The Revelation of Novel Mutations in Human Luteinizing Hormone Beta Subunit Related to Polycystic Ovary Syndrome among Sudanese Women

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Introduction: Polycystic ovary syndrome (POCS) is a mystery disorder with mysterious multiple players characterized by their mystic effects on disease pathophysiology resulting in various phenotypic pictures among the PCOS population. The Luteinizing hormone beta subunit (LH-B) (protein ID P01229) is a gonadotropin hormone secreted from the anterior pituitary belongs to the glycoprotein family, mapped on (chr19p13.3) and consists of three exons. It has a central role in promoting ovulation via stimulation of ovarian steroidogenesis.

Objectives: This is a prospective laboratory- based cross-sectional study to determine genetic mutations associate with polycystic ovary syndrome (PCOS) among (30) Sudanese families ((cases n=35 families, 90 females and (controls n= 11 families, 30 females) in Khartoum State, Sudan.

Methods: Quantitative Enzyme- Linked Immuno-Sorbent Assay (ELISA), enzymatic methods and polymerase chain reaction (PCR) used to analyze both the biochemical parameters and polymorphism detection followed by Sanger sequencing for genotyping in addition to bioinformatics software for protein structure and function.

Results: All the biochemical parameters levels of (FBG, LH, Testosterone, Insulin and lipid profile) elevated from the control group were statistically significant except for the serum FSH (cases=5.4±4.6, controls=5.3±2.8) and PRL ((cases=12.4±8.2, controls=8.0±6.1)) which were statistically insignificant p=0.94 and p=0.06. After Sanger sequencing; (5) single nucleotide polymorphisms ((rs5030775, A18T), rs746167425, R22K), (rs1800447, W28R), (rs35270001, H30R) and (rs34349826, I35T)) located on (exon 2) of *LHβ* gene were statistically significant with serum LH, Testosterone and insulin levels among PCOS families.

Conclusion: This is the first molecular identification of a family- based study in Sudan exploring the genetic of *LHB* gene and interrelated its serum level with PCOS manifestation. The revelation of these mutations will give a clue to the genetic inheritance mode links and might explain the abnormal poor response of controlled ovarian stimulation tests in some PCOS women.

Keywords: PCOS, Luteinizing hormone, SNPs, infertility, family studies

Introduction:

Polycystic ovary syndrome (PCOS) is a disorder that associated with abundant ovarian cysts combined with hormone imbalance and different metabolic disorders.[1,2] We have to admit that PCOS is a disease of contradictory even on his name which has been proposed to renamed completely to a magical name that reflects its complex interchangeable background as an epigenetic as well as metabolic disorder and bring the three common diagnostic criteria (1990 National Institutes of Health's (NIH), the Rotterdam criteria and the Androgen Excess Society) to a one diagnostic platform that fit and cover all phenotypic features of PCOS which could plays a role in reducing the prevalence of PCOS (1.6-18%) among women within the reproductive age.[2-4] Notably, various immuno-assay methods which lack their sensitivity and specificity on determine the accurate results of hormone profile increase the complexity of diagnosing PCOS, therefore, high-quality techniques such as liquid chromatography tandem mass spectrometry (LC-MS/MS) will be preferable to support the accuracy of diagnostic biochemical parameters. Consequently, a better understanding of disease pathophysiology with consentient diagnostic criteria will contribute on developing intellectual community towards PCOS on different levels; PCOS patients who will be more peaceful with disease complications such as type 2 diabetes (T2DM), cardiovascular disorders, and even fertility problems. In addition to increase family awareness towards its genetic background and perspective fertility plans which consequently result in a community with a low prevalence of obesity, dyslipidemia, and infertility.[5, 6]

In general, binding of GnRH to his GPCR on the gonadotropic cell actives the Gαq/11 protein which activate phospholipase Cb, followed by subsequent activation of second messenger inositol trisphosphate (IP3) and diacylglycerol (DAG), via cleavage of phosphatidylinositol 4, 5-bisphosphate. Both IP3 and DAG mobilize intracellular calcium from the endoplasmic reticulum stores, which is essential for beta subunit expression of both LH and FSH. Regarding the ovarian theca cells, LH binds to LHCGR to stimulate androgen synthesis during the follicular phase, stimulate ovulation and production of progesterone from corpus luteum. Steroidogenesis begins with the movement of mitochondrial cholesterol to the inner mitochondrial membrane, where

the cytochrome P450-associated enzyme removes the side chain of cholesterol, producing pregnenolone. [7] Thus excessive theca cell androgen secretion lead to vary of hormonal and metabolic abnormalities such as hirsutism, anovulation, clinical and/or biochemical hyperandrogenism, hyperinsulinemia, insulin resistance (IR), and obesity which are more common signs among PCOS population.[5, 8]

Moreover, the natural origin of PCOS might switch on during fetal life because of abnormal developmental programming associated with the presence of excess glucocorticoids and/or androgens from their mothers which results on dynamic changes in gene expression, resulting hyperinsulinemia, adipocyte dysfunction and childhood visceral obesity, and PCOS.[9, 10] Additionally, various familial aggregation studies suggested that PCOS is an autosomal dominant syndrome and both sisters and brothers of PCOS women might be associated with infertility problems. Recent genome-wide association studies (GWAS) in Han Chinese ladies with PCOS incontestable eleven genetic loci that related to PCOS that are accountable for steroidogenesis, steroid hormones action, gonadotrophin action, and regulation, internal secretion action and secretion, energy physiological state, chronic inflammation, and others.[11-16]

The recent study focused on *LHB* gene to determine the relation between single nucleotide polymorphisms (SNPs) of the *LHB* gene and the clinical features of PCOS among Sudanese women within the reproductive age.

2. Methodology:

This is a prospective laboratory based cross-sectional study carried out in different fertility centers as well as different localities (families' homes) in Khartoum State which approved by the Ethical committee - Federal Ministry of Health (3-12-19). According to the inclusion Criteria: the target population of this study is families (n=90, 35 families) whom have one or two sisters diagnosed as PCOS patients within the reproductive age (20-40) years (according to 2003 Rotterdam criteria). Matching criteria for control group selection was putted in consideration with the special circumstances of the study (n=30 11 families). Any Sudanese ladies below the reproductive age, females with family history of adrenal hyperplasia,

hyperprolactinemia, Cushing syndrome, thyroid disorders, ovarian cancer or breast cancer and causes of infertility rather than PCOS were excluded from the study.

For hormonal profile estimations we used quantitative Enzyme Linked Immuno-Sorbent Assay (ELISA), enzymatic methods for measurement of fasting blood glucose and lipid profile.[17-24]

Then DNA extraction from whole blood according to manufacture instructions and Primer 3 used to design the LHB primers to apply the polymerase chain reaction (PCR) and gel electrophoresis used for band amplification and visualization (Table 1). The PCR Protocol for LHB gene as follow; an initial denaturation at 95°C for 5 minutes, followed by 30 cycles at 95°C for 15 seconds, 54°C for 30 seconds, 72°C for 1 minute and final extension at 72°C for 5 minutes in the final cycle and the Sanger dioxnucleotidue sequencing method used for genotyping.[25, 26]

Bioinformatics software used for protein structure, function and predication. The Basic Local Alignment Search Tool (BLAST) [27] used to discover regions of similarity between genetic sequences in compare to reference genome database. GeneScan software used for detection and annotation of sequence variants [28]. Both Raptor X and USCF Chimera were used for protein modeling and visualization and the web server Project Hope used for the protein 3D structure and the effect of nucleotide variants on the structure and function of protein.[29-31]

Table (1): The *LHB* gene primers for PCR protocol.

Gene	Forward	Length	Reverse	Length	Produc length
LH-β	GGAGGCCTCTTTCTGGAGGG	20	GGAAGCCCTCTGTTCCTGC	19	571

3. Results:

Descriptive and biological information about study groups collected by self-administrated questionnaire and analyzed by IBM SPSS Statistics for Windows, Version 23.0.using descriptive analysis for frequencies of (age, years), BMI, HWR, signs and symptoms and biomedical elements. In addition to independent t-test and analysis of variance for statistical significance, Chi square test and Person correlation for statistical association. [23]

Table (1): elucidated that; the mean and standard deviation of the biochemical parameters among study groups, All the biochemical parameters (Age, FBG, LH, insulin test and lipid profile) were within the normal range and their elevation from the control group were statistically significant except for the serum FSH (cases=5.4±4.6, controls=5.3±2.8) and PRL ((cases=12.4±8.2, controls=8.0±6.1)) which were statistically insignificant (0.94 and 0.06).

Parameters	Study groups	mean± Std. Deviation	P-value
FBG/mmol/l	Case(n=90)	6.2 ±1.8	0.02
	Control(n=30)	5.4±0.9	
LH (ng/dl)	Case(n=90)	17.7±12.3	0.00
	Control(n=30)	9.7±5.4	
FSH (ng/dl)	Case(n=90)	5.4±4.6	0.984
	Control(n=30)	5.4±2.9	
Prolactin (ng/dl)	Case(n=90)	12.4±8.2	0.068
	Control(n=30)	8.0±6.2	
LH FSH ratio	Case(n=90)	5.5±6.1	0.00
	Control(n=30)	1.9±0.8	
TG (mmol/l)	Case(n=90)	1.5±0.6	0.03
	Control(n=30)	1.1±0.3	
T.Chol (mmol/l)	Case(n=90)	4.1±1.5	0.00

	Control(n=30)	2.2±0.4	
HDL(mmol/l)	Case(n=90)	1.2±0.2	0.012
	Control(n=30)	1.4±0.2	
LDL(mmol/l)	Case(n=90)	2.3±1. 6	0.00
	Control(n=30)	0.27±0.5	
INS	Case(n=90)	15.2±8.4	0.01
	Control(n=30)	7.7±6.2	
TesT	Case(n=90)	33.6±18.0	0.00
	Control(n=30)	10.5±7.0	

Table (2): elucidated that; the mean and standard deviation of the biochemical parameters among study groups in regards to the mutations, the Luteinizing hormone (ng/dl) (cases=17.7± 12. controls=9.7±5.3); The mean serum LH level among women who has the 5 SNPs through LHB gene is 17.7. The mean serum LH among women who is free from mutation is 9.7. The mean difference is 8.06. The difference is statistically significant p=0.000. This means LHB mutations influences the level of serum LH level among study group.

Parameter	Mutation		P-value
	Present	Absent	
LH (ng/dl) Mean+ Std. Deviation	17.7±12.3	9.7±5.3	0.00

Table (3): In a sample of 90 PCOS cases, the association of BMI and biological values of LH, FSH, PRL, INS, and TesT.

There is a statistical significant intermediate negative relation among over weight group with LH (r=-0.444, p=0.018) and TesT (r=-0.446, p=0.014) levels. While there is a statistical significant intermediate positive relation of test among lean PCOS women.

Parameters		Normal	Overweight	Obese
LH(ng/dl)	Pearson Correlation	0.406	-.444*	-0.156
	Sig. (2-tailed)	0.095	0.018	0.41
FSH(ng/dl)	Pearson Correlation	0.241	0.249	-0.216
	Sig. (2-tailed)	0.335	0.201	0.252
PRL (ng/dl)	Pearson Correlation	0.2	-0.177	-0.049
	Sig. (2-tailed)	0.427	0.367	0.798
INS	Pearson Correlation	-0.18	0.224	-0.079
	Sig. (2-tailed)	0.474	0.252	0.677
TesT	Pearson Correlation	.594**	0.116	-.446*
	Sig. (2-tailed)	0.009	0.558	0.014

Table (4): In a sample of 90 PCOS cases, the association of BMI, FBG and biological values of LH, FSH, PRL, INS, and TesT, There is a statistical significant strong positive relation between FBG and LH among lean group (r=-0.757, p=0.000), while other groups it is an intermediate correlation as well as for serum TesT level among normal weight (r=0.587, p=0.01).

Parameters		Normal	Overweight	Obese
LH	Pearson Correlation	.757**	.589**	.504**
	Sig. (2-tailed)	0	0.001	0.005
FSH	Pearson Correlation	0.027	-0.219	-0.158
	Sig. (2-tailed)	0.917	0.264	0.404
PRL	Pearson Correlation	.482*	0.026	-0.001
	Sig. (2-tailed)	0.043	0.896	0.997
INS	Pearson Correlation	-0.043	-0.239	-0.182
	Sig. (2-tailed)	0.864	0.221	0.335
TesT	Pearson Correlation	.587*	0.171	.361*
	Sig. (2-tailed)	0.01	0.385	0.05

4. Discussion:

In this recent study different biochemical parameters were investigated associated with *LHB* gene among (25) Sudanese families who had one or more females with PCOS and (11) families as control group. This performed study revealed that the prevalence of menstrual irregularity is (46.8%) irregular menstrual cycle with (54.5%) and (42.9%) had acne and facial hirsutism respectively. More than (70%) of this present study group had increased BMI (30) or higher while (23.4%) were lean and all the biochemical parameters were within the normal range but above the control group of the study except serum HDL, similar to (Shoaib et al., 2015) and disagree with (Sadeghi et al., 2014). Reports from multicenter study with (1466) subjects, of whom (363) had PCOS and (79) polycystic ovaries (PCO) without other symptoms of PCOS associated the homozygotes with recurrent spontaneous abortions, menstrual irregularities with infertility, and PCOS and elevation of testosterone, whereas the Finnish subjects whom were homo- or heterozygous for the polymorphic *LHB* allele were largely healthy. In contrast to the first report of this mutation among male population showed a male patient with HH and provided clinical and experimental evidence, confirming that this novel mutation causes selective LH deficiency whereas other results showed significantly higher serum level of LH in V-LH carriers among the young Baltic men and Estonian male infertility patients. Also, it is observed that sisters of affected women have irregular menstrual cycles which agreed with the Turkish study showed that sisters were having menstrual irregularity and larger ovaries with higher serum androstenedione and dehydroepiandrosterone sulfate levels than sisters without PCO, suggesting a spectrum of clinical phenotypes in PCO families.[24-30]

More than (70%) of this present study population had increased BMI (30) or higher with increased level of serum insulin which is responsible for the central adiposity and on the other hand the findings of the present study were in agreement with (Patel, 2018) [31].

This recent up to date performed study highlighted the metabolic relationship between the occurrence of genetic mutations on exon 2 of *LHB* and its association with PCOS,

moreover, the present results exposed that the presence of (5) single nucleotide polymorphisms of *LHB* gene, that shared between the investigated cases were with homozygous (AA) inheritance mode, located on the signal peptide and the homologue glycoprotein parts could have significant influence on serum level of the hormone but not inactivating or damaging its biological function. The most reported mutations of this research findings were (rs1800447 (W28R) and rs34349826 (I35T)) which associated with protein glycosylation, controlled ovarian stimulation, male infertility and PCOS, while rs5030775 (A18T) associated with more potent in stimulating IP3 response than wild type LH. [32-34] No literature found regarding to rs746167425 (R22K) and rs35270001 (H30R) but in regards to their physiochemical properties; Arginine mutated into a Lysine at position 22 which is smaller but not affecting protein stability. Whereas Histidine located very close to a residue that makes a cysteine bond and supposed to form hydrogen bond with Tryptophan on position 28 mutated to Arginine with positive charge and small in size. Interestingly, this newly positive charge is already mutated on the present cases to Arginine that created an extra glycosylation on the mutated type and might cause loss of hydrophobic interactions with other molecules on the surface of the protein [30].

Signal peptides is a short peptides located in the N-terminal of gene-proteins, responsible for protein recognition, secretion, and often cleaved to generate the mature protein [31]. Therefore, presence of rs5030775 mutation (A18T) within the leader sequence that has been reported as potential stimulator of IP3 more than cAMP pathway could emphasis that alteration of GnRH pulse due to overstimulation of IP3 which in turn increase *LHb* gene expression and might influence abnormal recognition of the prohormone within the endoplasmic reticulum when binding with SRP and thus affect its interaction with LHCGR in contrast to point mutation associated with autosomal recessive familial isolated hypoparathyroidism caused due to improper cleavage by signal peptidase at the normal position[33]. Remarkably, this mutation ((historically reported as Thr-3A) A18T) was also found in 3 (3/100) Rwanda population with heterogeneous pattern might be a sign of evolutionarily background of PCOS origin despite the difference on their inheritance mode and support the idea that high level of serum *LHB* associated

with increased pulse frequencies because high GnRH pulse frequencies favor *LHb* gene expression whereas low GnRH pulse frequencies favor FSHb gene expression[34-36].

Protein glycosylation is one of the most frequent and relevant post-translational modifications and plays a key role in the modulation of protein properties and function mainly on the half- life of the hormone and heterodimer stability and in the intrinsic bioactivity. The carbohydrate content of LH is approximately 15% (N50), that of FSH 20% (N25, N42), and that of hCG 30% (N33, N50) and the circulatory half-lives of the gonadotropins are about (20 mins) for LH, (2 hrs.) for FSH, and (12–24 hrs.) for hCG. Molecular alterations of V-LHB, due to rs1800447 (Trp28Arg) and Ile35Thr (rs34349826) create an extra glycosylation signal (Asn-X-Thr) into the *LHB* chain, which introduces an oligosaccharide side chain into Asn13, similar to that present in HCG, which might increase the affinity binding between the peptide portion of the hormone and the receptor and also increase the life span of the hormone (Shafaghi *et al.,* 2019). Findings on serum samples of variant homozygotes showed that V-LH is more active than WT-LH in bioassays in vitro, but it has a shorter half-life in circulation. The shorter half-life of V-LH is possibly compensated for by about (40%) higher promoter activity of the gene, explained by (8) SNPs in the (50-flanking regions) of *V-LHB.* They were reported as modulatory effect on hyperandrogenemia phenotype of PCOS. More recently, **Alviggi *et al***. have confirmed that *v-LHβ* polymorphism produces a less active form of the hormone which is not able to support satisfactorily FSH activity during controlled ovarian stimulation, and that the *v-LHβ* carriers experience a considerable decrease of the number of transferred embryos, thereby highlighting the essential role of LH/FSH cooperation in the latest stages of follicle maturation.

In conclusion, giving a patient the diagnosis of PCOS makes the patient aware of possible fertility concerns, obesity, diabetes, dyslipidemia, hypertension, and theoretical increased risk of cardiovascular disease. Since PCOS could be genetic, it may bring awareness to family members and future children. Given that insulin resistance is heavily associated with PCOS, these individuals require increased screening and will likely have better long-term outcomes with early lifestyle interventions, as well as

insulin sensitizing medication, such as metformin when indicated. In addition, screening for hyperlipidemia could lead to earlier lifestyle/medical intervention could likely help reduce one's cardiovascular outcomes.

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Revelation of Novel Mutations in Human Luteinizing Hormone Beta Subunit Related to Polycystic Ovary Syndrome among Sudanese Women

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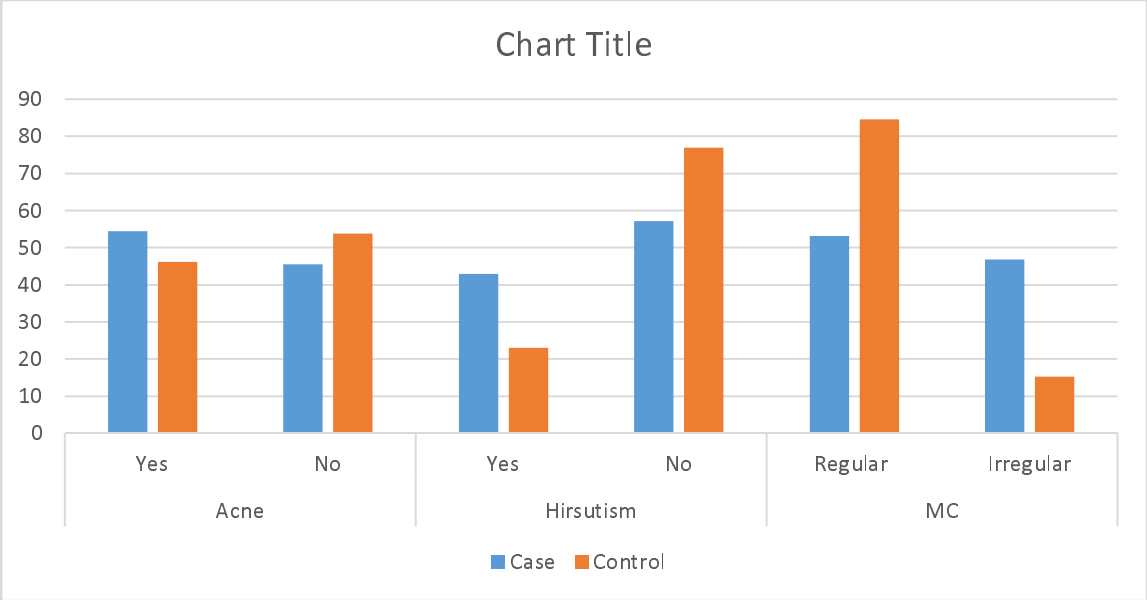


Figure (1): The frequencies of presence of Acne, hirsutism, and status of MC between study groups; (54, 5) but 57.1% of them had no facial hirsutism similar to 77% of control group.84.6% of our control had regular menstrual cycle and only 15.3 had irregular menstrual cycle, while the case group had the higher distribution than control group.

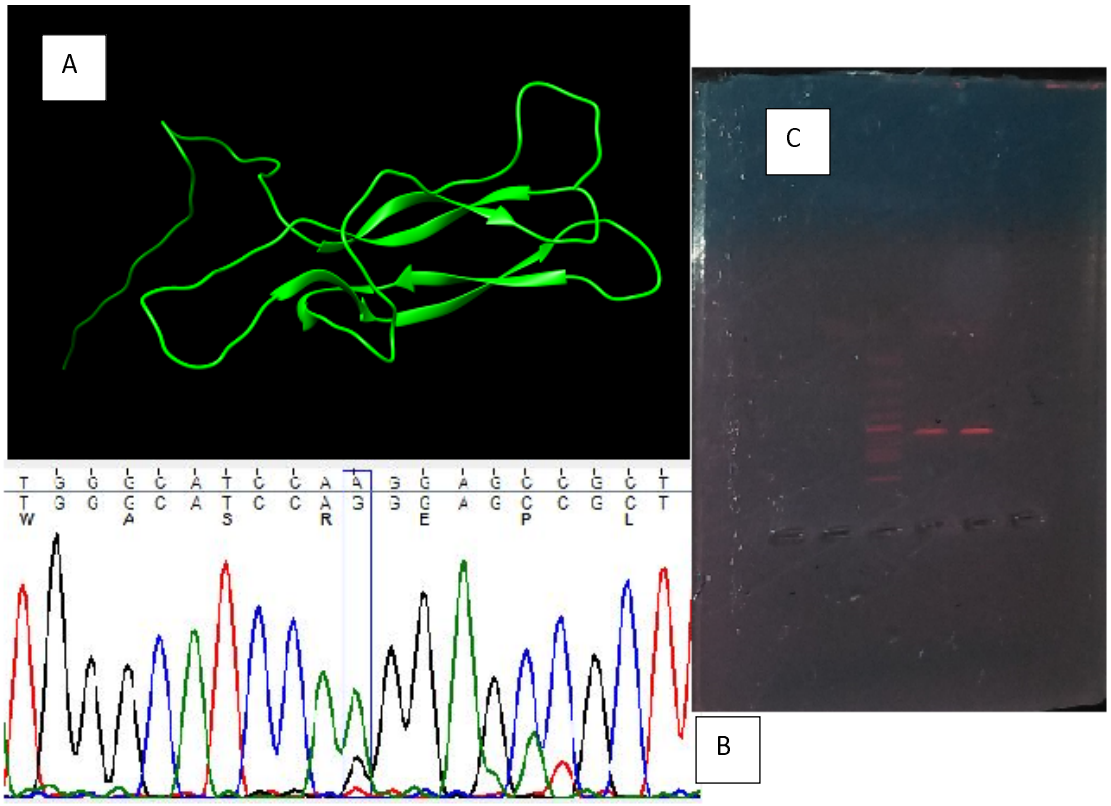
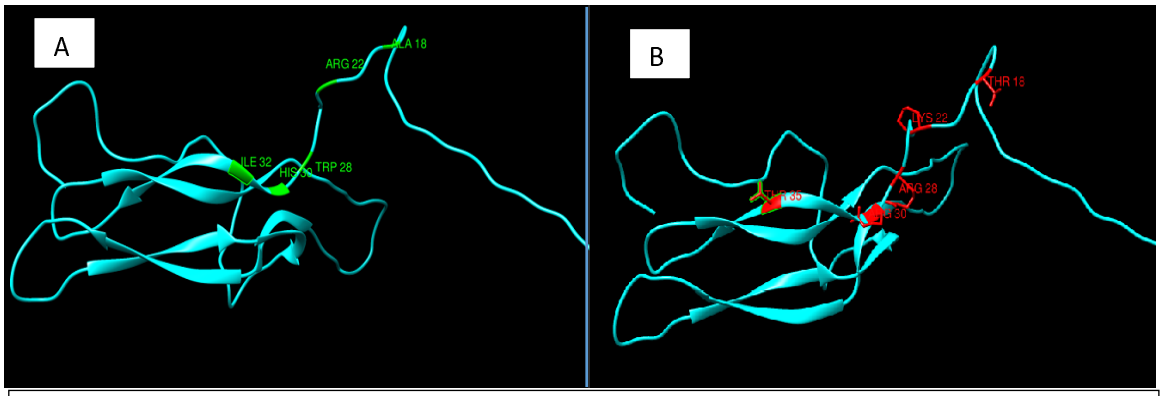


Figure (2) (A)The normal chemical structure of LH protein, (B) The chromatogram graph for the sequenced protein, (C) The gel electrophoresis showing the PCR product band (571bp).



>sp|P01229|LSHB_HUMAN Lutropin subunit beta OS=Homo sapiens OX=9606 GN=LHB PE=1 SV=3
MEMLQGLLLLLLLSMGGAWASREPLRPWCHPINAILAVEKEGCPVCITVNTTICAGYCPT
MMRVLQAVLPPLPQVVCTYRDVRFESIRLPGCPRGVDPVVSFPVALSCRCGPCRRSTSDC
GGPKDHPLTCDHPQLSGLLFL

Figure (3) illustrated both normal and mutated amino acids on sequenced exon (2) LHβ gene