Single Nucleotide Polymorphism SNP Analysis of Exon6Follistatin Gene Relatedtopolycystic Ovarian Syndrome inThiQar Governorate.

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Abstract

Follistatin has been identifiedas a nominee gene for polycystic ovary syndrome (PCOS) from relationship studies.

Acting to control the developmentof ovarian follicles and as an antagonist to aromatase action, changes in Follistatinrole or expression may affect main features of PCOS alteration.

The aim of the recent study was to study the association of *FST* gene to define if genomicdifferencesarerelated with phenotypic structures of PCOS patients in a case–control suggestion study.

Methods: This study involved 30 women with PCOS and 20 healthy women, whowerematched by their ageand anthropometric and biochemical profiles.The study was designed to examine theseparation of deoxyribonucleic acid (DNA), and genotype analysis was done for all the study participants using PCR-SNP.

Results: Polymorphisms (SNPs) in the exon 6 *FST* gene region were determined for PCOS patients and controls, it was found that PCOS patientshad17 SNP in different samples, and after performing amino acid sequences alignment for these SNPs, it was observed that the last two SNPs of sample C12 constituted a non-synonym mutation, and the functional conformation of the produced FST gene weremodified with regard to exon 6 in the genotype C12. When this exonic SNP appears in the three-dimensional structure of the resulting proteins of C12 genotypes, the positively charged amino acid, Asp, is converted into another positively charged amino acid, His, in position 331 of the whole amino acid sequence of *FST* protein.**The conclusion**is that this, in turn doesn't change the final phenotypic manifestation of the protein, and there was no alteration in the phenotypes of the PCOS patients.

Keywords: polycystic ovary syndrome PCOS, in silico , SNPs, exon 6 *FST* gene

الخلاصة

يعرف الفولستاتينعلى انه من اكثر الجينات ارتباطا بمرض تكيس المبايض اذ انه يعمل على السيطرة على نمو الحويصلات المبيض وكذلك السيطرة على فعالية انزيم الاروماتيز وان أي تغيير في هذا الجين يؤدي الى زيادة امراضية النساء المصابات بتكيس المبايض وان الهدف من الدراسة الحالية هو دراسة العلاقة بين الجين المذكور ومرض تكيس المبايض مقارنة مع النساء الطبيعيات.

تضمنت هذ الدراسة 30 امراة مصابة بتكيس المبايض و20 امراة غير مصابة و تمت المقارنة بينهن من الناحية المظهرية وقياس المعايير الهرمونية وكذلك دراسة التغيرات الجينية.

لقد وجد من نتائج الدراسة ان النساء المصابات بتكيس المبايض يعانين من الزيادة في الوزن وكذلك ظهور شعر في مناطق غير مرغوب بها وزيادة في هرمون اللوتيني ونقصان في الهرمون المولد للحويصلات اما من الناحية الجينية فقد لوحظ وجود 17 تغير جيني عند النساء المصابات بتكيس المبايض وبعد مطابقة هذه التغيرات الجينية مع التغيرات المحتملة في البروتين وجد ان فقط 2 من التغيرات الجينية التي تسمى الطفرة الوراثية الصامتة التي تؤدي الى التغير من بروتين موجب الشحنة الى بروتين موجب الشحنة ايضا الا انه هذا التغير لا يؤثر على التغيرات المظهرية او امراضية تكيس المبايض .

الكلمات المفتاحية: متلازمة تكيس المبايض، جين الفولستاتين، تتابع النيوكليوتيدات.

Introduction

Polycystic OvarySyndrome (PCOS) is considered one of most common endocrine complaints among femalesof childbearing age. This syndrome was first described by Leventhal and Stein, in1952. At thattime, it was known to be related to ovarian cysts.In 1990,the National Institute of Health estimated that this disorder affects 6-8 % of women in the world(March *et al*., 2010). This syndrome results in many complications, which includereduced fertility, obesity, excess hair growth, and an increased risk of developing type 2 diabetes (Kaushal *et al*, 2004) .DevelopingPCOS depends on the interaction of genetic and environmental factors (Wijeyaratne, 2011).Many genetic metabolic pathways have interacted in PCOS, like the steroid hormone metabolic pathway, the action of gonadotropins, and the metabolism of the hormone, insulin (Ewens *et al*., 2011).Many studies have been done,for example, one included 150 families to examine the genetic role in the occurrence of the disease. Researchers found that there are 37 genes that contribute to PCOS, evidence has indicated that the most common disease-causing gene is Follistatin (*FST*) (Urbanek *et al*, 1999).Follistatincontrolsactivin, which is responsiblefor follicular ovaryexpansion, reticence of theca cell androgen manufacture and increasesin bothinsulin secretionand the follicle stimulating hormone FSH secretion (Calvo *et al*., 2001). FST is mainly produced in the granulosa cells of theantral follicle of the ovary, Follistatin mRNA is present within the main follicle during growth and declines during the atreticdevelopment (Lin *et al*., 2003).Follistatinplays a main role in aromatase action in the granulosa cell, and also LH-stimulated progesterone relief from theca cells (Phillips and Krester, 1998).

High levels of Follistatinin mice has resulted in a decline in follicular levels of FSH, both important phenotypes of PCOS (Urbanek *et al*., 2000). Many studies have found that there is a strong relationship between Follistatin and PCOS, as Follistatin islinkedto activin and impedes its work, which is stimulating FSH as they havefound that the genetic changesin the gene FST causes a relative reduction in FSH concentration in serum (Odunsi *et al*., 1999). On the other hand, *FST*controls the growth of follicles in the ovariesand as an opponent to aromatase action, changes in Follistatinrole or utteranceresulting in maintopographies of PCOS, for example, decreasedthe levels of FSH, and weakenedthe growth of follicular and androgen synthesis . Follistatin, or activin-binding protein is a monomeric encoded by a single gene, *FST*, this gene is located on chromosome 5 q11.2 and receptors of this protein exist in most of the body tissues. However, the essential role of Follistatin is binding toActivin, which is a protein that works on the secretion, stimulation, and synthesis of follicle-stimulating hormones. Activin works also to regulate the menstrual cycle. Contrary to Activin, another protein called Inhibin works to inhibit the production of FSH (Jones *et al*, 2007).When first discovered, Follistatin was called FSH Suppressing Protein(FSP). It was isolated from the Follicular fluid, and it was noticed that it has an inhibitory effect on the anterior pituitary, and specifically on the secretion of follicle-stimulating hormone (Nakatani *et al.*, 2008). Follistatin is secreted by cells in the anterior pituitary called folliculostellate cells (FS). Those cells are in contact with the other cells of the anterior pituitary, including Gonadotropic cells. In tissues, a cell proliferation is induced by Activin, and that's why Follistatin role is to regulate cell proliferation to prevent over proliferation of cells. Also Follistatin contributes tocell differentiation; the main role of Follistatin is inducing the differentiation of ovarian follicular cells, and also to work on the differentiation of estrogen producing granulosa cells (EC) from the grown follicle. Follistatin also

contributes to producing progesterone through stimulating progesterone producing large lutein cells (LLC) (Reis *et al*., 2007).

Many studies have demonstrated that there's a strong connection between Follistatin and Polycystic Ovaries Syndrome, as Follistatin binds to Activinand affects its work which is to stimulate FSH,it was found that the excessive genetic changes of FST cause a decrease in the concentration of FSH in the blood serum. (Odunsi *et al*, 1999). The purpose of thisresearch was to study the change in the nucleotide bases sequencing of exon 6 toFollistatin gene FST, and how that relates to PCOS.

Materials and Methods

Blood Sampling and Biochemical Study

5 ml of blood was collected from a sample of 30 women who have PCOS, and who have been coming regularly to Obstetrics & Gynecology hospital of Bint-Alhoda (Thi–Qar province) duringthe period between (November 2015 –April 2016). The ages of these women ranged from twenty to thirty years . Based on Rotterdam criteria (ASRMS ESHRE), these women underwent the following examinations. An ultrasound exam was done onthe twelfth day of the menstrual cycle to check for ovarian cysts. For diagnosing PCOS, the size of the ovary and the number of the follicles were examined in two groups (healthy women and patient) . They were also checked for lack or irregularity of ovulation, excessive secretion of Androgen (androgen excess), and menstrual irregularity (26 to 30 days). They also had no history of endocrine disorders and didn't use drugs or contraception. A sample of 20 normal women also underwent the sameexaminations and proved to have normal ovaries. The blood samples weresplit into two portions. 3 ml of blood was put into atubewithout anticoagulant (EDTA), and lift it 30 minutethen the sample was centrifuged at room temperature to separate the blood serum (20 min/3000 rpm). Then the serum was stored under -20˚C until the examination was performed. The level of the hormones FSH, and LH weremeasured during the follicular phase,according to manufacturer's instructions (BioMérieux, France). The other 2 ml portions of blood wereplaced in an anticoagulant (EDTA) tube.

Genomic DNA Extraction

The genomic DNA was isolated from blood using a Genomic DNA Mini Kit (Geneaid, Taiwan). The extracted genomic DNA was evaluated by 0.8% agarose gel electrophoresis in 1X TAE (40 mMTris acetate; 2 mM EDTA, pH 8.3) using Ethidium bromide (0.5 mg/ml), and quantified using a nanodrop (BioDrop µLITE, Biodrop, UK).

PCR Primers Design

One pair of primers was successfully designed to amplify the exon 6 of FST genetic locus using the NCBI primer BLAST online software (GenBank Acc. No. NG_028911.1). The designed oligonucleotide primer pair is; forward 5′-GAACACAAGAGCGCTTTTTATCTA -3′, and reverse 5′-TGCCATGTTTGAAAATATAAAATTG -3′, which was designed to amplify 1344bp bp to partially cover exon 6 of FST gene.

PCR Analysis

The PCR reaction was performed using AccuPower PCR premix (Cat # K-2012, Bioneer - Korea). The optimum annealing temperatures were determined empirically in our extracted DNA template using gradient PCR (ver. Mastercycler-nexus,

Eppendorf, 22331Hamburg). The amplification was startedby initial denaturation at 94°C for 5 min, followed by denaturation at 94°C for 30 secondsto30 cycles of, annealing at 58.5°C for 30 seconds, and elongation at 72°C for 30 seconds, and was concluded with anending extension at 72°C for 5 minutes. After performing PCR thermocycling, PCR products were verified by electrophoresis on a 1.5% (w/v) agarose gel in 1X TBE buffer (2 mM of EDTA, 89 mM of Tris–Borate, pH 8.3), using a 2000-bp ladder (Cat # D-1010, Bioneer – South Korea) as a molecular massindicator for approval of the size of the PCR products. Gels were stained with ethidium bromide (0.5 mg/ml) and visualized with the gel image documentation system (ChemiDoc, Bio-Rad, USA). All SSCP non-suitable amplicons bands were eliminated.

DNA Sequencing and Sequencing Analysis

Each unique sample's pattern for the amplified FST gene (1344bp) fragment was purified and sequenced from both ends (Macrogen Inc. Geumchen, Seoul, South Korea). Only vibrant chromatographs found from ABI sequence records were further examined, confirming that the annotation and differences are not because of PCR or sequencing artifacts. The reference sequences of exon 6/FST gene (GenBank acc. No. NG_028911.1) were recovered from the NCBI website (http://www.ncbi.nlm.nih.gov). The sequencing outcomes of the PCR products of different DNA sequence patterns were edited, aligned, and associated with their reference sequences using BioEdit Sequence Alignment Editor Software Version 7.1 (DNASTAR, Madison, WI, USA).

Protein Structure Designing

The primary structure designing of each *FST* genotype was begun by mutating the available reference NCBI DNA sequences of exon 6/*FST* gene, by substituting each observed SNP into its reference sequences using BioEdit /Lasergene software to represent the non-synonymous C12 genotype. The whole amino acid sequences of the bovine FST protein were retrieved online from the protein data bank (www.rcsb.org). Thesetwo nucleic acid sequences were translated into amino acids in a reading frame correspondingto the reference *FST* amino acid sequences using the Expasy online program (http://web.expasy.org/translate/). Multiple amino acid sequence alignments weremade between the reference exon 6/*FST* amino acid sequences and its observed genotype using UniprotKB software (www.uniprotkb.org), and the results were represented using BioEdit /Lasergene software. The three-dimensional arrangement of the FST gene was constructed from the online three-dimensional model prediction software; protein homology/analogy recognition engine (Phere2), version 2.0 (www.sbg.bio.ic.ac.uk/phyre2). The virtual proposed changes within its corresponding mutants wereperformed by using PyMOL-v1,7.0.1 software (www.shrodinger.com).

Finding deleterious nsSNP(s) by the SIFT program

sorting intolerant from tolerant (SIFT) software forecasts whether replacement with any of the other amino acids is accepted or damaging for eachsite in the succumbed sequence (Patel *et al*., 2015). The SIFT prediction was given a tolerance index (TI) score ranging from 0.0 to 1.0,which was the normalized probability that the amino acid change was tolerated. A nsSNP with a TI score of V0.05 was considered to be deleterious *i.e*. amino acids with probabilities

< 0.05 were predicted to be harmful. The amino acid sequence of *FST* along with nsSNPs with corresponding amino acid positions were submitted to the SIFT program (http://sift.bii.a-star.edu.sg/www/SIFT_seq_submit2.html).

Validation of the Functional Characterization of nsSNPs(s) by the PANTHER Program

The SIFT predicted nsSNPs were validated by the PANTHER (protein analysis through evolutionary relationship) program (http:// www.pantherdb.org/tools/csnp). This tool guesses the probability of a particular non-synonymous coding SNP to create a foundationfor a functional influence on the protein using Hidden Markov Models (HMM) based modeling and evolutionary relationship. It analyzes the subPSEC (substitution position-specific evolutionary conservation) mark based on an alignment of evolutionarily related proteins. The PSEP (position-specific evolutionary preservation) measures the length of time (in millions of years) a position of thecurrent protein has been preserved by tracing back to its reconstructed direct ancestors. The longer a position has been preserved, the more likely that it will have a deleterious effect. The thresholds we chose were: "probably damaging" (time > 450my, corresponding to a false positive rate of ~0.2 as tested on HumVar), "possibly damaging" (450my > time > 200my, corresponding to a false positive rate of ~0.4) and "probably benign" (time < 200my) (Tang and Thomas, 2016).

Statistical Analysis

The results were statistically analyzed using Minitab (program) and with one ANOVA way test to calculate the mean ± Standard deviation (SD) and the results were compared between the women with PCOS and the control group under a significance level of 0.01.

Results and Discussion

The study was designed to engage the Follistatin (*FST)*gene and polycystic ovary syndrome (PCOS) because there's a significant indication that there is a connection between *FST* gene and PCOS , as Follistatin was considered a likely cause.

Follistatin is an activin-binding protein that deactivates the action of activin in vivo and in vitro and is stated in varioussites, countingthepituitary, adrenal cortex,ovary, and pancreas. Activin, a member of the transmuting growth element superfamily, controls the manufacture of androgens by thecal cells of the ovary, the growth of follicular, and the excretion of FSH by the pituitary and insulin by the pancreas. SinceFollistatinprevents the action of activin, changingFollistatin'saction would be a probableway to affect ovarian follicle growth, androgen synthesis, FSH release, and insulin secretion. All these events have been shown to be disturbed in PCOS links to activin and affect its work, which is to stimulate FSH, it was found that the excessive genetic change of FST causes a decrease of the concentration of FSH in the blood serum (Jones *et al*., 2007).

Biochemical Study

The results of ultrasound examinations of ovaries of women with PCOS showed that there was a largenumber of sacs that contained immature eggs sized 2-9 mm. What is supposed to happen is that; each menstrual cycle, a sac grows to produce one mature egg sized 18-22 mm. But what actually happens here in the sampleis that a

largenumber of sacs grow at the same time and then they all stop growing midway, and so, none of these eggs ever reach the right size and pregnancy doesn't happen. Also the figure shows an enlarged uterus and the sacs appear as a string of pearls as shown in figure(1)

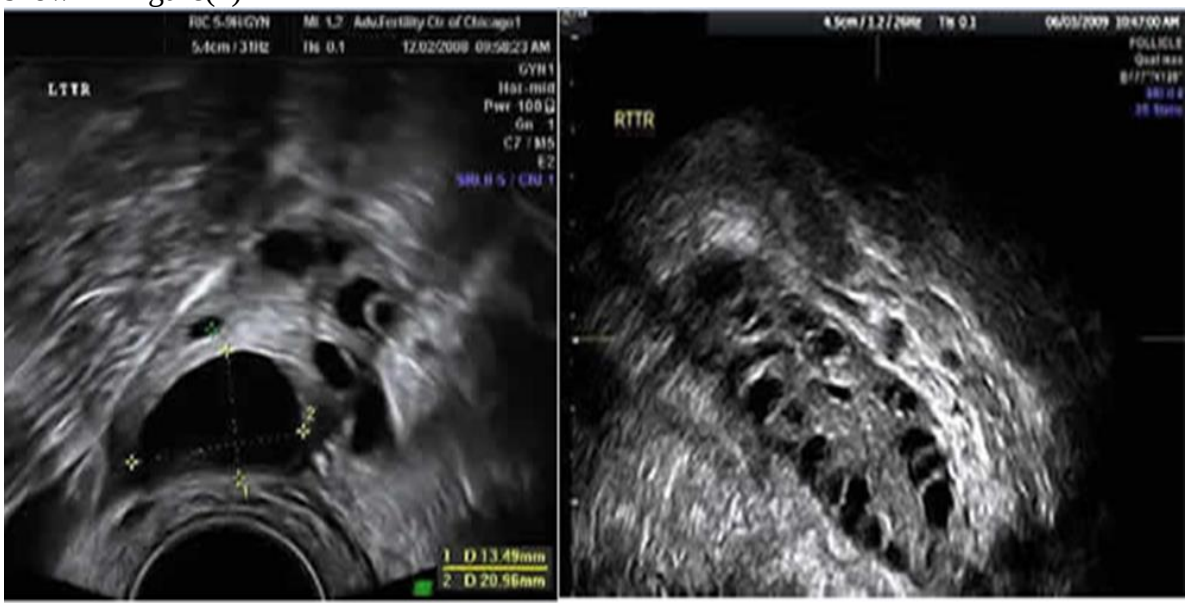

Figure (1): shows a normal ovary on the left and a polycystic ovary on the right

Table (1) shows the body mass of women with PCOS compared to women with normal ovaries. We notice that there is a calculated probability of the body mass of the women with PCOS54.70 ± 11.37on the significance level of p≤ 0.01.

Table (1) shows the body mass of women with PCOS compared to healthy women

	groups	Mean±Std. deviation	Significant
Body mass	PCOS women	54.70 ± 11.37	0.00
	Healthy women	48.31 ± 12.19	

p≤ 0.01

The remarkable increase of the body mass which may reach the degree of obesity, is attributed to the fact that most women with PCOS have insulin resistance which leads to an excess of body fat and an increase in the body mass index,especially in the abdomen (Venkatesan, Dunaif and Corbould, 2001), the study assumed that free fatty acids released from fat cells affect the extraction of the insulin hormone from the liver, and consequently prevents the body cells from absorbing glucose (Kelley, 2002; Holte *et al*., 1994). This study's resultsagreed with of the research done byWijeyaratneand his colleagues (2011) who noticed an increase in the body mass of women with PCOS and attributed it to a deficiency in body metabolism , also the study of Maryam *et al* (2015) showed an increase in the body mass of women with PCOS. The study was done on women aged over 35 years old. This increase is a result of a deficiency in metabolism and may lead to a deficiency in ovulation, and they suggested reducing the body weight to improve ovulation and regulate the menstrual cycle.Table(2) shows a comparison between women with PCOS and healthy women on the basis of the concentrations of pituitary hormones; follicle stimulating hormone

FSH,and luteinizing hormone LH, we notice in this table the relative difference in the concentrations of these two hormones, as the LH concentration is above the normal when compared to the concentration of FSH (elevated LH to FSH ratio). The level of LH in the women with PCOS was 6.51 ± 2.96 before the release of the egg. This refers to a probability of ($P<0.01$) in the level of LH in the women with PCOS when compared to the normal women's group.

Table (2) shows a comparison between the level of LH and of FSH in women with PCOS and healthy women.

Parameters	groups	Mean±Std. deviation	Significant
Follicle stimulating hormone (FSH)	PCOS women	5.08 ± 0.98	0.001
	Healthy women	8.62 ± 3.1	
Luteinizing hormone (LH)	PCOS women	6.51 ± 2.96	0.00
	Healthy women	3.88 ± 1.44	

$p \leq 0.01$

as a result, the ovulation doesn't happen and the egg becomes a cyst and consequently the menstrual cycle and the pregnancy are delayed. (Asuncion, 2000; Louis, 2007). The increase in LH happens because the ovary couldn't secrete Gonadotropin hormones like Estrogen through negative feedback which is under the control of (hypothalamic- pituitary axis) (Louck *et al*., 2000).

Asthe levels of LH hormone stay high in patients with PCOS, this leads to the level of FSH becoming lower when compared to the level of LH. Becausethe LH/FSH ratiobecomes high in blood, the secretion of estrogen will decrease and the secretion of Androgens will increase. One of these Androgens is Testosterone, which is secreted from the ovary and causes the ovary to fail in stimulating the growth and maturity of ovarian follicles and producing graafian follicles,with normalsizes of 18-22 mm, and so, ovulation becomes rare and the menstrual cycle becomes irregular and pregnancy is delayed. (Altuntas *et al*., 2006). This study agreed with the study of (Anlakash *et al*., 2007; Legros*et al*., 2002;GulabKanwar *et al*., 2015) at a remarkable elevation of luteinizing hormone (LH) level was found when compared to follicle-stimulating hormone (FSH), and this elevation causes imbalance of the other hormones relating to the hypothalamus-pituitary-ovary axis.In another study an elevation in the level of LH was found in the serum of women with PCOS. This elevation may cause oversecretion of androgens by the theca interstitial cells (Dhanalakshmi *et al*., 2012) .

When comparing the concentrations of FSH in women with PCOS with womenin thehealthy group 5.08 ± 0.98,alternatively the results showed that there's a probability value of ($P > 0.01$) between the concentration rate of LH in women with PCOS and women with normal ovaries, and this study agreed with (Iwasa *et al*., 2009; Fakhoury *et al*., 2012; Saxena *et al*., 2012) but FSH stimulates the growth of ovarian follicles and so, its level drops below the normal levels, it leads to ovarian failure because it is related to estrogen through a negative feedback mechanism in women with PCOS (Robert *et al*., 2009). The current study agreed with the study of (Fakhoury *et al*., 2012), as this study found an elevation in the LH / FSH ratio and at the same time a reduction in the level of FSH hormone in the serum of Saudi women with PCOS.

Also, in the study of Holteand his colleagues (1994) they noticed that the level of Follicle-stimulating hormone was lower than normal through measuring its concentration in the follicular phase, which indicates the importance of its role in ovulation and that its reduction causes irregularity of ovulation and leads to PCOS.

Genotyping of FST SNPs

Nucleotide sequences from all eight observed genotypes revealed several SNPs. The pattern and nature of each SNP detected by sequencing indicated that the genotype of healthy women beginning with the sample 1 that was called C1 has five SNPs (C 860 G, T 894 C, C 911 G, C 914 G, and T 949 G) (Fig. 4, A), C2 has three SNPs (A 959 T, T 1202 G, and A 1136 T) (Fig. 4, B), C4 has only one SNP (T 1202 G) (Fig. 4, C), C6 has two SNPs (T 529 G, and T 1202 G) (Fig. 4, D), while the genotype of PCOS women in samples : C7 has only one SNP (T 1177 C) (Fig. 4, E), C8 has two SNPs (C 804 A, and C 1095 A) (Fig. 4, F), C9 has only one SNP (A 1167 T) (Fig. 4, G), while C 12 has two SNPs (G 334 C, and G 339 C) (Fig. 4, H). However, after performing amino acid sequences alignment for these SNPs, it was found that the last two SNPs of C12 constitute a non-synonym mutation (Fig. 5), and the functional conformation of the produced *FST* gene is modified with regard to exon 6 in the genotype C12 (Fig. 6). When this exonic SNP appears in the three-dimensional structure of the resulting proteins of C12 genotypes, the positively charged amino acid, Asp, is converted into another positively charged amino acid, His, in the position 331 of the whole amino acid sequences of *FST* proteins. This, in turn doesn't change the conformation in the final phenotypic manifestation of the protein (Fig. 6, A and B). Conversely, the conversion of the uncharged amino acid, Gln, into a positively charged amino acid, His, in the position 322 of the whole amino acid sequences may have a drastic effect on the final confirmation of the *FST* protein and its consequent function(Fig. 6, A and B). To determine the functional effects of nsSNPs in *FST* genes, two widely used *in silico* tools, specifically SIFT and PANTHER were utilized. If a marker is found to be associated with the disease, and the marker is a nsSNP, prediction tools can provide independent evidence as to whether the nsSNP itself contributes to disease. The two examined nsSNP substitutions wereidentified as tolerated by the SIFT program with a score of 0.08 and 0.16 for both D331H and 0.16 respectively (table 3), and this result is differentfrom another result that found another exon not be exon 6 that the FASTA format of protein was assumedto the nsSNP Analyzer device and the difference E152Q andC239S was specified as a response in the SNP data field. E152Qalteration is neutral, as well asthe C239S sources disease.

PANTHER – Evolutionary analysis of coding SNPs: theprotein sequence was analyzed for theE152Q and C239S SNPs. The probability that a givenvariant will affect a harmfulresultof aprotein functionis projected by $P_{\text{deleterious}}$ such that a subPSEC score of −3corresponds to a *P*deleterious of 0.5. (Palanisamy *et al*., 2010).

These two nsSNPs were further validated by the PANTHER program. However, it was found by the later program that both nsSNPs were classified as possibly damaging. Unfortunately, and according to our knowledge, no previous proteomic studies were done thatfocused on bovine exon 6 of the *FST* protein so it is impossible to compare our results with any other study. Thus, thisdata provides the first evidence about the nature of such possiblemutations in this gene. However, it is not necessary that all variants have a maindamaging functional influence and some may be well tolerated. However, nsSNPs which are linked to illnesses or other phenotypes often have some molecular significance (Johnson *et al*., 2005). They may alter enzyme

activity, undermine protein structures, or unsettle protein connections. In the current study, after the extraction of DNA and replication through Polymerase Chain Reaction technique, the gene was multiplied with the use of a particular primer. The size of the gene was 1344 bp in both the patients' samples and the normal women, as this gene is important for it produces the protein of Follistatin which is very important. Any change or mutation in one base (SNP) leads to a change inthe gene and of its production of proteinaccording to our knowledge. Another study found many mutations were found in this gene and were related to PCOS (Liao*et al*., 2000). Another study, which related to SNP of Exon 6 of gene FST it was noticed that the base of 243 changed from T to A in women with PCOS. (MARGRIT *et al*., 2015).

A recent study disagrees with the study of Urbanek *et al*(2000) that detectedthat coding areas and some introns of the Follistatin gene were sequenced, disclosing at least 17 polymorphisms; however, 16 of them were erratic, making a significant influence of these alternatives to the pathogenesis of hyperandrogenismdoubtful. Moreover, the merelypublic polymorphism found, situated in exon 6 but not translated, was not linked with PCOS when correcting for multiple analysis, and the authors concludedthat aids to the etiology of PCOS from the Follistatin gene, if any, are minor.

In theoreticaldisagreement, no mutations in the Follistatingene have been found in Chinese PCOS patients definite bymenstrualdysfunction, hyperandrogenism, and polycysticovaries (Liao *et al*., 2000), and the only mutation wasestablished in a chain ofpatients from Spain, a silent G951A variant, was equallyconcentrated in PCOS patients and in vigorous women (Calvo *et al*., 2001).

In another study,the exon 6 variant (T-to-A) alteration is located 78 nucleotides downstream of the end codon in the 39 untranslated area of exon 6 and, therefore, are not translated. Exon 6 is then again spliced to products of two transcripts. One transcript holds the exon 6 variant site, whereas the other transcript does not. Thus, the functional magnitude of the exon 6 variant is indistinct; andthis variant has been earlierpronounced by Shimasaki *et al*. (1988).

In previous studies,that roster of intronic and identical SNPs, with theirnucleotide site, amino acid alteration information, anddbSNP link, is provided for additionalinvestigation. Scientistswere unable to recognize any transmutation of the stimulating orpreventing type in the whole coding region of Follistatingene in 64 patients with PCOS. Therefore, transmutations in thecodingsites of the Follistatin gene may not be a publicreason of PCOS in the populace studied. However, it isthinkable that mutations may be present in the controllingareaof the gene, which should be partitioned once its sequenceis known. Also, it would be abundantly helpful tostudythe presense ofmutations in PCOS patients inother culturalareas, principally of European background, as itwas in this people that the connection between the Follistatingene and PCOS was recognized(Liao *et al*., 2000).

The conclusions in this study are that awide sequencing of the Follistatin gene hasknown variants at 17 sites in eight testers, but none of these appear to be probable etiological causes in PCOS. Only sample 8, called C12 variant has two nsSNPs compared with the other seven variants, which are not interpreted, and other strictlyconnected polymorphic sites external the Follistatin gene,. Even at exon 6, where the strongest proof was found, the verdicts are, at most, a touch significant; and they are not significant whenalteredfor testing a larger sample. Even though this discovery reduces provision for the Follistatin gene that linked with PCOS patient itself.

Table (3):shwos Functional validations of nsSNPs inFST gen (D, Q amino acid) using SIFT and PANTHER programs.

Amino acid change	SIFT prediction	SIFT score	PANTHER prediction	PNATHER score (PSEC)
D331H	Tolerated	0.08	possibly damaging	361
Q332H	Tolerated	0.16	possibly damaging	455

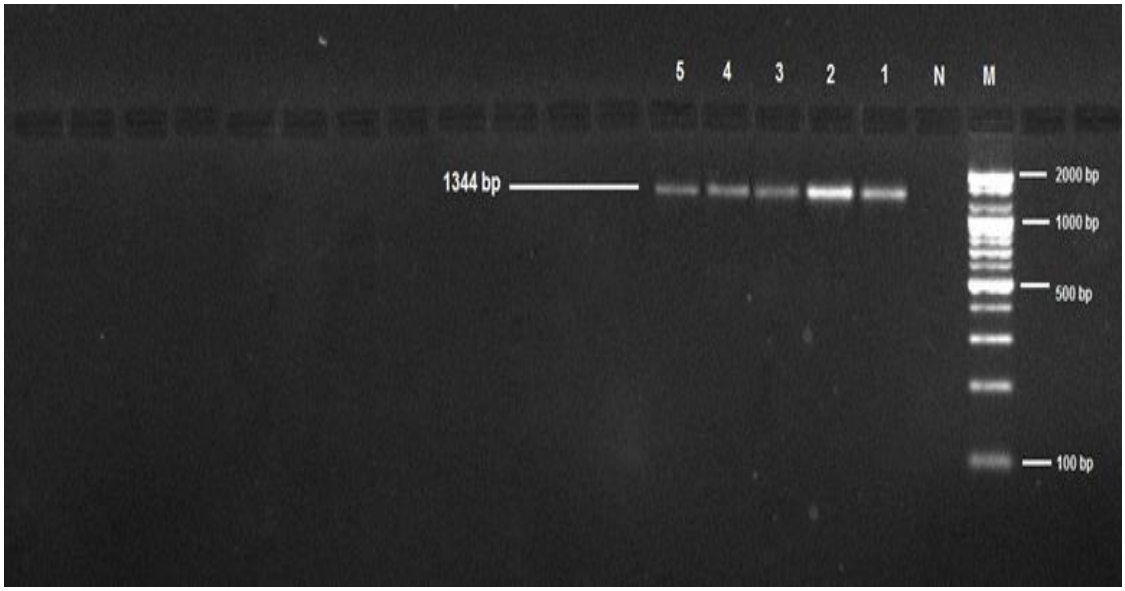

Figure (2) represents Agarose gel electrophoresis the result of gene FST of the normal women group. With a concentration of 1.5 %) volts=70, amps= 50, time= 1hour)

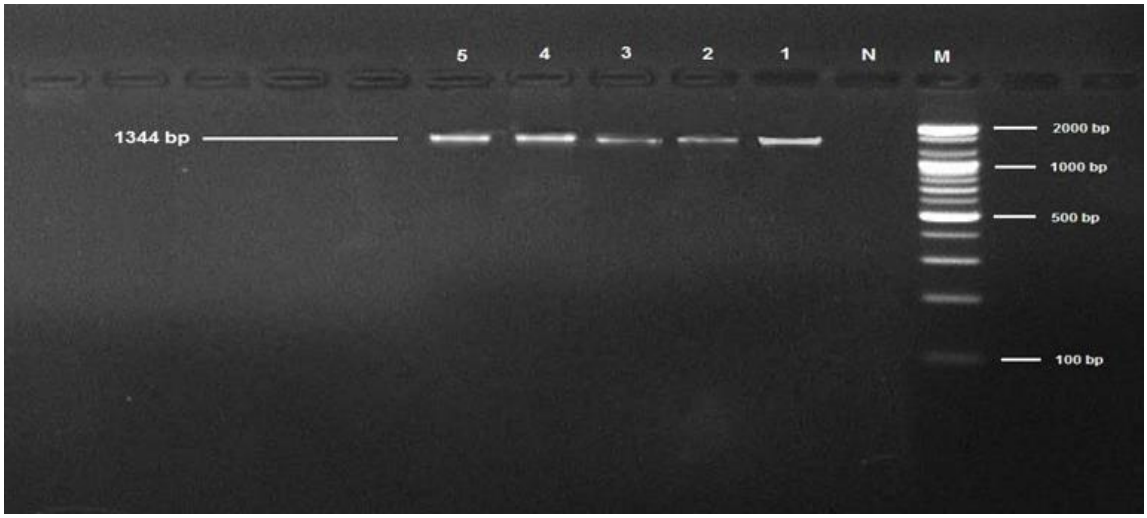

Figure (3) represents Agarose gel electrophoresis the result of gene FST of the group of women with PCOS with a concentration of 1.5 % (volts=70, amps= 50, time= 1hour)

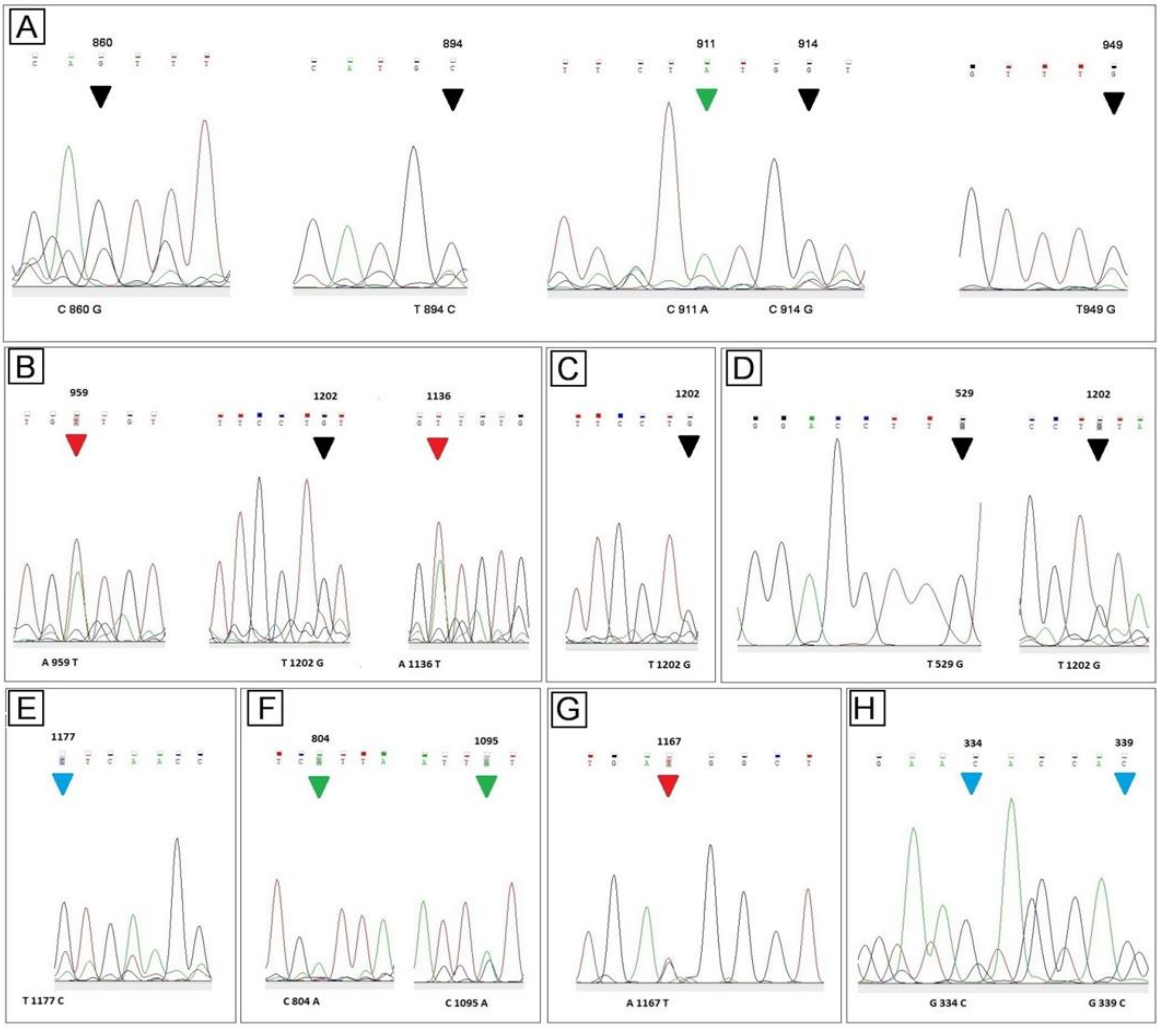

Fig. (4): chromatogram sequencing results of Homo sapiens FST gene, exon no. 6. A) C – G, T – C, C – A, C – G, and T – G SNPs at positions 860, 894, 911, and 949 respectively in C1 genotype of Homo sapiens FST gene, exon no. 6. B): A – T, T – G, and A – T SNPs at positions 959, 1202, and 1136 respectively in C2 genotype of Homo sapiens FST gene, exon no. 6. C): T - G SNP at position 1202 in C4 genotype of Homo sapiens FST gene, exon no. 6. D): T – G SNPs at positions 529, and 1202 respectively in C6 genotype of Homo sapiens FST gene, exon no. 6. E): T – C SNPs at positions 1177 in C7 genotype of Homo sapiens FST gene, exon no. 6. F): C – A SNPs at positions 804 and 1095 in C8 genotype of Homo sapiens FST gene, exon no. 6. G): A – T SNP at position 1167 in C9 genotype of Homo sapiens FST gene, exon no. 6. H): G – C SNPs at position 334 and 339 respectively in C12 genotype of Homo sapiens FST gene, exon no. 6.

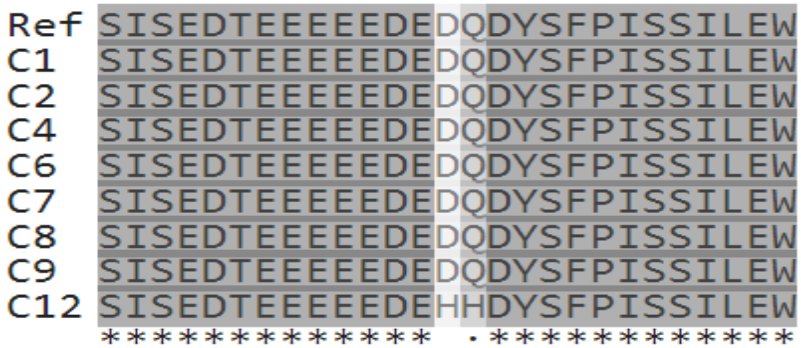

Figure: (5) Multiple sequence alignment of amino acids sequences of FST protein of *Homo sapiens*. The dark grey shaded sequences refer to the similar amino acid sequences. The dots refer to the nature of differences in the amino acid sequences. Only C12 variant has two non-synonym SNPs compared with the other seven variants.

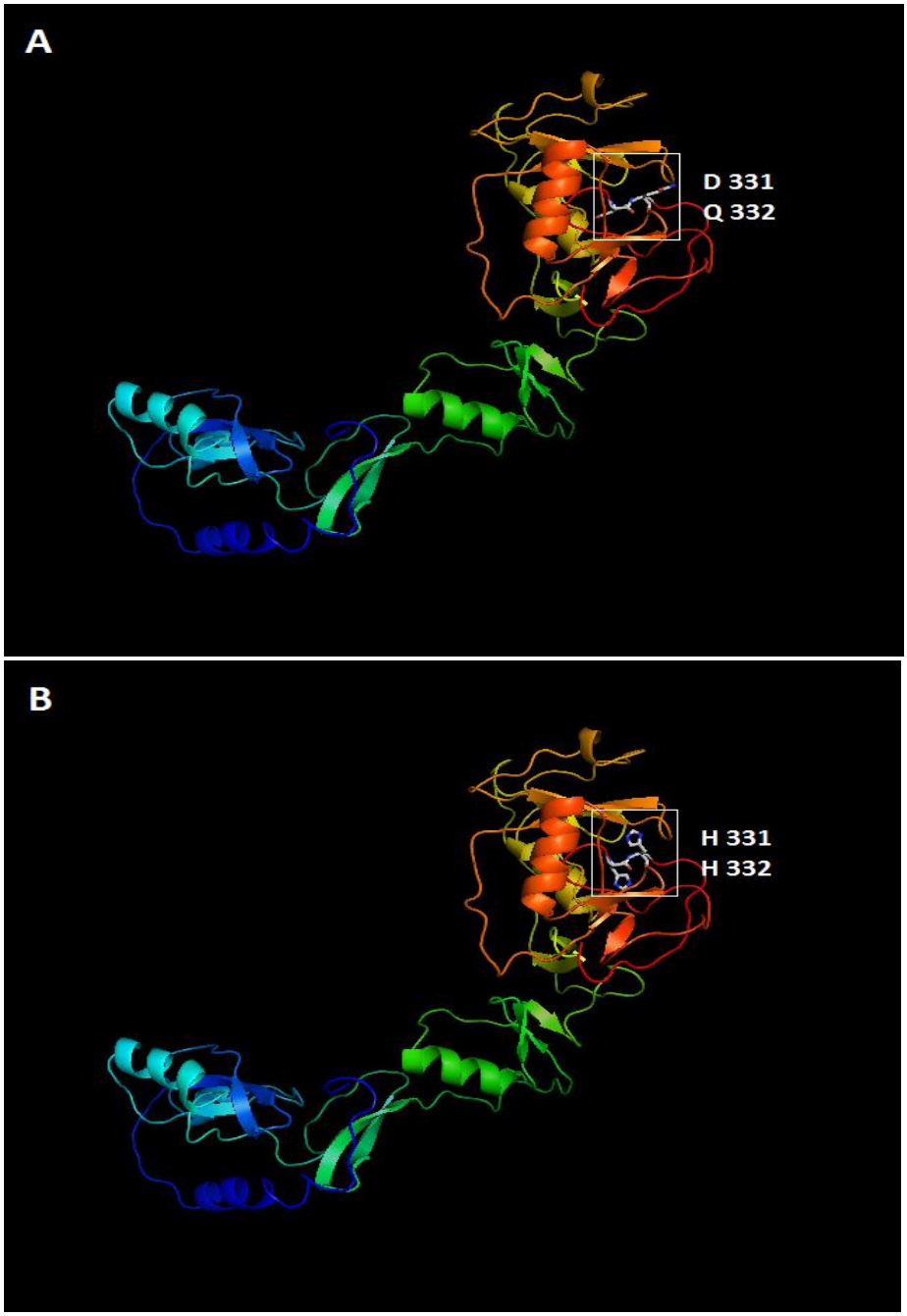

Fig. 6 Postulated three-dimensional structure of the human *FST* protein showing the change of amino acid "D" or "Asp" (in the position 331) and the amino acid "Q" or "Gln" (in the position 332) of the reference protein (A), into amino acid "H" in both positions in only C12 protein variants.

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