PCOS 04	2.8 X 3.1 X 2.7	3.5 X 2.7 X 2.6	12	11	4	6
PCOS 05	3.7 X 2.8 X 1.7	4 X 2.5 X 2	8	11	5	5
PCOS 06	40 X 26.1 X 2	44.1 X 23.5 X 2	9	10	3	3
PCOS 07	47 X 2.5 X 17	42 X 17 X 25	8	9	6	5
PCOS 08	3.5 X 2.4 X 2	3.1 X 2 X 1	10	12	5	5
PCOS 09	3.3 X 2.8 X 1.7	4 X 2.7 X 2	7	9	3	5
PCOS 10	4.5 X 3.5 X 2.9	3.7 X 3.1 X 2.7	11	13	4	4

4. Discussion

Follicle development and maturation are important for fertility in females and this depends upon the coordination of FSH and its receptor. The differing capacity of the receptor to attach and trigger signal transduction pathway is the result of a genotypic change in FSHR [11].

Any mutation or the genetic alteration which causes loss of function or reduced function of a protein is called inactivating mutation. Inactivating FSHR mutations are classified as autosomal recessive and clinical features are seen in homozygous and compound heterozygous individuals. In females this hormone is necessary for follicle development, greater loss of function mutation leads to ovarian digenesis, amenorrhea, and infertility [12].

Linda et al. in 2003 described inactivating Pro³⁴⁸Arg mutation in FSHR in a subject having primary Amenorrhea [2]. Other inactivating mutations discovered include; T⁴⁷⁹C, C¹⁷¹⁷T, A⁶⁷¹T, C¹⁸⁰¹G, G¹²⁵⁵A, A¹⁵⁵⁶C, T⁶⁶²G and are single case reports [11]. Women with Ovarian dysgenesis (ODG) were reported with an inactivating mutation in FSHR gene [13].

Besides the inactive mutations identified in the extra cellular domain, there are various other mutations identified in the Trans membrane domain as well. In women with PCOS, a mutation at position Ile411Asn position was identified [14]. By studying the genotypic and phenotypic analysis and in vitro observations of inactivating mutations of FSHR it offers insight into the mutated residue and the responsible site of hormone receptor binding and signal transduction. When there is a defect at the level of maturation of the receptor, certain mutations are involved in cell surface trafficking Janovick et al. (2009) [15]. Our study was restricted to a small native population of Vellore, South India. The PCR-RFLP analysis which was performed on the subjects of PCOS showed that there was the absence of inactivating mutations. Since the enzyme DdeI, cuts at its recognition site C[^]TNAG between C and T, variation in bands were not observed.

5. Conclusion

As our study comprised of a smaller population, polymorphism in the gene could not be studied. In our study known inactivating mutation in FSHR gene was absent in the subjects with PCOS.

Acknowledgments

The authors would like to thank the management of VIT University for providing the excellent facilities to carry out this work.

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