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Molecular and Biochemical Aspects of Hyperprolactinemia in infertile Women

A Thesis

Submitted to the Council of College of Science, Al-Nahrain University, in Partial Fulfillment of the Requirements for the Degree of Doctor in Philosophy of Biotechnology

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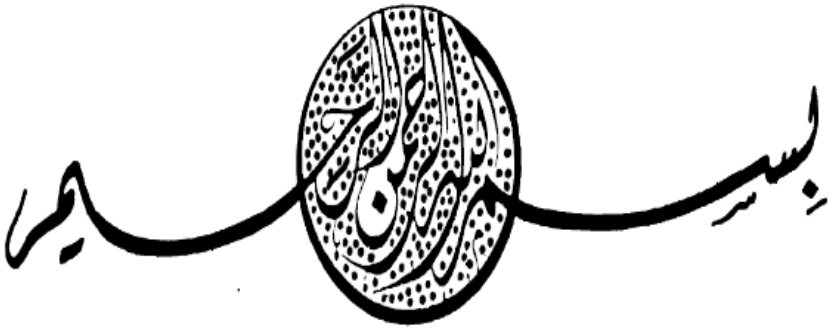

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Summary

This study was constructed to investigate hyperprolactinemia related infertility through a biochemical and molecular base associated with single nucleotide polymorphism (SNP) at prolactin gene and prolactin receptor gene in hyperprolactinemic patients.

The study included one hundred fifty blood samples from patients suffering hyperprolactinemia and infertility during the period from March 2014 to September 2014, collected from Kamal Al-Sammaraee and Al-Alwyaa Hospital. Fifty blood samples from women were collected serving as the control group. The average ages of patients were 20 to 50 years.

The biochemical study utilized the 150 samples that were first divided into 2 groups according to two infertility groups (primary and secondary) so as to study in which group the effect of hyperprolactinemia was manifested. Then, the subjects were divided into three age groups, (20-30),(31-40) and (41-50), years old in order to identify the effect of higher prolactin hormone level on age group. Serum samples for all hyperprolactinemic patients were analyzed to detect the fertility hormones Luteinizing hormone (LH) , Follicle stimulating hormone (FSH) and prolactin hormone (PRL) which were performed in all subjects.

It was found that there is a significant difference in hormone concentration in serum patients when compared to the normal. Hormones (Luteinizing hormone)(LH) and (Follicle stimulating hormone) (FSH), recorded a significant decrease, while prolactin recorded a significant increase when compared to the normal. The decrease of the two fertility hormones FSH and LH was in the second fertile group while PRL increased more in this

group. The greatest decrease of the two fertility hormones LH and FSH and the greatest increase of PRL hormone was in the age group (31-40) years old.

The variation in gene responsible for the synthesis of prolactin was conducted using samples of hyperprolactinemic patients. The study confirmed the incidance of SNPs detected in prolactin gene of hyperprolactinemic patients. Polymerase chain reaction (PCR) was done using a specific set of primers. Eight primers were selected to amplify the exons region of the gene (2 to 5) in addition to intron 1. Another 4 primers were designed to amplify exon 1 of prolactin receptor gene (PRLR). After optimization of the amplification condition, the product of (489, 533, 719, 475, 307, 306 and 436 bp), was sent for DNA sequencing which was the tool for the detection of variation within patients, genes which may reveal the association of this variation of prolactin gene to hyperprolactinemia. It was found that the percentage of substitution mutation was 88.46%, while the deletion mutation percent was 11. 54% in which the highest mutation number was in exon 2, which was 9 mutations This number is significant. All mutations in this exon were substitutions ,while the less mutation number was in exon 3 and exon4 which was 2 for each exon, one substitution and one deletion mutation in exon 3 while the two mutations in exon 4 were substitution only. This result is also significant. No mutation was detected in exon 5. But in intron 1 of gene, seven mutations were detected by using primer 1, in which two of them were deletion mutations and 5 were substitutions, while in the same intron of the gene using primer 2, 6 substitutions mutations were detected . The results of mutation detected in the PRL gene exons and intron region showed a presence of

mutations in the samples of hyperprolactinemia patients. Such mutations were common between patient samples, i.e., substitution mutations in exon 2 and exon 4. Besides, exon 1 of prolactin receptor was sequenced and it was found that there were one SNP was detected in hyperprolactinemic patients. It was found that SNPs in the exons and introns of the PRL gene were detected and these polymorphisms alter the expression attributable to altered transcription factor gene binding.

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List of Abberviations

Abbreviation	Full name
Bp	Base pair
DNA	Dioxy ribo nucleic acid
D.W	Distilled water
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylene diamine tetra acetic acid
FSH	Follicle stimulating hormone
GH	Growth hormone
Jak	Janus kinase
Kd	Kilo dalton
LH	Luteinizing hormone
LSD	Least significant difference
µl	Milliliter
mg	Microgram
NCBI	National center for biotechnology information
OD	Optical density
PCR	Polymerase chain reaction

pmol	Picomole
Primer F	Primer forward
Primer R	Primer reverse
PRL	prolactin
PRLR	Prolactin receptor
rpm	Rotation per minute
SD	Standard deviation
SNP	Single nucleotide polymorphism
Stat	Signal tranducer and activator of transcription
TBE	Tris-Borate EDTA
UV	Ultraviolet

Chapter One

Introduction and Literature Review

1.Introduction and Literature Review.

1.1. Introduction.

Prolactin (PRL) is a polypeptide hormone of a pituitary origin, whose production is controlled by dopamine .This prolactin hormone has many biological activites such as lactation and reproductive functions (Bernichtein *et al*., 2010). It is produced mainly by the anterior pituitary gland and also produced locally by multiple extra pituitary sites where it acts in an autocrine/paracrine manner (Terasaki *et al*., 2010).

Hyperprolactinemia is a condition of the presence of abnormally high levels of prolactin in the blood in which the normal levels are 10-21 mIU/ml. This condition is present as a pathological condition (Davis, 2004).

An excessive prolactin secretion decreases the level of gonadotropin releasing hormone impairing the pituitary production of follicle stimulating hormone and luteinizing hormone. It also impairs directly the endocrine activity of ovarian follicles so it will affect the ovulation (Grosignani, 2012). Thus hyperprolactinemia represents a common problem in the reproductive dysfunction, in which it leads to high circulating levels of prolactin and hypogonadism which lead to lack of gonadotrophin cyclicity and to infertility. The clinical feature can range from irregular cycle, oligomenorrhea, amenorrhoea and galactorrhoea. Mild hyperprolactinemia can cause infertility even when there is no abnormality in the menstrual cycle (AbdElghani *et al*., 2013).

The human prolactin gene is present as a single copy on chromosome 6 it is about (12.215 kb). It contains 5 exons and 4 introns and the transcription of it is regulated by two promoters. Upstream, it is used in extra pituitary

cells and tissues and down stream promoter that directs the transcription in pituitary lactotrophs (Rui and Nevalainen, 2000).

The effects of prolactin are mediated by the interaction with its receptor (PRLR). The binding of prolactin activates the pre-dimerized prolactin receptor and results in the activation of prolactin receptor-associated signaling cascades such as Jak2/Stat5 resulting in the transactivation of prolactin-responsive genes (Fang *et al*., 2010).

As the prolactin is an essential regulator of mammary development. The primary cells targeted by prolactin are the breast tissue cells which are involved in the development of mammary gland and in cellular growth and differentiation as well as in the initiation and maintenance of lactation (Mong *et al*., 2011).

This study aims to:

1- Identify the effect of high prolactin hormone level (hyperprolactinemia) on two other fertility hormones (LH) and (FSH) in women patients.

2- To demonstrate if genetic polymorphisms in PRL and PRLR genes can lead to variations in plasma levels leading to excessive secretion of prolactin (hyperprolactinemia), then to find out the relation between infertility caused by hyperprolactinemia and prolactin gene and prolactin gene receptor.

1.2. Literature Review.

1.2.1. The Endocrine system.

The endocrine system is an integrated system which consists of several glands, hormones and scattered hormone secreted cells (Kester *et al*., 2004). Al-though vertebrate endocrine systems vary, they consist of the same basic glands and hormones (Lewis *et al*., 2002).

This system helps maintain homeostasis, integrate and coordinate many diverse physiological functions. It coordinates with the nervous system in other vital communication functions within the animal's body.These two systems work together to produce a variety of responses from sexual and reproductive behavior to control the growth and development and to adjust the delicate chemical balance of body fluids (Lewis *et al*., 2007).

The major endocrine glands include the pineal gland, pituitary gland, parathyroid gland, hypothalamus and adrenal glands. These glands produce different types of hormones that evoke a specific response in other cells, tissues and/or organs located throughout the body. Many endocrine glands discrete organs whose primary functions are the production and secretion of hormones (Fox, 2006).

Hormones are of different classes based on their chemical composition and they can be divided into chemical classes such as amines which are derived from the amino acids tyrosine and tryptophan such as hormones derived from the thyroid glands, such as triiodothyronine (T3) and tetraiodothyronine (thyroxine, T4) which make up a subset of this class because they derive from the combination of two iodinated tyrosine amino

acid residues. Polypeptide and proteins in which polypeptides such as antidiuretic hormones. Proteins are polypeptides with more than 100 amino acids like growth hormone. Glycoproteins consist of a long polypeptides . All hormones secreted by the pituitary gland are peptide hormones, such as FSH and LH. Steroid hormones are lipids derived from cholesterol such as the leptin (Fox, 2004).

1.2.2. Pituitary gland.

This gland is under the control of the hypothalamus which decides how the hormones are released either through hormonal or electrical messages. It is located at the base of the brain and it is a hormone secreting gland compartmentalization into the anterior pituitary and posterior pituitary as is shown in figure (1-1) (Lewis *et al*., 2007).

The anterior pituitary contains five major hormone secreting cell types. Corticotrophs produce adrenocorticotropic hormone (ACTH), gonadotrophs secrete follicle-stimulating hormone and luteinizing hormone, thyrotrophs secrete thyroid stimulating hormone, somatotrophs secrete growth hormone(GH) and lactotrophs secrete prolactin (PRL). Dasen and Rosenfeld (2001) differentiated five cell types that do not occur at the same time during development. Usually, the first differentiated cell is the corticotroph, followed by the differentiation of gonadotrophs, thyrotrophs, somatotrophs, and lactotrophs in that order (Simmons *et al.*, 1990).

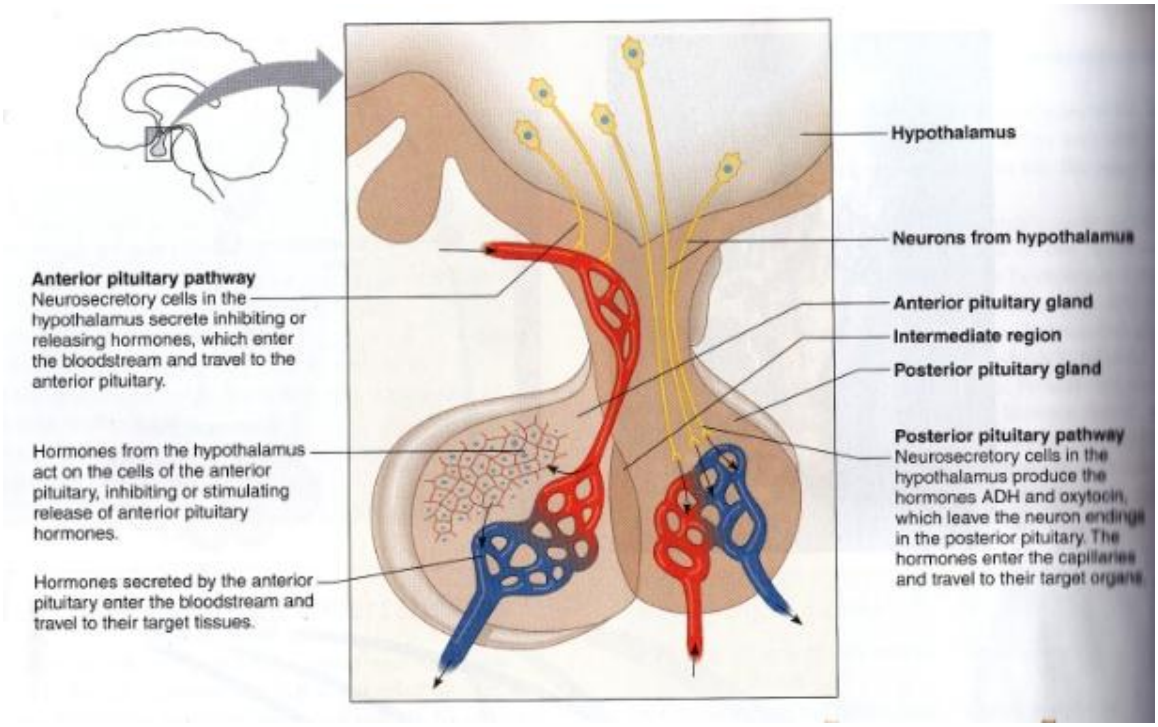


Figure (1-1):Endocrine system control (Lewis *et al*., 2007).

Although the anterior loop secretes at least eight hormones, only six have well established functions (Forsyth and Wallis, 2002).

- Growth hormone (GH):

  It promotes growth in childhood. For adults, it helps to maintain healthy muscle and bone mass.

- Adernocorticotropic hormone (ACTH):

  This hormone promotes the production of cortisol which helps to reduce stress, maintain healthy blood pressure and more sensation like hungry and thirsty.

- Thyroid stimulating hormone (TSH):

This hormone helps to regulate the body's thyroid, which is crucial in maintaining a healthy metabolism T3, T4.

- Luteinizing hormone (LH):

  It is a glycoprotein hormone, and it is essential for reproduction females. Its function is to regulate estrogen, and at the time of menstruation, it initiates follicular growth, specifically affecting granulose cells (Ross and Vande-Wiele, 1974). But in men it regulates testosterone.

- Follicle stimulating hormone (FSH):

  It is found in both men and women. It regulates the development, growth, pubertal maturation and reproductive processes of the human body.

  In females, its function is to initiate follicular growth, stimulating the releasing of eggs, and in men it helps to ensure the normal functions of sperm production (Buckman and Maire, 1981).

- Prolactin (PRL):

It is unique among the anterior pituitary hormones in which its major function is not to exert control over the secretion of a hormone by another endocrine gland. Its most important action is to stimulate the development of the mammary glands and milk production. It has direct effects upon the breasts (Widmaier *et al*., 2006).

The back part of the pituitary gland is called posterior pituitary. It produces the following two hormones:

❖ Oxytocin:

This hormone causes pregnant women to start having contractions at the appropriate time. It also promotes milk flow.

❖ Antidiuretic hormone (ADH):

It is commonly referred to as vasopressin. This hormone helps to regulate water balance in the body. The hormones produced by the pituitary gland is shown in figure (1-2), (Lewis *et al*., 2007).

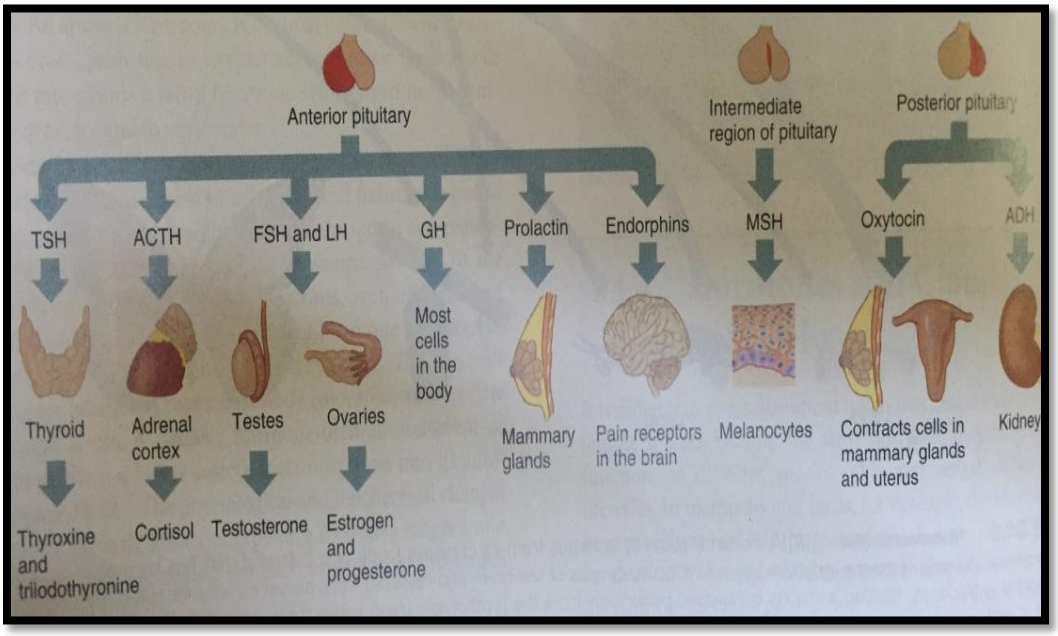


Figure (1-2): Hormones produced by the pituitary glands and their targets (Lewis *et al*., 2007).

1.2.3. Prolactin hormone.

1.2.3.1 Structure.

Prolactin hormone is a multifunctional hormone discovered by Stricker and Grueter as a pituitary factor that could induce milk secretion in rabbit mammary glands, and crop milk production in pigeons. The factor was purified and given the name prolactin shortly thereafter (Riddle and Braucher, 1931); (Riddle *et al*., 1933).

The entire amino acid sequence, including a 28 residue single peptide, was discovered by a cook and his colleagues from the nucleotide sequence of human cDNA (Riddick *et al*., 1978).

Prolactin is a polypeptide hormone composed of 199 a.a (23KD), that is synthesized and secreted by specialized cells of the anterior pituitary gland (lactotroph) (Yen *et al*., 1999; Fitzgeralda and Dinan, 2008).

It circulates mainly in a monomeric form but variants of prolactin become of post translational modifications such as proteolytic cleavage, dimerazation, polymerization, phosphorylation and glycosylation (Freeman, 2000).

The human prolactin circulates in blood in various sizes, monomeric PRL (little PRL 23KD), dimeric PRL (big PRL, 48-56 KD), and polymeric forms (big-big PRL,100 KD). The monomeric form is the most bioactive PRL, and it has over 300 separate biological activates (Baban *et al* ., 2008).

In general, these PRL variants have reduce the biological activity. Large molecular isoforms (>150 kD) are termed macroprolactin due to complexes of PRL and IgG (De Schepper *et al*., 2003).

A variation in the levels of prolactin was noticed in mammals for instance during pregnancy. High circulating concentrations of estrogen and progesterone inhibit the action of prolactin on milk production. After delivery, reducing estrogen and progesterone production allows prolactin to induce lactation (Serri and Ezzat, 2004). The levels of prolactin after childbirth, fall as the internal stimulus for them are removed, but sucking by the baby on the nipple then promotes further prolactin release This maintains the ability to lactate. The sucking activates mechano receptors in and around the nipple, then the signals are carried by nerve fibers through the spinal cord to the hypothalamus, where changes in the electrical activity of neurons that regulate the pituitary gland cause an increased prolactin secretion.The suckling stimulus also triggers the release of oxytocin from the posterior pituitary gland, which triggers milk let-down. Prolactin controls milk production (lactogenesis) but not the milk-ejection reflex; the rise in prolactin fills the breast with milk in preparation for the next feed (Nelson and Cox, 2005).

1.2.3.2.Prolactin gene.

In mammals, the prolactin PRL gene family is a large family of paralogous genes encoding hormones and cytokines (Soares *et al.,* 2007).

The human prolactin gene is present as a single copy per haploid genome. It is located on chromosome 6 and divided into 5 exons and 4 introns (Truong *et al*., 1984).

The molecular size of it is about 10.215 (kb) and the transcription is regulated by two independent promoter regions; the proximal 5000 bp

region directs pituitary –specific expression , while more upstream (distal) promoter region is responsible for extrapituitary expression (Berwear *et al* ., 1994).

The human prolactin mRNA is 914 nucleotides long and contains a 618-nucleotide open reading frame translated prolactin prohormone of 227 amino acids. The 28 amino acid signal peptide is cleaved and the mature human prolactin is formed (199 amino acids)(Binart *et al* ., 2010).

1.2.3.3. Prolactin receptor.

The actions of prolactin are initiated through an interaction with a specific cell surface high affinity prolactin receptor (PRLR)(Omelka *et al* ., 2008). It is a member of the largest class-1-cytokine receptor super family(Marc *et al* ., 2000).Only a single isoform of the prolactin receptor has been identified in humans (Boutin *et al*., 1989). It is located on chromosome 5 and is approximately 180 kb in length and originally has 10 exons of which (3-10) coding exon(Arden *et al*., 1990),(Hu *et al*., 2001).The PRLR is composed of an extracellular ligand-binding domain which consists of 210 amino acids (Boutin *et al*., 1988). This receptor can be further divided into NH2-terminal D1, which has two pairs of disulfide bonds between cysteins (Cys12-Cys22 and Cys51-Cys62)and membrane proximal D2 domains which have conserved region "WS" motif(Trp-Ser-x-Trp-Ser).

Both disulfide bond and "WS" motif are necessary for a proper folding and trafficking of the receptor. The second part consist of a transmembrane domain which is a 24 a.a hydrophobic domain and an intracellular domain which is essential for initiation of the signal transduction mechanisms associated with the prolactin receptor.

The two intracellular conserved regions within the PRLR are termed Box1 which is a rich proline and it is necessary for the consensus folding of the molecule, and Box2 which is missing in a short isoform of the prolactin receptor (Kelly *et al*., 1989); (Bole-Feysot *et al*., 1998).

Receptors for prolactin are widely expressed in the mammary glands, ovaries pituitary glands, hearts, lung, thymus, spleen, liver, pancreas, kidney, adrenal glands, uterus, skeletal muscle and skin (Mancin *et al*., 2008).

This receptors has many actions in different tissues and this biological action happens because of the expression and regulation of different PRLR isoforms and the utilization of different signaling pathways (Bole, 2008).

Like many other members of this family, the first step in receptor activation is generally believed to be a legend-induced dimerization whereby one molecule of PRL is bound to two molecules of receptor (Elkins *et al*., 2000). Recent reports suggest that PRLR pre-assembles at the plasma membrane in the absence of ligand (Gadd *et al*., 2006), suggesting that ligand-induced activation involves conformational changes in preformed PRLR dimers (Broutin and Isabelle, 2010). The genetic polymorphism in prolactin receptor genes can lead to a variation in plasma levels of encoded proteins (Dunning *et al*., 2004).

1.2.3.4. Regulation of prolactin hormone.

Prolactin hormone synthesized and secreted from lactotroph cell, in the anterior pituitary, which compromises about 15-22% of functioning anterior pituitary cells (Melmed and Kleninberg, 2007). The secretion of it is mainly under the tonic inhibition of hypothalamic dopamine (Freeman, 2000).

Dopamine reaches the pituitary via the hypothalamic-pituitary portal system and inhibits PRL by binding to type 2 dopaminergic receptors on the lactotrophs leading to a rapid suppression of PRL release from secretary vesicles, inhibition of PRL gene expression and lactotroph proliferation, PRL exerts a negative feedback on its own release by stimulating hypothalamic dopamine synthesis (Ben-jonathan and Hnassk, 2001) Although the control of PRL secretion is mainly inhibitory, there are several known PRL-releasing factors, including thyrotropin releasing hormone (TRH), vasoactive intestinal polypeptide, oxytocin and endothelin. Also estrogens stimulate lactotroph cell proliferation as well as PRL secretion (Vician *et al*., 1979). Moreover, estrogens activate secondary responses that may influence PRL gene transcription, i.e. inhibiting dopaminergic hypothalamic activity and upregulating TRH receptors. Furthermore, PRL secretion is increased by different forms of stressors.

A summary of the regulation of PRL secretion is presented in Figure (1-3),(Berinder, 2011).

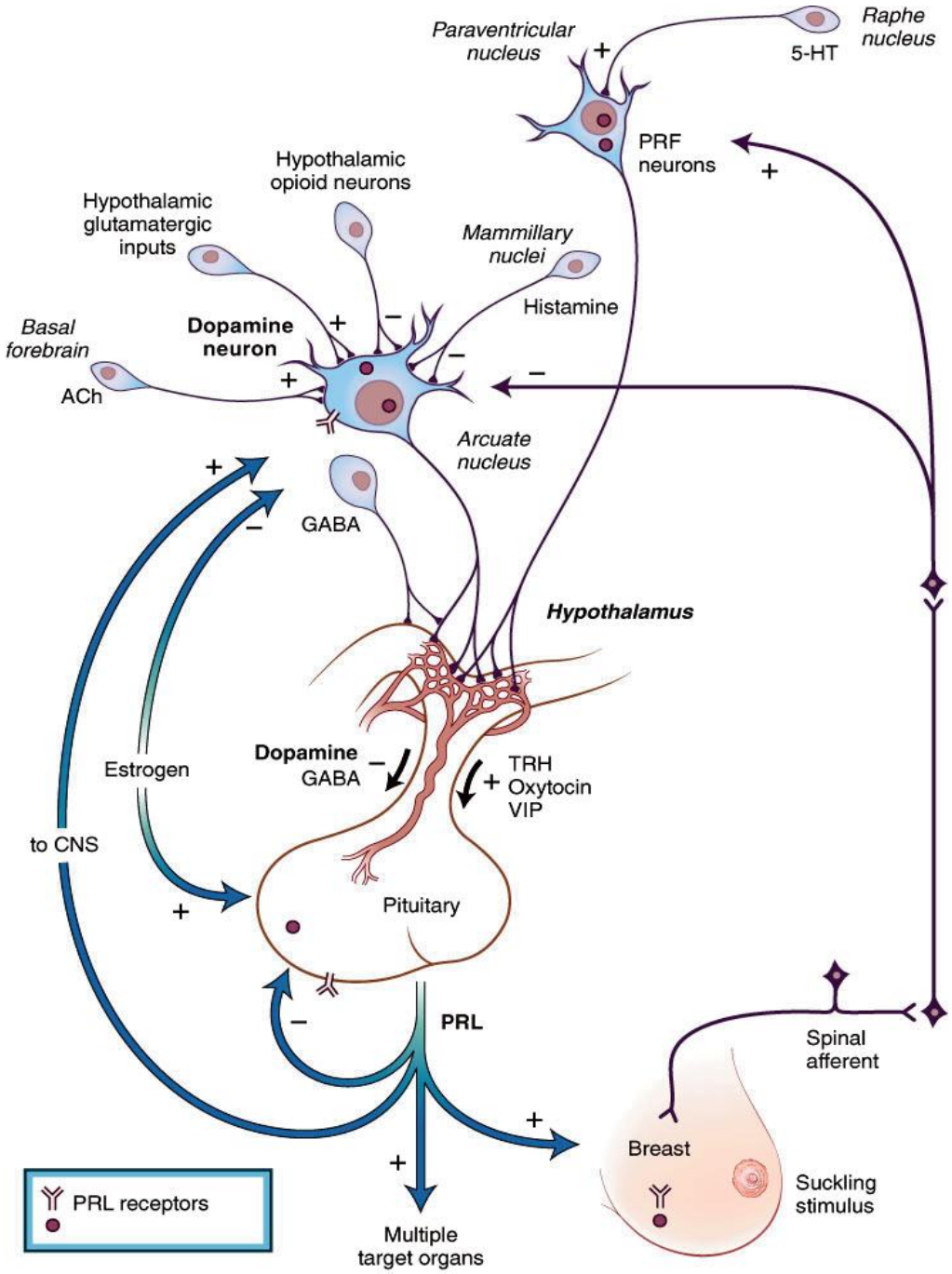
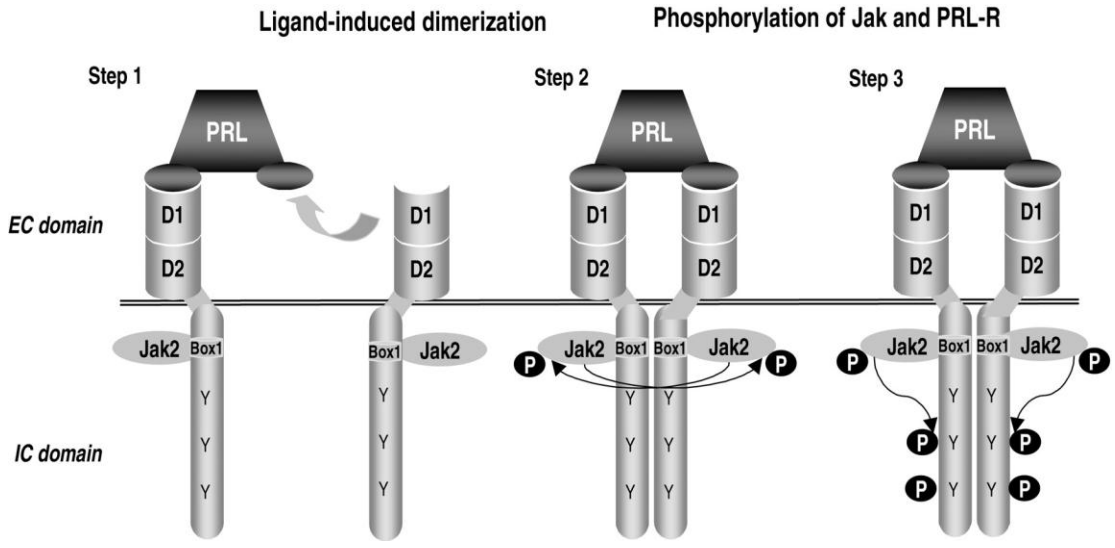


Figure (1-3): Regulation of prolactin secretion (Berinder, 2011).

1.2.4. Hormone signal transduction.

The function of PRL hormone in cells and tissues has been related to expression of PRLR on the cell surface and the utilization of signaling pathways. The PRLRs are non-kinase receptors whose activation of signaling pathways requires participation of receptor-associated kinases, such as Janus kinases or Src kinases (Ihle, 1994). The signal transduction of PRLR involves mainly JAK/Stat pathway as it is the most important signaling pathway used by cytokine receptors. The JAK2 activity, induced by PRLR dimerization, is necessary for PRL action (Finidori and Kelly, 1995).

The PRL molecule contains two receptors-binding sites; PRL binding site 1 interacting first with one PRLR. This leads to a complex formation which is (PRL-PRLR), then PRL bind site 2 intracting with the second PRLR, resulting in a PRLR dimerazation and activation (Freeman *et al*., 2000), as shown in figure(1-4).



Figure(1-4) The activation of prolactin receptor(Freeman, 2000).

After a ligand stimulation of the receptor, JAK2 activation occurs within 1 min. The ligand-induced receptor dimerization will bring two receptors associated JAK molecules close together. This results in activation by transphorylation of JAK tyrosines. Activated JAK2 and phosphorylation of tyrosine residues are on PRLR. All PRLR isoforms can activate this JAK2 but only tyrosine residues of the long and intermediate PRLR isoform are phosphorylated after JAK2 activation(Clevenger and Klin, 2001).

The phosphorylation of tyrosine residues is important because it is considered as a potential binding site for transducers most of which contain Ser homology regions2 domains. This SCH2 domain is found in the signal transducer and activator of transcription (state) proteins as it contains DNA binding domain and a c-terminal trans activity domain. The state family consists of eight members. State 5 has two isoforms which are state 5a and state 5b, which are initially identified as PRL induced mammary gland transcription factors (Teglund *et al*., 1998).

The major difference between state5a and state 5b isoforms lies in their serine/threonine phosphorylation sites (Beadling *et al*., 1996).

The SH domain of state interacts with the phosphorylated tyrosine residue of the activated long prolactin receptor isoform. This will make a complex PRLR/JAK2/STAT5, being stated with phosphorylate by the receptor associated with JAK kinase (Freeman *et al*., 2000).

These phosphorylated states dissociate from the receptor and dimerize with the SH2 domain of another phosphorylated state molecule through their phosphorylation residues (Brooks, 2012).

Finally, this dimer tranlocates to the nucleus and activates a stat DNA-binding motif in the promoter of a target gene (Carter-su and Smith, 1997).The consensus DNA motif recognized by Stat1, Stat3, and Stat5 homo

or heterodimers is termed GAS (gama_interferon activated sequence). GAS consists of a palindroic consensus sequence. Once bound, state engages several elements of the transcriptional machinery, stimulating gene expression (Freeman, 2000).

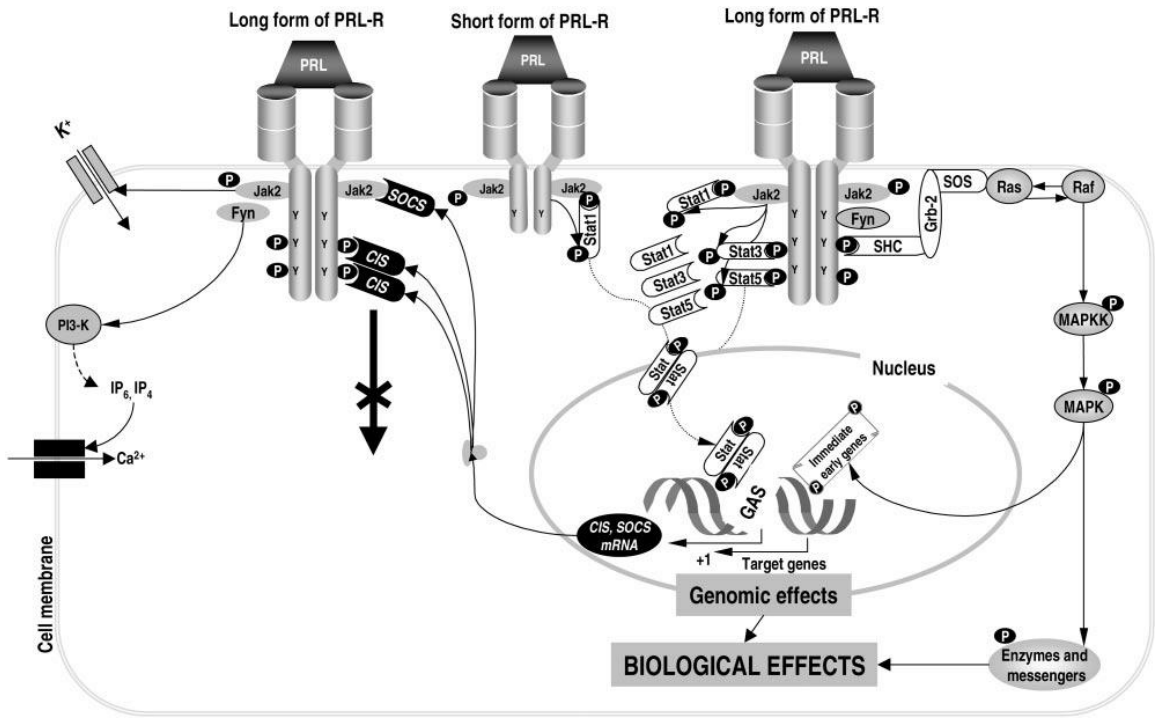


Figure (1-5): Signal transduction pathway (Freeman, 2000).

Apart from lactotrophs in the anterior pituitary, PRL is also produced by different organs and tissues. It is synthesized in many extra pituitary sites e.g., reproductive organs, immune cells and brain where it may function as an autocrine/ paracrine functions(Yen *et al*., 1999),(Ben-jonathan *et al*., 2008).

1.2.5. Biological action of prolactin hormone.

More than 300 different biological functions have been attributed to prolactin (Bole-feyrsot *et al*., 1998). The actions of this anterior pituitary hormone in the body include immunoregulation and protection. This is because its widely recognized as an important physiological modulator of the immune response by acting in acytokine-like manner (Dorshkin and Horseman, 2000, Bole-Feysot *et al*., 1998). It stimulates T-cell proliferation (Clevenger *et al.,* 1990) and supports interferon alfa production (Schwarz *et al* ., 1992). Prolactin is synthesized and secreted by human peripheral blood mononuclear cells and it functions in an autocrine manner as a growth factor for lymphoproliferation (Sabharwal *et al*., 1992).

It is also involved in regulating monocyte/macrophage function *in vitro* (Aziz *et al*., 2008). Macrophage activation and superoxide anion production responsible for killing pathogenic organisms are effects mediated by the PRLR in inflammatory pathways (O' Neal *et al*., 1991).In reproduction, the actions of PRL represent the largest group of different functions in which it exerts effects on the mammary gland development of females during pregnancy by stimulating the growth of it to allow the pregnant to prepare for breast feeding. This is one of the important things for the initiation and maintenance of lactation in the female (Bole-Fysol, 1998; Sinha, 1995). It

also exerts effects on the targets important to the reproduction of the mammalian species(Ben-Jonathan, 2008). It exerts effects on targets important to reproduction and many autocrine /paracrine functions (Fujikawa *et al*.,2000). PRL receptor is expressed in cells in testis prostate gland, seminal vesicles and ovary (Cook, 1995).

The synthesis of milk (lactogenisis), stimulates the uptake of some amino acids, the synthesis of milk proteins casein and alfa lactoalbumin uptake of glucose and synthesis of milk sugar lactose also milk fat (Benker *et al*., 1990). Prolactin regulates a variety of brain functions including the suppression of adenocorticotrophin secretion during the stress response (Torner *et al.,* 2001).

One of the least understood actions of prolactin is the regulation of solute and water transport across mammalian cell membranes (Shimon *et al*.,1997). Studies in this area were motivated by the finding in lower vertebrates that prolactin stimulates solute transport across cell membranes and thus could be an osmoregulatory hormone (Besson *et al.*,1986).

Bole-Feysot *et al*, (1998) summarizes the broad biological functions of PRL in five categories:

- water and electrolyte balance.
- growth and development.
- endocrinology and metabolism.
- brain and behavior.
- reproduction.

1.2.6. Hyperprolactinemia.

A condition in which excess prolactin circulates in the blood stream of non lactating and non pregnant women and in males called "hyperprolactinemia" . Its probably one of the most common endocrine disorders related to pituitary function, and it is more commonly diagnosed in women than in men (Willims *et al* ., 2003).

Normally prolactin is present in small amounts throughout the blood stream of non pregnant females and in males kept under control of another hormone called "prolactin inhibiting factor" (dopamine) (Fitzgerald and Dinan, 2008), in which the normal levels are typically 10-35 ng/ml in females and 5-10ng/ml in males, each 1 ng is equivalent to 21.2 mIU/ml(Crosignani, 2012). But there are numerous conditions that may cause elevated prolactin levels in females, such as secretion which may happen with physiological causes, such as increases mildly with sleep, stress, exercise, nipple stimulation, lactation and pregnancy(American society for reproductive medicine, 2009). Besides, there are a pathological reasons that cause prolactin secretion increase the hypothalamic disorders such as tumors, or infiltrative disease like tuberculosis. It may also relate to pituitary disorders such as prolactinoma, macroadenoma, or may be of other reasons such as polycystic ovarian disease, primary hypothyroidism, chronic renal failure, liver cirrhosis or some medication (Melmed and Kleinberg, 2007). Hyperprolactinemia may

result in hypogonadism, infertility, and galactorrhea, or it may remain a symptomatic (Gillam *et al*., 2006), (Schlechte, 2003).

The signs of hyperprolactinemia are represented by irregular menstrual, milky discharge from the breast (amenorrhea), headache, sometimes change in vision also estrogen level can be decreased to a point where the loss of bone calcium can occur (Melmed *et al*., 2011).

1.2.7. Hyperprolactinemia and infertility.

Infertility is the inability to conceive after one year of marriage without using the contraception. It representes a common condition with many implications. The infertility evaluation is usually of different causes which include either male infertility or female infertility or both (Pasqualotto *et al*., 2005). The reasons of it are many and may be environmental factors, weight change, age, life style, hormonal imbalance, ovarian functional problem, uterine factors, thyroid disease, sexually transmitted disease and may be of hyperprolactinemia (Olooto *et al*., 2012).

This common problem of reproduction dysfunction affects about one-third of infertile women (Nilsson and Helberg, 2006).

Hyperprolactinemia results in a variety of reproductive dysfunctions and cause female infertility through many ways:

- Decline in the body's production of progesterone during the luteal phase after ovulation.
- Irregular menstruation

  Prolactin affects women's menstrual cycles. That is why women who are breast feeding rarely get pregnant. This is done by inhibiting two hormones necessary for ovulation which are follicle stimulating hormone(FSH) and gonodotropin releasing hormone (Bernstein *et al*., 2012).

This leads to suppressing the ovulatory cycle by inhibiting the secretion hormones by impairing the pituitary production of FSH and LH.

- Furthermore, it may directly impair the endocrine activity of ovarian follicles; as a consequence defective luteal phase in constant ovulation and chronic ovulation are conditions frequently observed in hyperprolactinemic patients (Solomon *et al*., 2013).

- Decrease of sexual hormone levels will not only cause infertility, but also oligomenorrhea, menorrhea and less often galactorrhea (Gomez *et al*., 1977).
- In males, it can lead to reducing body hair and muscle, decreasing libido, impotence and causing inefficient sperm production and infertility (Colar *et al*., 2004).

1.2.8. The genetic variation in genes of prolactin and its receptor in relation to hyperprolactinemia.

Studies found three germ line heterozygous miss sense SNPs in the PRLR gene (Courtillot *et al*.,2010).

Exploration of genetic variants in PRL and PRLR has identified single nucleotide polymorphisms (SNPs) that alter the transcription factor binding (Stevens, 2001) and modify prolactin receptor activity (Bogorad, 2008). This might associate with breast cancer risk which are also with circulating prolactin levels (Courtillot *et al*., 2010).

One of the studies that screened promoter regions of the PRL and PRLR genes for polymorphisms observed significantly an increased risk for

carriers of the variant alleles of the PRL promoter SNP and for the TGTG haplotype which contain these variant alleles(Vaclavicek *et al*., 2006).

Furthermore, there are several hormones and growth factors that are shown to participate in the development of both normal and carcinogenesis of breast epithelium e.g. hormones of estrogen, progesterone, prolactin, growth hormone and insulin (Wennbo *et al*., 1997).

1.2.9. DNA sequencing.

In the early 1970s, the first DNA sequences were obtained through extremely laborious techniques. An example is the sequencing of the two dozen base pairs of the lac operator (Gilbert and Maxam, 1973). Then the first revolution in the DNA sequencing field took place in the second half of the 1970s with the methods published by Allan Maxam and Walter Gilbert (Maxam and Gilbert, 1977) and Frederick Sanger and Colleagues in which both of these technique were increased the throughput of DNA sequencing (Sanger *et al*., 1977).

This technique refers to the sequencing methods for determining the order of the nucleotide bases-Adnine, Guanine, Cytosin and Thymine, in a molecule of DNA. It allows DNA sequences of a given short segment of DNA that contains nucleotides to be determined in few hours. The entire genome of many organisms and large DNA sequences projects can also be sequenced, in addition to gaining more information by knowing, base, by base the entire sequence of a gene or even the entire genome of an organism (Nelson and Cox, 2004).

Knowledge of DNA sequences has become indispensble not only for basic biological research, but also for other search branches utilizing DNA

sequencing and in numerous applied fields such as: medical diagnostic, biotechnology, forensic biology, virology and biological systematic (Hutchison, 2007 ; Peterson *et al*., 2009).

But still there is some disadvantage of the Maxam-Gilbert method which is still reagent and time consuming. This was a trigger to investigate alternative methods that are more efficient. So it was rapidly replaced by the chain terminator method as it was more efficient and used fewer toxic chemicals and lower amounts of radioactivity and it was called next generation sequencing (NGS) or second generation sequencing method (Pareek *et al*., 2011).

There are many applications for the next generation sequencing which can be summarized in :

- As important application includes a full genome sequencing or more targeted discovery of mutations or polymorphisms (Shendure and Hanlee, 2008).
- Mapping of structural rearrangements which may include copying the number variation, balanced translocation breakpoints and chromosomal inversions (Sudmant *et al*.,2010).
- Chip-seq. or genome-wide mapping of DNA-protein interactions by deep sequencing of DNA fragments being bulled down by a chromatin immunprecipitation (Blecher *et al*.,2004).
- Large scale analysis of DNA methylation by a deep sequencing of bislfite treated DNA (Taylor *et al*., 2007)

Chapter Two
Materials and Methods

Chapter two

2.Matrerials and methods.

2.1. Materials.

2.1.1. Equipments and apparatus.

The equipments and apparatus used in this study are listed in table (2-1):

Table (2-1): Apparatus used in this study

No.	Equipments	Manufacturing company	Origin
1.	Autoclave	HIRAYAMA	Japan
2.	Bench centrifuge	Uni-media	Korea
3.	Electrophoresis equipment	Bio Rad	USA
4.	Eppendorff-cooling centrifuge	Eppendorf	Germany
5.	Gel documentation	Bio-red	USA
6.	Microcentrifuge	Hettich	Germany
7.	Microwave	LG	Korea
8.	Minividas	Biomerarex	France
9.	Nanodrop spectrophotometer	Techne	UK
10.	Oven	Sanyo	Japan
11.	PCR master cycler gradient	Techne	UK
12.	PCR thermal cycler	Techne	UK
13.	pH-meter	Hanna	Romania
14.	Sensitive Balance	Mettlev	Switzerland

19.	UV transilliuminator	Flowgen	UK
20.	Vortex	Scientific Industries	USA
21.	Water bath	Grant	UK

2.1.2. Chemicals.

The chemicals used in this study are listed in table (2-2):

Table (2-2): Chemicals

No	Chemicals	Manufacturing company	Origin
1.	Green master mix		
2.	TBE		
3.	Proteinas k		
4.	DNA marker 100-1500 (bp).		
5.	Ethidium bromide	Sigma	USA
6.	Agarose		
7.	Loading solution		
8.	Absolute ethanol	Phamacia	Sweeden
9.	Primers	Shine Gene	Korea
10.	Deionized water		

2.1.3 Kits.

Table (2-3) Kits used in this study

Table (2-3): Lists of kits

No.	Kit description	Company	Origin
1.	DNA extraction miniprep system (from blood)	Promega	USA
2.	LH	Bio-merieux	France
3.	FSH		
4.	Prolactin		

2.1.4. Primers

2.1.4.1 Sequences of the primers used to amplify a portion of the human prolactin gene

Primers used to amplify the gene

No	Oligonucleotide	Oligosequnese	Prod.Size (bp)	GC%	Tm	Ref.
1	Forward primer	CGTAGGCTGGATTTGAAGGGT		52.38	54.36	
		AGCGATAGATCAGGGTGCCT	312			NCBI
	Reverse primer		Intron 1	55.00	53.83	
2	Forward primer	AGGGGGTAACATGCATAGCAG		52.38	54.36	
		TCCCTGGATGGAGAGAGTCTG	416			NCBI
	Reverse primer		Intron 1	57.14	56.31	
3	Forward primer	ATCCCGGGAAGTAAGCATGG		55.00	53.83	
		TTGCTAGGGCTTTGGAGGTC	618			
	Reverse primer		Intron 1	55.00	53.83	NCBI
4	Forward primer	ATGTGTGACAACTCACTGCG		50.00	51.78	
			489			NCBI
	Reverse primer	GGCCAATCCACATTAGAGGC	Exon 2	55.00	53.83	
5	Forward primer	GCTGAATCCATGGTGGGGAA		55.00	53.83	NCBI
		TCTCTGTGGAGGCCCTTGAT	533			
	Reverse primer		Exon 3	55.00	53.83	
6	Forward primer	AAACGGTATACCCATGGCCG		50.00	53. 83	
		AGTGGCAACTGTAGCTGTGA	719			NCBI
	Reverse primer		Exon 4	55.00	51.78	
7	Forward primer	AGCCTCCTGGTGCTTCTTTG		55.00	53.83	
			475			NCBI

	Reverse primer	TGCTCCTCCCTGACAAGTCT	Exon 5	50.00	51.88	
8	Forward primer	ACAGTAAATTTTGTCTTAGGGC TCA	375	36.00	52.76	NCBI
	Reverse primer	AAACCAGGAAAGCCCAGAGG	Intron 8	55.00	53.83	

2.1.4.2 Sequences of primers used to amplify human prolactin gene receptor:

The Primers used to amplify gene receptor:

No.	Oligonucleotides	Oligosequance	Prod. Size (bp)	GC%	TM	Ref.
1	Forward primer	TCTTCGCAGGATTCCAGCTC	307	55.00	53.83	NCBI
	Reverse primer	CGCGAACGGTCGGTAAAATC		55.00	53.83	
2	Forward primer	GCCCTAATCATGCAAAACCG A	306	47.62	52.40	NCBI
	Reverse primer	GAAAGCCCAGCCCAGAAAA C		55.00	53.83	
3	Forward primer	GGCAGGCTCTGGACGTTTT TCCTCAGTGTTCGCCTCCAT	382	57.00	53.25	NCBI
	Reverse primer			55.00	35.83	
4	Forward primer	TCATGCAAAACCGATCTGGG	436	50.00	51.78	NCBI
	Reverse primer	GTTTTGCCAGGGAGCAAAGT		50.00	51.78	

All primers in this study were designed by Ass. Prof. Dr. Rebah Najah Jabbar /Biotechonlogy research center at Al-Nahrain university using primer 3 software at NCBI, this primers were categorized with special features to make these highly suitable for genes amplification by PCR and further sequencing.

2.2. Study subjects.

This study included 150 blood samples of patients suffering from hyperprolactinemia and infertility during the period from march 2014 to Sep 2014, from Kamal Al-Sammaraee and Al-Alwyaa Hospital . Fifty blood samples from healthy women were collected, they were selected to be used as a control group. The main ages of patients were 20 to 50 years old.

2.3. Collection of Samples.

The blood samples were collected after diagnosis as infertile women by gynecologist consultants. They were collected from one hundred fifty (150) primary and secondary infertile women suffering from hyperprolactinemia and their ages ranged between (20-50) years. A volume of 5 ml of peripheral blood was collected by vene puncture and divided into two tubes, 1ml to EDTA tube and stored at -20 ^{0}C for DNA extraction. The 4 ml was transferred to a plain tube, for a biochemical analysis, and subjected to centrifugation at 2000 RPM for 10 min. The serum was separated and stored at -20 ^{0}C.

2.4 Solutions and buffers

DNA Extraction miniprep system kit

This kit is ready to use and it contained the following components:

- GT buffer
- GB buffer
- W1 buffer
- Wash buffer
- Elution buffer
- Colletion tubes and colums

2.5. Green Master mix.

Go Taq Green Master Mix is apremixed, ready to use solution containing bacterially derived Taq DNA polymerase, dNTPs, $MgCl_2$ and reaction buffers at optimal concentrations for effective amplification of DNA templates by PCR. GoTaq Green Master Mix contains two dyes (blue and yellow) that allow monitoring of progress during electrophoresis.

2.6. DNA ladder (100 bp) (FAVORGEN BIOTECH CORP).

DNA ladder 100- 1500 bp containing 11 fragments was supplied in storage buffer 10 mM Tris-HCL pH (7.5), 0.1 mM EDTA.

2.7. Methods.

2.7.1 Sterilization methods.

- Autoclaving: Buffers and solutions were sterilized by pressure vessel (autoclave) at 121 ^{0}C and 15 bar for 15 minutes.

- Dry heat: A laboratory oven was used for glassware sterilization. A glassware was placed in the oven at 200 ^{0}C for 2 hours.

2.7.2. Hormonal assay.

The hormones concentrations were measured with the VIDAS, (biomerieux, France) (Butt and Blunt, 1988)

2.7.2.1. Follicle stimulating hormone.

FSH levels were measured by the Bio – Merieux kit marcy I' Etoile – France, as instructed by the manufacturer.

2.7.2.2. Luteinizing hormone.

LH levels were measured by the Bio – Merieux kit marcy I' Etoile – France , as instructed by the manufacturer.

2.7.2.3. Prolactin.

Prolactin levels were measured by the Bio – Merieux kit marcy I' Etoile – France , as instructed by the manufacturer.

2.7.3. Specimen preparation.

- Preparation of blood sample:

  Blood sample was left for thawing then mixed thoroughly by a rotisserie shaker at room temperature.

2.7.4. Extraction of DNA from samples.

Protocol for the extraction of DNA from blood samples.

The extraction was briefly carried out as follows:

- The blood sample was mixed thoroughly for at least 10 min, in a rotisserie shaker at room temperature:
- Aliquate of 20 µl of the protinase k solution was added
- Aliquate of 200 µl of blood was added to the proteniase k solution and mixed briefly.
- Aliquate of 200 µl of cell lyses buffer was added to the tube and mixed for at least10 seconds, by vortexing, then incubated at 56 ^{0}C for 10 minutes.
- Aliquate of 250 µl of binding buffer was added to the tube and mixed for 10 seconds by vortexing.
- The sample mixture was transferred to a filter column set and centrifuged at maximum speed for 1 min.
- The column tube containing the supernatant was discarded and replaced by a fresh collection tube.
- Aliquate of 500 µl of the column wash solution was added to the column and centrifuged for 3 minutes at max speed and the supernatant was discarded.
- Aliquate of 50 µl of nuclease free water was added to the column and centrifuged for 1 min at a max speed.
- The column was discarded and the elute was stored at -20 ^{0}C.

2.7.5. Measurments of extracting DNA concentration and purity.

2.7.5.1. Blanking.

- After lifting the arm of nanodrop, 2 µl of elution buffer was pipetted on to the measurement pedestal.
- The sampling arm was laid down and the solution was observed bridging the gap between both optical fibers.
- When the Blank button was clicked, the apparatus measured the solution with both 1 mm and 0.2 mm path lengths, and then the system recorded both results automatically.
- When the measurement completed, the sample arm was opened and then the blanking buffer was wiped from pedestal using laboratory wipe.

2.7.5. 2. Sample Measurement.

- Two µl of the sample was pipetted onto the measurement pedestal while the sample arm was opened.
- The sampling arm was laid down and the solution was observed bridging the gap between both optical fibers.
- The button (Measure) was clicked and the measurement result appeared on the screen within 3 sec.
- When any sample gave purity less than 1.5 and /or concentration less than 10 ng/µl DNA, the extraction from the blood of that sample re-performed until the desired purity and concentration were obtained .

2.7.6. Polymerase Chain Reacction Amplification.

2.7.6.1. Preparation of primers solution.

The lyophilized primer was dissolved using sterile distilled water to have the final concentration of 10 pmol/ µl .

2.7.6.2 .PCR mixture and PCR program conditions.

Polymerase Chain Reaction were performed in PCR tubes under sterile conditions. All volumes of the reaction mixture were completed to 25µl using nuclease free water. All amplification experiments included a negative control blank (which contained all PCR materials with the exception of the target DNA). Mixture and program conditions for PCR amplification of PRL gene and PRLR gene are listed as follows:

- The Go Taq® Master Mix was thawed at room temperature. The master mix was mixed by vortexing then it was spinned briefly in a micro centrifuge

Component of Go Taq® Master Mix are illustrated in table (2-4).

Table (2-4) Components of Go Taq® Master mix

NO.	Component	Quality / concentration
1-	Taq polymerase	2.5µl
2-	dNTP (dATP, dCTP, dGTP, dTTP)	250 µM
3-	Tris – HCL (pH 9.0)	10 mM
4-	KCl	30mM
5-	MgCl	1.5mM

- The reaction mix was prepared by combining the following:

The optimal values of various steps in the amplification**.**

Components	Volume	Concentration
Go Taq® Green Master Mix	12.5 µl	2x
Forward primer	1.0 µl	0.2 µM
Reverse primer	1.0 µl	0.2 µM
DNA template	3 µl	< 250 mg
Nuclease free water	7.5 µl	-
Final volume	25 µl	

- The mixture then put in microcentrifuge for a better mixing.

- The reaction was placed in a thermal cycler using the protocol shown in the table below:

Program of PCR used for amplification of prolactin gene using primer 1:

Thermal cycler protocol	No. of cycle	Temperature –time
Initial Denaturation	1 cycle	94^0C for 5 minutes
Denaturation	35cycle	94^0C for 1 min
Annealing		58^0C for 1 min
Extension		72^0C for 1 min
Final Extension	1 cycle	72^0 C for 10 mins.

Program of PCR used for amplification of prolactin Gene is using primer 2:

Thermal cycler protocol	No. of cycle	Temperature –time
Initial Denaturation	1 cycle	94^0C for 5 minutes
Denaturation	35cycle	94^0C for 1 min
Annealing		59^0C for 1 min
Extension		72^0C for 1 min
Final Extension	1 cycle	72^0 C for 10 min.

Program of PCR used for amplification of prolactin gene using primer 3:

Thermal cycler protocol	No. of cycle	Temperature –time
Initial Denaturation	1 cycle	94^0C for 5minutes
Denaturation	35cycle	94^0C for 1 min
Annealing		58^0C for 1 min
Extension		72^0C for 1 min
Final Extension	1 cycle	72^0 C for 10 mins.

Program of PCR used for amplification of prolactin gene using primer 4:

Thermal cycler protocol	No. of cycle	Temperature –time
Initial Denaturation	1 cycle	94 ^{o}C for 5 minutes
Denaturation	35cycle	94 ^{o}C for 1 min
Annealing		55 ^{o}C for 1 min
Extension		72 ^{o}C for 1 min
Final Extension	1 cycle	72 ^{o} C for 10 mins.

Program of PCR used for amplification of prolactin gene using primer 5:

Thermal cycler protocol	**No. of cycle**	**Temperature –time**
Initial Denaturation	1 cycle	94 °C for 5 minutes
Denaturation	35cycle	94 °C for 1 min
Annealing		57 °C for 1 min
Extension		72 °C for 1 min
Final Extension	1 cycle	72 ° C for 10 mins.

Program of PCR used for amplification of prolactin gene using primer 6:

Thermal cycler protocol	**No. of cycle**	**Temperature –time**
Initial Denaturation	1 cycle	94 °C for 5 minutes
Denaturation	35cycle	94 °C for 1 min
Annealing		59 °C for 1 min
Extension		72 °C for 1 min
Final Extension	1 cycle	72 ° C for 10 mins.

Program of PCR used for amplification of prolactin gene using primer 7:

Thermal cycler protocol	No. of cycle	Temperature –time
Initial Denaturation	1 cycle	94 °C for 5 minutes
Denaturation	35cycle	94 °C for 1 min
Annealing		58 °C for 1 min
Extension		72 °C for 1 min
Final Extension	1 cycle	72 ° C for 10 mins.

Program of PCR used for amplification of prolactin gene using primer 8:

Thermal cycler protocol	No. of cycle	Temperature –time
Initial Denaturation	1 cycle	94 °C for 5 minutes
Denaturation	35cycle	94 °C for 1 min
Annealing		59 °C for 1 min
Extension		72 °C for 1 min
Final Extension	1 cycle	72° C for 10 min.

Program of PCR used for amplification of prolactin receptor gene using primer 1:

Thermal cycler protocol	No. of cycle	Temperature –time
Initial Denaturation	1 cycle	94 °C for 5 minutes
Denaturation	35cycle	94 °C for 1 min
Annealing		61 °C for 1 min
Extension		72 °C for 1 min
Final Extension	1 cycle	72 ° C for 10 mins.

Program of PCR used for amplification of prolactin receptor gene using primer 2:

Thermal cycler protocol	No. of cycle	Temperature –time
Initial Denaturation	1 cycle	94 °C for 5 minutes
Denaturation	35cycle	94 °C for 1 min
Annealing		57 °C for 1 min
Extension		72 °C for 1 min
Final Extension	1 cycle	72 ° C for 10 mins.

Program of PCR used for amplification of prolactin receptor gene using primer 3:

Thermal cycler protocol	No. of cycle	Temperature –time
Initial Denaturation	1 cycle	94 ^{o}C for 5 minutes
Denaturation	35cycle	94 ^{o}C for 1 min
Annealing		57 ^{o}C for 1 min
Extension		72 ^{o}C for 1 min
Final Extension	1 cycle	72 ^{o} C for 10 mins.

Program of PCR used for amplification of prolactin receptor gene using primer 4:

Thermal cycler protocol	No. of cycle	Temperature –time
Initial Denaturation	1 cycle	94 ^{o}C for 5 minutes
Denaturation	35cycle	94 ^{o}C for 1 min
Annealing		57^{o}C for 1 min
Extension		72 ^{o}C for 1 min
Final Extension	1 cycle	72 ^{o} C for 10 mins.

2.8.Gel electrophoresis (Sambrook and Russell, 2001)

1- Agarose gel (2%), were prepared by mixing 100ml of 0.5X TBE buffer and 2 gm agarose in a glass bottle. A glass bottle was heated in a magnetic stirrer with a heater until the agarose was dissolved.

2-This solution was cooled to 70^{o}C, 5 µl ethidiumbromide was added from stock solution and mixed thoroughly.

3-The clean glass mold (17X12X4 cm) was set on a horizontal section of the bench. The comb was set in position 0.5-1.0 mm above the surface of the mold so that a complete well was formed when agarose was added.

4-The warm agarose – solution was poured into the mold.

5-After the gel was completely set (20-30 min. at room temperature), the comp was carefully removed and the gel mounted in the electrophoresis tank which contains previously small amounts of 1X TBE buffer.

6-A volume of 600ml of 1X TBE was added to cover the gel in depth about 1mm.

7-A volume of 10 µl of the sample of DNA (PCR product) was added slowly into the slots of the submerged gel using an automatic micropipette.

8-A volume of 5 µl of DNA marker was mixed with 1 µl of loading buffer. The mixture was set slowly into the slots of the submerged gel using an automatic micropipette.

9-The gel was subjected to electrophoresis at 70 volts until the bromophenol blue tracking dye migrated at least two-thirds of the way down the gel (around 2 hours).

10-The gel was examined by ultraviolet light using a UV transilluminator to utilize the DNA bands. Then, gel documentation system was used for document bands.

2.9. Measurements of DNA concentration before sequencing:

Before sequencing DNA concentration of the prolactin gene, PCR products from 50 blood samples and 20 healthy were measured using nanodrop (techno UK). Nearly all products gave a concentration more than 100ng/µl which is a good concentration required for DNA to be sequenced.

2.10. DNA sequencing of purified PCR product.

The purified PCR products of the analyzed PRL and PRLR gene regions and primers were sent by Macrogen company (U.S.A) for DNA Sequencing. The obtained sequences of these samples were aligned using (Mega-6) software. Furthermore, the nucleotide sequences were compared to the information in gene bank of the National Center for Biotechnology Information (NCBI) web site databases using the BLAST search tool and examined for the presence of SNPs.

2.11. Statistical analysis.

The statical analysis of any study is very essential for outlining the final outcome of the results. Data were translated into a computerized database structure. The statical significance of the association between all categorized variables was assessed by a linear regression. The statistical analysis was done using Minitab 15 statistical analysis software. Two way ANOVA test was used to compare different groups among each other and with the control. All values were expressed as Ch square, LSD and Mean ± Standard Deviation (M ± SD). P value < 0.05 and < 0.01 was regarded as statistically significant.

Chapter Three
Results and Discussion

3. Results and Discussion.

3.1. The distribution of the studied groups.

Table (3-1) shows a total of two hundred samples was taken. One hundred fifty (75%), samples from hyperprolactinemic infertile women. This group was screened as a case group and the remaining fifty (25%) fertile healthy group as a control. The case group was distributed as 97 (64.6%) were primary infertile women, 53 (35.4%) as secondary infertile women and all group ages ranged from (20-50) years old.

The p Value of the Chi-square test was 0.0001 which is less than 0.01; there is a significant relationship between primary and secondary infertility of hyperprolactemic patients with that of the healthy. The details of the distribution of the studied groups are shown in figure (3-1).

Table (3-1): Frequency and percentage of the whole group, control group, primary and secondary infertility case groups.

Sample	Frequency	Percent
Study group	200	100%
Control	50	25%
Hyperprolactinemia	150	75%
Primary infertility	97	64.6%
Secondary infertility	53	35.4%
Chi-square (χ^2)	---	9.673 **
P-value	---	0.0139

**** (P<0.01).**

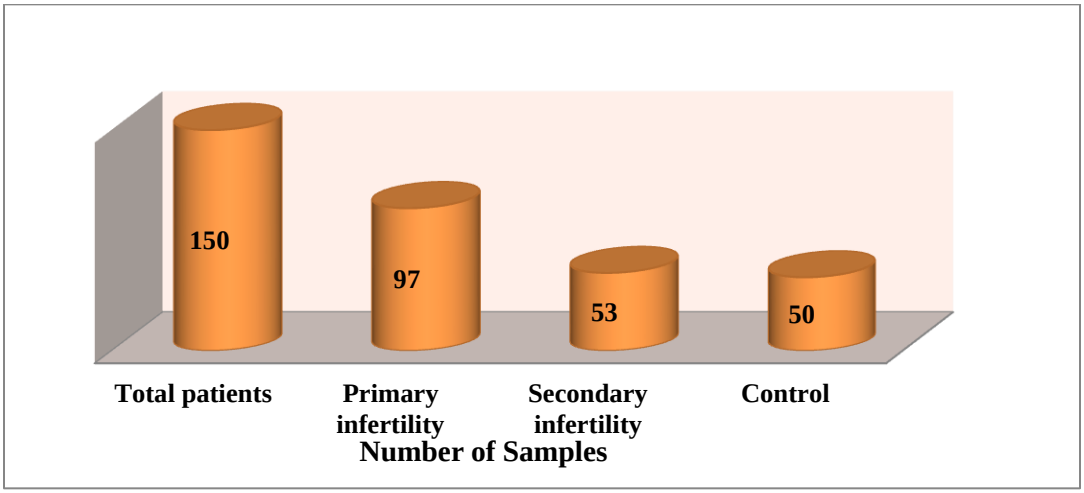


Figure (3-1): Distribution of study groups.

3.1.1. Effect of hyperprolactinemia on fertility hormones.

The screening of hyperprolactinemia according to the fertility hormones (LH, FSH) was affected by the increased prolactin level, the results are shown in table (3-2).

Table (3-2): Serum prolactin (ng/ml), LH, FSH (mIU/ml) concentrations in females with hyperprolactinemia.

Hormone type	Normal concentration	Measured concentration	No. of total cases.	Ages
Prolactin	1.3-25 ng/ml	30 -114 ng/ml	150	20-50
LH	1.5-8 mlU/ml	1- 4.5mlU/ml		
FSH	3.9-12 mlU/ml	0.8 -3.59 mlU/ml		

Prolactin level increased in hyperprolactemic patients and also it had an effect on decreasing the two fertility hormones (LH and FSH). This is clear in table (3-3). From the same table it is found that the mean of prolactin hormone level in hyperprolactemic patients is 42.18 ± 12.64, while for control healthy is 10.84±4.67. This higher difference is statistically significant as the difference between the mean value of two groups (control and patients) is higher than the LSD value which is 8.306. For LH hormone, the decrease in hormone level of hyperprolactemic patient is highly significant as the difference of the mean value for both groups (patients and control) is higher than LSD for this hormone which is 1.941. The same was also for the other fertility hormone (FSH), in which it decreased in hyperprolactemic patients rather than in the healthy control group.

Table (3-3): Serum prolactin, LH and FSH in hyperprolactinemic patients.

Hormone	Mean ± SD		LSD value
	Control	Patients	
Prolactin ng/ml	10.84±4.67	42.18 ± 12.64	8.306 **
LH mIU/ml	5.700±1.809	2.60 ± 0.937	1.941 **
FSH mIU/ml	8.251±2.477	3.71 ± 1.077	2.853 **

** ($P<0.01$).

3.1.2 Hyperprolactinemic patients, according to type of infertility

The comparison between prolactin levels and the fertility hormones in primary and in secondary infertile women , and the p value were all listed in table (3-4). an increase was found in prolactin level in secondary infertile women rather than in primary infertile women when the mean was 41.1±11.8 in the primary infertile group and 44.0±16.1 in the secondary infertile group respectively, but the difference of PRL hormone

level between two groups was statistically non significant as. For FSH hormone, it is clear that the level of it's decrease in the secondary infertile group was of the mean 3.55±1.43, but it is higher in the primary infertile group when its mean was 3.80± 1.13. This increase is not significant as the difference between the levels of these two hormones was 0.25, and that was less than T-value (0.972). Also for the other (LH), it is clear in from the table that the level is higher than that in the primary infertile group in which the mean was 2.61± 1.05 but in the second group, it was 2.59±1.18, and the difference between these two groups was 0.02 but it was less than T-value 0.619. thus the decreases in the hormones level in the second infertile women were not significant.

Table (3-4): Group Statistics of primary and secondary infertile women by mean, SD. Deviations and T- Value >0.05, N= number).

Fertility type		N	Mean	St.Dv	T- value
Prolactin	Primary	97	41.1 ng/ml	11.8	8.029 NS
	Secondary	53	44.0 ng/ml	16.1	
FSH	Primary	97	3.80 mIU/ml/	1.13	0.972 NS
	Secondary	53	3.55 mIU/ml	1.43	
LH	Primary	97	2.61 mIU/ml	1.05	0.619 NS
	Secondary	53	2.59 mIU/ml	1.18	

NS: Non-significan . A graphical representation of fertility hormones according to type of infertility is shown in figures (3.2),(3.3) and (3.4) regarding PRL, FSH and LH respectively.

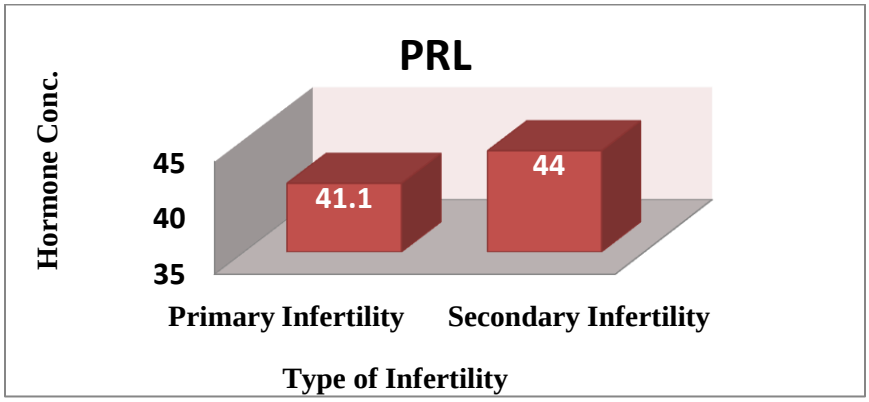


Figure (3-2): PRL levels in hyperprolactinemic infertile women categorized according to nfertility type.(35,40 and 45 ng/ml)refers to hormone range.

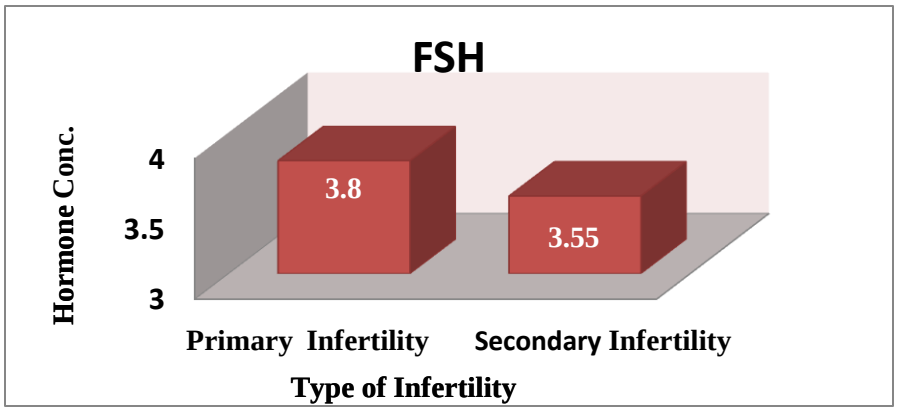


Figure (3-3): FSH levels in hyperprolactinemic infertile women categorized according to infertility type.(3, 3.5 and 4 mlU/ml) refers to hormone range.

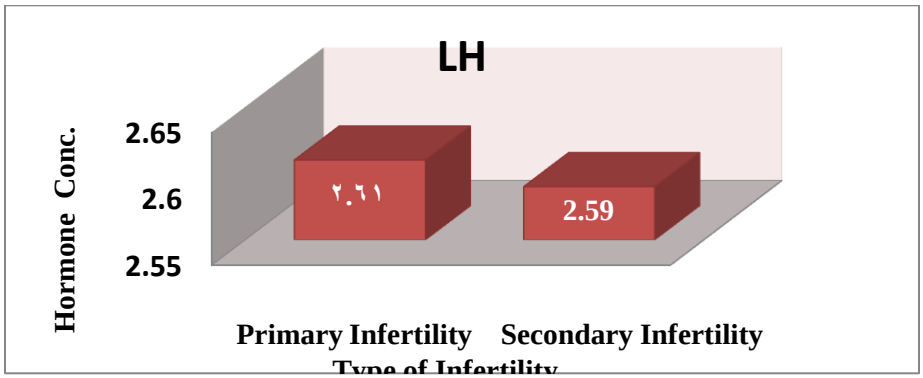


Figure (3-4): LH levels in hyperprolactinemic infertile women categorized according to infertility type.(2.55, 2.6 and 2.65 mlU/ml) refers to hormone range.

3.1.3. Hyperprolactinemic patients according to age groups and types of fertility hormones.

The results in table 3-5, show that the measurements of prolactin hormone increased with each infertile group while the fertility hormones (LH and FSH) decreased compared with the control group.

The serum prolactin means and SD were 38.56±12.59, 44.10±14.98 and 43.87±12.12.49 in the three age infertile groups while it was 10.84±4.67 in the control group. It was shown from the table (3- 5) that the elevation in prolactin hormone was more in the age group (31-40) years old among the other two groups (20-30), (41-50) years old, with a mean ± SD of 44.10±14.98, but the elevation was less in group 20-30 in which the mean was 38.56 ±12.59. In the age group (41-50) years old the prolactin level was noticed with a mean of 43.87±12.49 in comparison to the age group (31-40) years old. This difference in PRL hormone level with the three age groups was not significant. For the LH hormone, the decrease in level of it was more in the age group (41-50) years old, with the mean 2.451±1.078, and also decreased in the other two age groups (20-30), (31-40) years old, but the decrease in the hormone level between the three age groups was non significant. In contrast, for the third hormone FSH it was noticed that this hormone decreased significantly in all age groups as the difference of level of this hormone was more than the LSD value which was 0.638.

Table (3-5):Statistical analysis of fertility hormones in serum of hyperprolactinemic females categorized according to age.

Hormone	Age group			LSD value	Control
	20-30 (Mean±SD)	31-40 (Mean±SD)	41-50 (Mean±SD)		(Mean±SD)
Prolactin ng/ml	38.56±12.59 A	44.10±14.98 A	43.87±12.49 A	9.337 NS	10.84±4.67
LH mIU/ml	2.756±0.925 A	2.594±1.256 A	2.451±1.078 A	0.502 NS	5.700±1.809
FSH mIU/ml	3.915±0.956 A	4.007±1.436 A	3.199±1.168 B	0.638 *	8.251±2.477

*(P<0.05), NS: Non-significant.

The graphical presentation of each hormone in patient and health group categorized according to age, is shown in figures (3-5),(3-6) and (3-7).

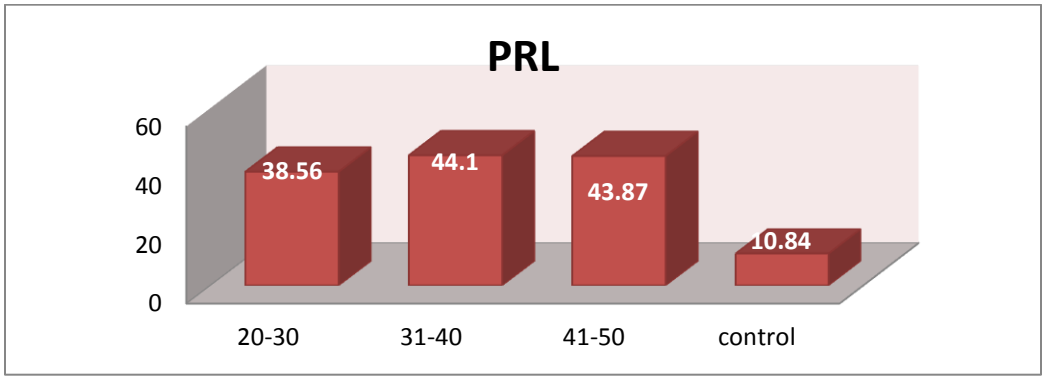


Figure (3-5): PRL level in three patient groups according to age and control group.(0, 20, 40 and 60 ng/ml) reffered to hormone range.

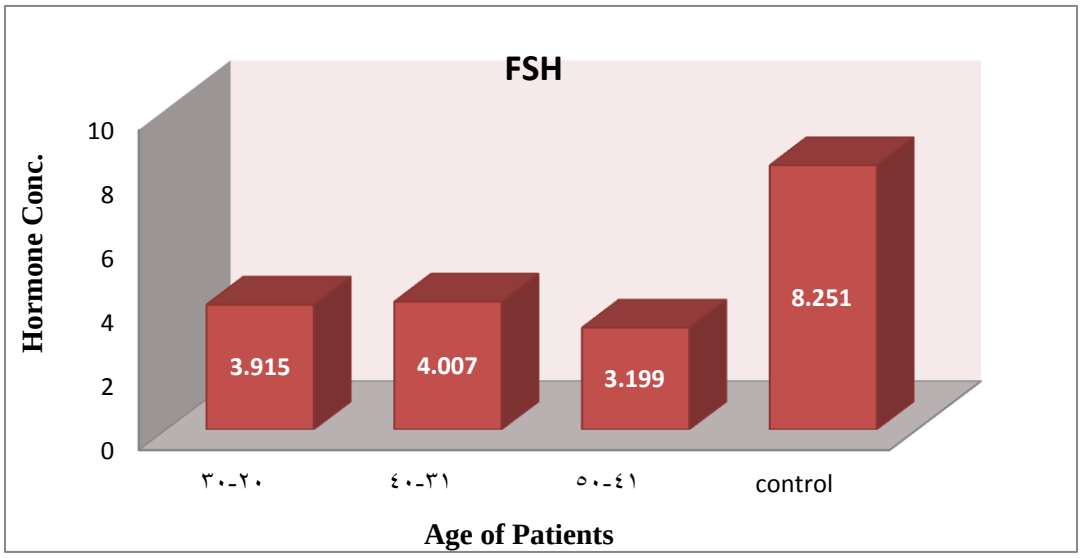


Figure (3-6): FSH level in three patient groups according to age and control group.(0, 2, 4, 6, 8 and 10 mlU/ml), reffered to hormone range.

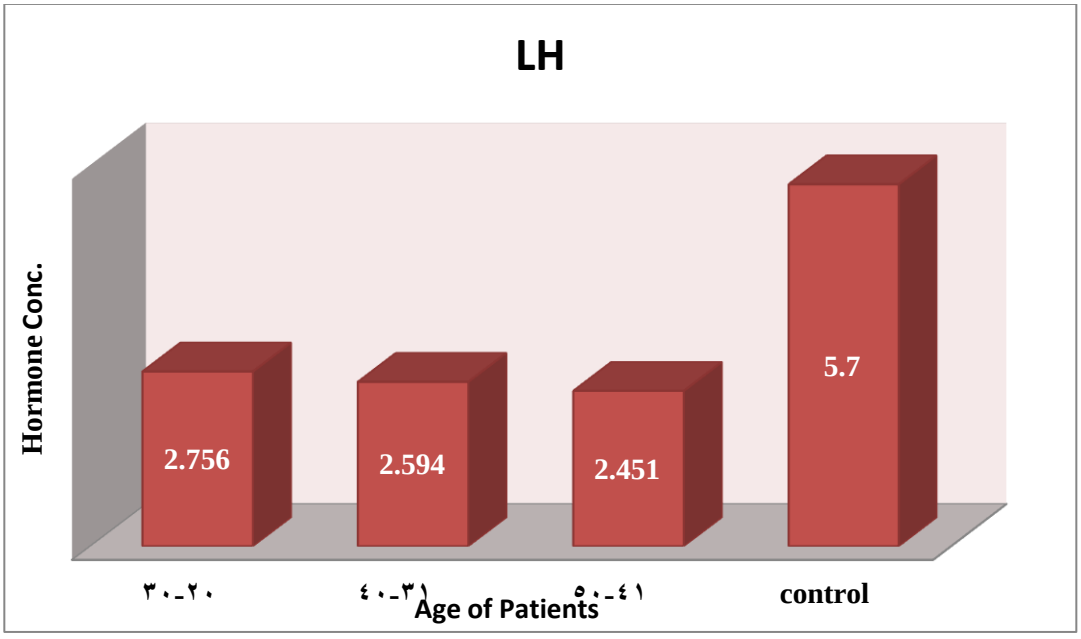


Figure (3-7): LH level in three patient groups according to age and control group.(0, 1, 2, 3, 4, 5 and 6 mlU/ml), refered to hormone range

The results of this study revealed elevated prolactin concentrations in the infertile subjects. By comparison between two infertile groups it was found that the prolactin value in secondary infertile women was higher than the prolactin value in primary infertile women . The result coincided with the study of Solomon *et al*., (2013) who stated that the concentration of prolactin has been always higher in secondary infertile women than in the primary infertile women. Also, according to Al-Muhammadi *et al*., (2012), this was probably due to the fact that the patients with secondary infertility consult a specialist less frequently than others because they are in less urge for seeking medical help since they were already got children. This delay in the treatment could be due to the fact that these cases had a marginal elevation of the prolactin and were not having any additional symptoms except infertility, so they waited for a longer period.

Furthermore the obvious results showed that the highly significant increase in serum prolactin level in both infertile groups will lead to a significant decrease in serum LH and FSH. Thus in different groups of hyperprolactinemic, women have an increase in serum prolactin level and a decrease in other fertility hormones and this was observed more in women with secondary infertility than women with primary infertility. Also in different age groups of hyperprolactinemic women, an increase in serum prolactin level and variation in other hormonal levels were observed. It is clear that this occurs more in women with the age group (30-40) years, as shown in figures (3-3) and (3-4) but the difference is not significant.

This result coincides with the study of Lee *et al*. (2012) who showed hyperprolactinemia decreases in the pulsatile secretion of gonadotrophin-releasing hormone impairing the release of LH and FSH . It is clear that

most of the increase in prolactin level among the different age groups happened in the age of thirties. It is well known that age plays an important role in female infertility, however, in this study the correlation between them is clear as the mean value of the increased prolactin level was found in one age group rather than in the other . This support the idea, that when the abnormal elevation starts, it will continue and may remain in women. (AbdElghani *et al*., 2013).

3.2. Molecular diagnosis of infertile hyperprolactinemic patients.

To study the genetic cause of hyperprolactinemia, the PRL gene and PRLR gene was diagnosed after extracting DNA from the above patients then analyzed by PCR amplifications using specific primers.

3.2.1. Concentration and purity of DNA extracted from blood sample.

Genomic DNA was extracted from all samples of patients using genomic DNA miniprep extraction kit, as shown in table (3-6).

Table (3-6): Concentration and purity of DNA from patient samples

Type of patient sample	DNA conc. ng/ µl	DNA purity
Frozen blood	50.8- 120.2	1.7-2.0

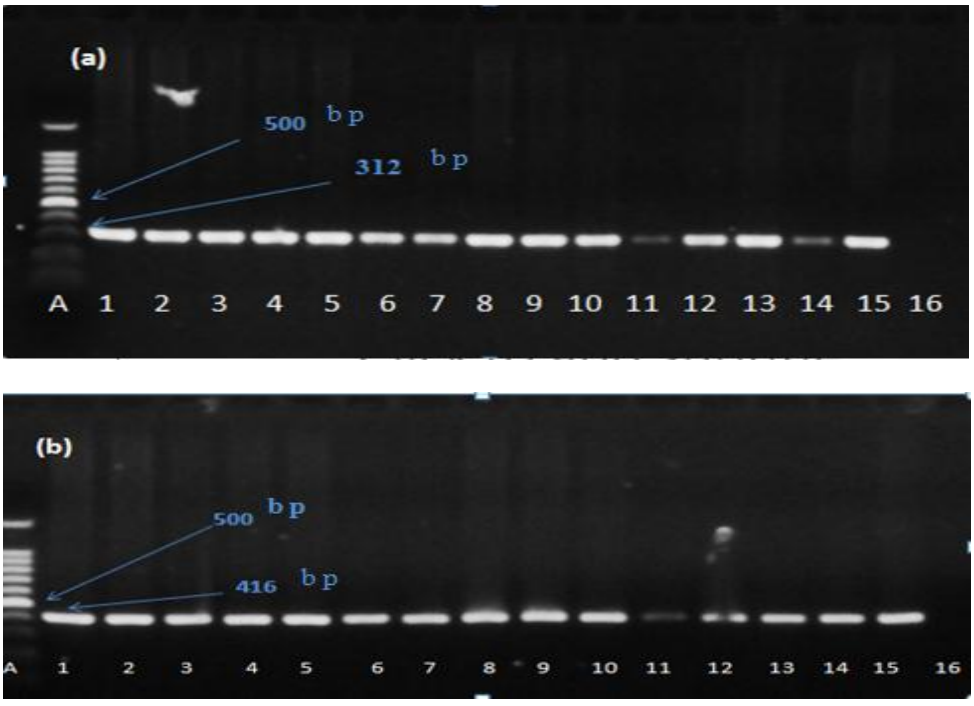
It is clear that this quantity and purity are suitable for amplification by PCR because higher amounts of DNA template increase the risk of generating nonspecific PCR products and lower amounts of product reduce the accuracy of the amplification. Also, DNA samples have been analyzed by gel electrophoresis in which the bands give an indicator that DNA pure and ready to be subjected to PCR amplification.

3.2.2. Molecular study of PRL and PRLR gene.

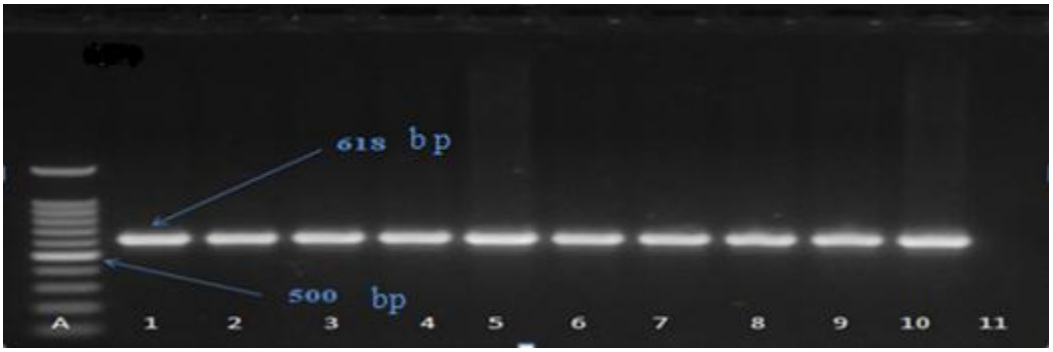
To select the molecular genetic markers related to women product of prolactin hormone, prolactin gene and prolactin gene receptor were adapted to be candidate genes. In this study, eight pairs of primers were designed to amplify PRL gene and 4 pairs of primers were designed to amplify PRLR gene using the NCBI Primer-Design online tool to detect any genetic variations of this target gene leading to a hyper expression of prolactin hormone.

3.2.2.1 Prolactin gene amplifications

All blood samples were subjected to molecular detection through PCR amplification of the PRL gene by using eight specific primers predesigned for this gene. The first 3 primers were used in this PCR technique (PRL1, PRL2, PRL3), specific for intron 1 region of the prolactin from NCBI primer design with product lengths(312, 416 and 618bp respectively) which is shown in figures (3-8 a, b), (3-9).

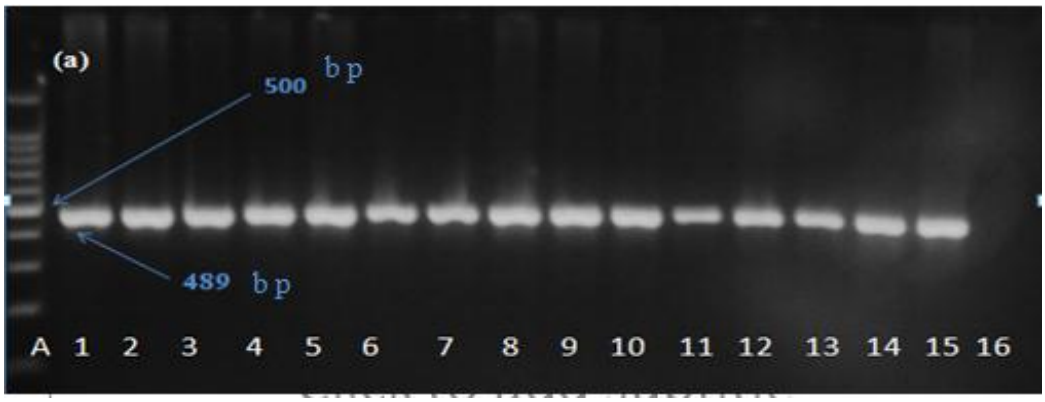


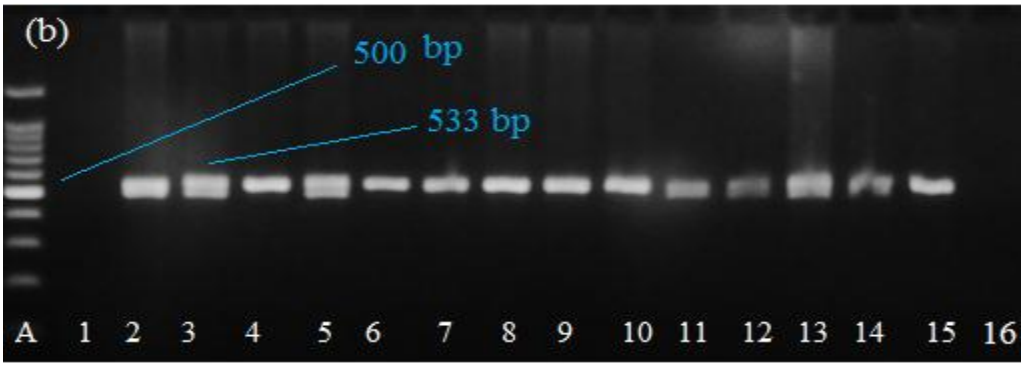
Figure(3-8, a, b): Gel electrophoresis for amplification of PRL gene of hyperprolactinemic and breast cancer patients by using primer 1, 2 which amplify intron 1 of the gene,(a) product size 312 bp and (b) 416 . Electrophoresis was performed on 1.5% agarose gel and run with a 70v/mAMP current for 50min.Line A=100bp ladder, line (1-14) DNA isolated from blood samples of patients, line (15) healthy, line (16) control negative.



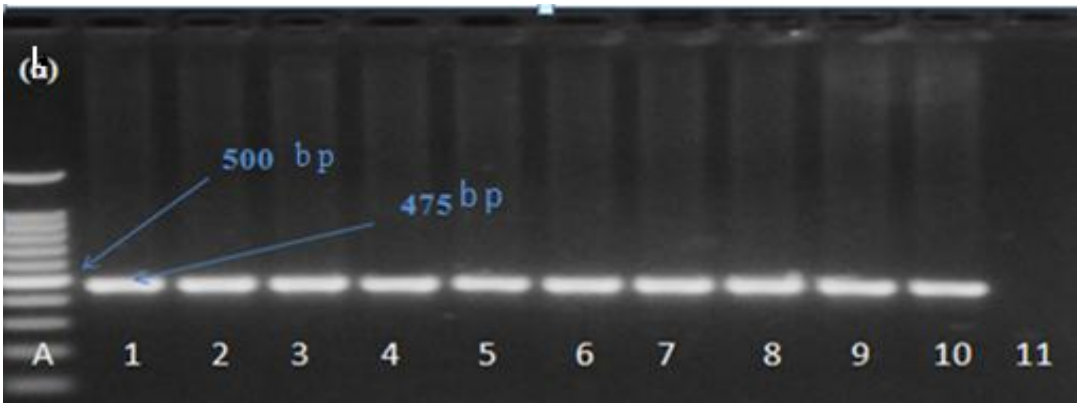
Figure(3-9): Gel electrophoresis for amplification of PRL gene of hyperprolactinemic patients by using primer 3 which amplifies intron 1 of the gene, product size 618 bp. Electrophoresis was performed on 1.5% agarose gel and run with a 70v/mAMP current for 50min.Line A=100bp ladder, line(1-9) DNA isolated from blood samples of hyperprolactinemic patients, line (10) healthy , line (11) control negative.

Another primers used in PCR technique(PRL4, PRL5, PRL6 , PRL7and PRL8) were specific to amplify exons region of prolactin gene. As can be seen in the figures below, the sizes of PCR products relevant to each exon were 489 bp for exon 2, 533 bp for exon 3, 719bp for exon 4 and 475bp for exon 5. Below, primer 4 was specific for the amplification of the second exon of the PRL gene, primer 5amplifies Exon 3, primer 6 amplifies Exon 4, primer 7 amplifies exon 5 and primer 8 amplify intron 4. Figures (3-10 a, b), (3-11 a ,b) and (3-12) show the PCR amplifications of some of hyperprolactinemic patients, in addition to the control samples, used in this study.





Figure(3-10, a, b): Gel electrophoresis for amplification of PRL gene of hyperprolactinemic patients by using (a):primer 4 which amplifies exon 2 of the gene, product size 489 bp. (b): primer 5 which amplifies exon 3 of the gene, product size 533bp. Electrophoresis was performed on 1.5% agarose gel and run with a 80v/mAMP current for 50min.Line A=100bp ladder, line(1-14) DNA isolated from blood samples of hyperprolactinemic patients, line (15) healthy ,line 16 control negative.

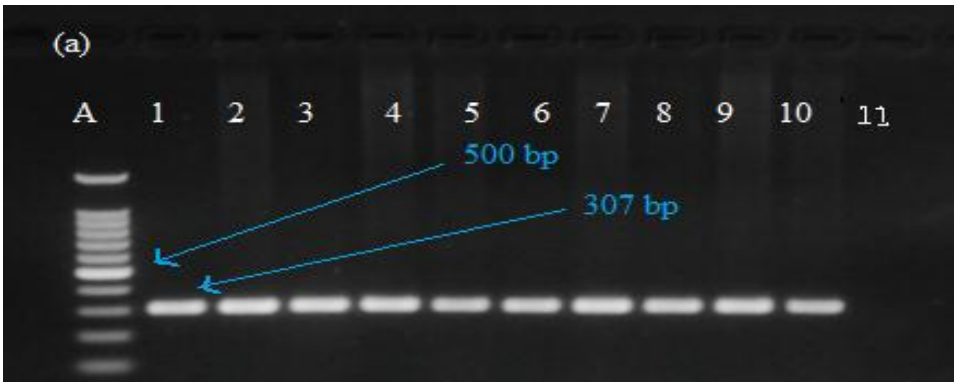


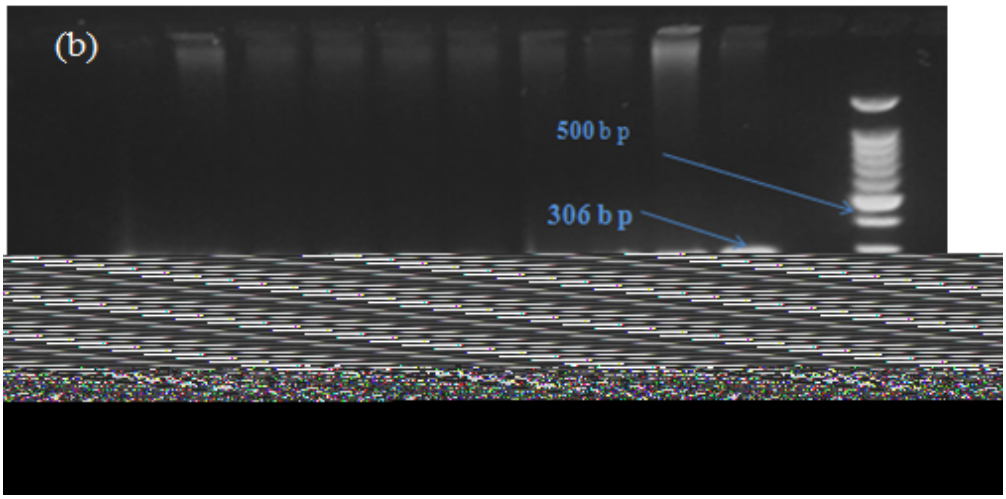
Figure(3-11, a, b): Gel electrophoresis for amplification of PRL gene of hyperprolactinemic patients by using (a):primer 6 which amplify exon 4 of the gene, product size 719 bp. (b): primer 7 which amplifies exon 5 of the gene, product size 475bp.Electrophoresis was performed on 1.5% agarose gel and run with a 80v/mAMP current for 50min.Line A=100bp ladder, line(1-9) DNA isolated from blood samples of hyperprolactinemic patients, line (10) healthy , line(11) control negative.

Figure(3-12): Gel electrophoresis for amplification of PRL gene of hyperprolactinemic patients by using primer 8 which amplifies intron 4 of the gene, product size 357 bp. Electrophoresis was performed on 1.5% agarose gel and run with a 70v/mAMP current for 50min.Line A=100bp ladder, line (1-9) DNA isolated from blood samples of hyperprolactinemic patients, line (10) healthy, line (11) control negative.

3.2.2.2. PRL receptor gene amplification.

The polymerase chain reaction technique was used to check the genetic cause of hyperprolactinemia and the relation with the PRLR activation. Four pairs were used in this study to amplify exon 1 of the prolactin gene receptor as shown in figures (3-13 a, b, c) and (3-14).





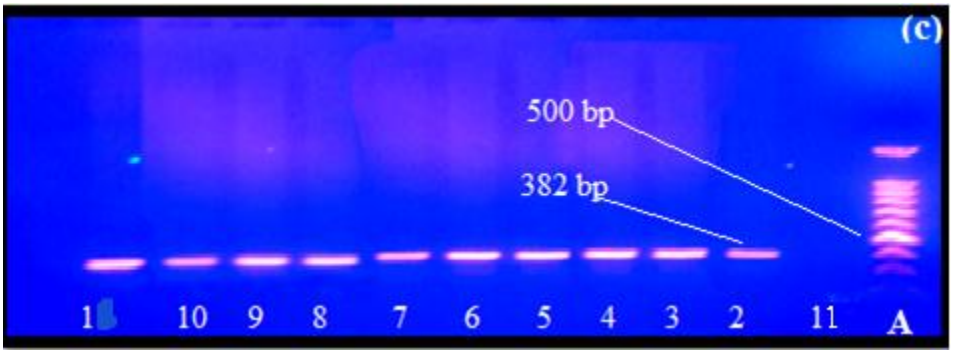


Figure (3-13 a,b and c): Gel electrophoresis of (a) PRLR 1, product size 307 bp. (b) PRLR 2 product size 306 bp.(c) PRLR3 product size 382 bp.Electrophoresis (1.5% agarose gel, at 80v/mAMP for 50min). Line A=100bp ladder, line (1-9) DNA isolated from blood samples of hyperprolactinemic patients, line (10) healthy , line (11) control negative.

The figures above show that the sizes of PCR products relevant to each primer was 307, 306, 328 and 436bp for this exon of PRLR gene. Two primers (306 and 436 bp) out of four were selected for further analysis of gene sequences.

Figure (3-14): Gel electrophoresis of amplification of PRLR4 of hyperprolactinemic and breast cancer patients product size 436 bp. Electrophoresis was performed on 1.5% agarose gel and run with a 80v/mAMP current for 50min.Line A=100bp ladder, line (1-10) DNA isolated from blood samples of hyperprolactinemic patients, (11) healthy and line (12) control negative.

3.3. Detection of PRL and PRLR gene mutations in hyperprolactemic samples by sequencing.

After amplification of genomic fragments corresponding to intron1 to exon 5 of the PRL gene and exon 1 of PRLR gene, the PCR products were (312, 618,416, 489, 533,719, 475, 357bp) for the prolactin gene and (307, 306, 436,382 bp) for the prolactin gene receptor shown in the figures. By using the DNA of the above cases good quality products (pure and concentrate) were selected to be sequenced in order to evaluate if any genetic variation in the PRL and PRL receptor gene were known as predictors of high prolactin levels.

The sequencing was done for patient women, i.e., 15 samples for each exon of hyperprolactinemic patients with 5 for control. The sequence involved part of the PRL gene spanning from nucleotide number 6169 to nucleotide number 17680 of chromosome 6.The results were directly compared with the Iraqi healthy, and compared to the information

in the gene bank of the NCBI web site databases at www.ncbi.nlm.nih.gov using the BLAST search tool and also by using Mega 6 program. The current study utilized a forward and reverse primer for sequencing PRL gene of blood sample of hyperprolactinemic patients. It was found that the mutations were found around all PRL gene regions involved in this study, i.e., (intron 1, exon2, exon3. exon4 and exon5). According to NCBI, this streach contains 26 SNPs.

The mutation frequency was different among the five studied regions of the gene. Table (3-7) shows the details of the number of these SNPs with their percentage. It is obvious that the highest mutation number was in exon 2, which was 9 mutations and this number is significant as the p-value of it is 0.0001, while the less mutation number was in exon 3 and exon4 which was 2 for each exon with p-value 0.0001 for each and this also significant. On the other hand, no mutation was detected in exon 5. In intron 1 of gene, 9 mutations were detected.

From table (3-7), it is clear that many samples showed mutations at different regions of the gene. Such sequencings of the coding regions of the amplified product of the PRL gene was done seeking for the detection of any polymorphisms within these sequences related to hyperprolinemic patients and the relation between the SNPs was seen. The percentages of mutation types that displayed substitution 88.46% and deletion 11.54% were present in hyperprolactemic patients, details in table (3-8).

Table (3-7): Prolactin gene mutations in hyperprolactinemic patients.

PRL gene mutation						
Type of mutation	**Exon2**	**Exon3**	**Exon4**	**Exon5**	**Intron1 by using p.1**	**Intron1 by using p.2**
Deletion	0 (0.00%)	1 (50.0%)	0 (0.00%)	0 (0.00%)	2 (28.58%)	0 (0.00%)
Insertion	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
Substitution	9 (100.00%)	1 (50.0%)	2 (100.00%)	0 (0.00%)	5 (71.44%)	6 (100.00%)
Total	9 (100%)	2 (100%)	2 (100%)	0 (0.00%)	7 (100%)	6 (100%)
Chi-square (χ^2)	15.00 **	12.75 **	15.00 **	0.00 NS	15.00 **	14.96 **
P-value	0.0001	0.0001	0.0001	1.00	0.0001	0.0001

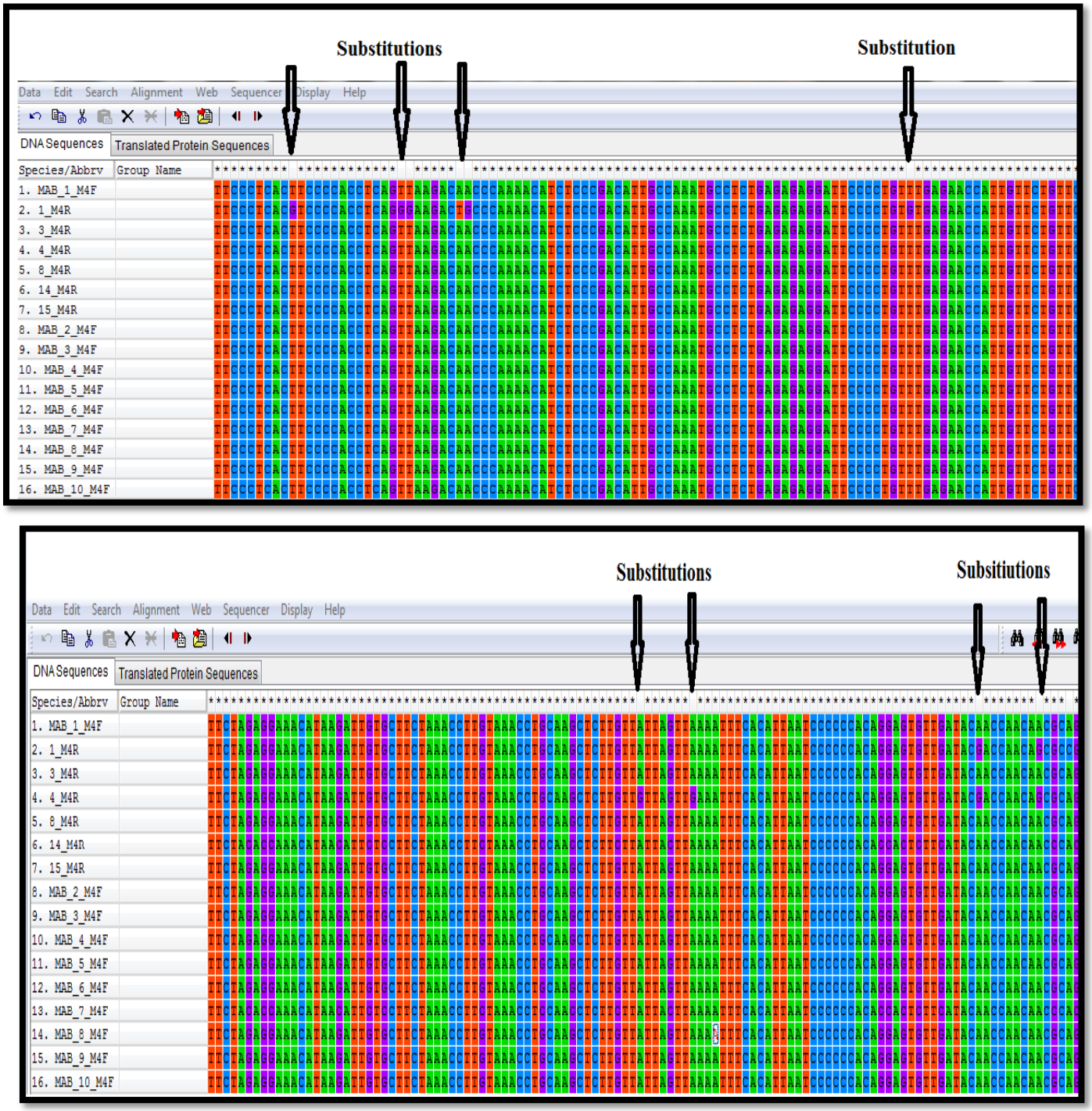
**** (P<0.01).**

Table (3-8): Percentages of mutation types in hyperprolactemic and breast cancer patients.

Type of mutation	Percentage
Substitution	88.46%
Deletion	11. 54%

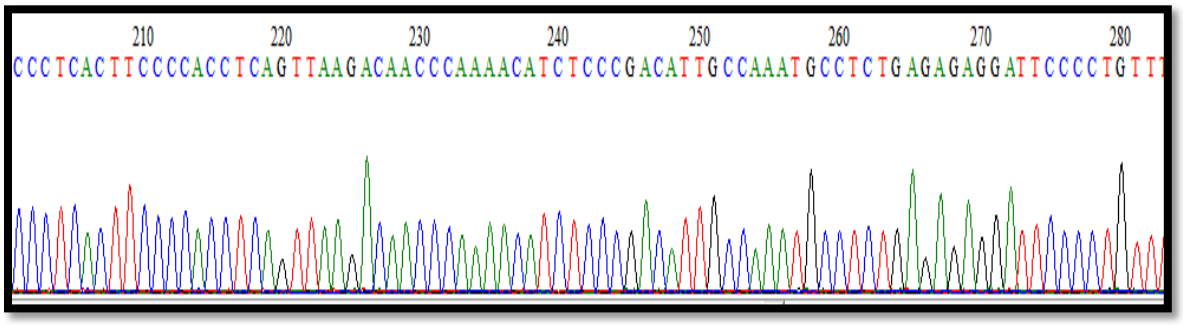
3.3.1. Detection of mutations in PRL gene.

The polymorphisms observed within the exon 2 of PRL gene are shown in figure (3-15), where patients with polymorphisms were compared to the healthy samples. All polymorphisms in this exon were observed in infertile hyperprolactemic patients while no mutation was detected in breast cancer patients. The substitutions are obvious in figure (3-16 a, b)**.** A homology with the PRL gene of *Homo sapiens* from the Gene Bank was done , 100% compatibility of that gene of healthy samples with standard genes of Gene Bank results are shown in figure (3-17 a, b). The polymorphisms that we observed within exon 2, show that the samples of patients have 99% identities with the wild type. The score of healthy samples when compared to *Homo sapiens* was 452, but the score was lowered compared to that of hyperprolactemic patients which was 442, 428 respectively. The nucleotide sequence profile of exon 2 was in appendix (3).



Figure(3-15): Comparision between control and infertile hyperprolactinemic patients in MEGA 6 program for (Exon 2).

(a)



(b)

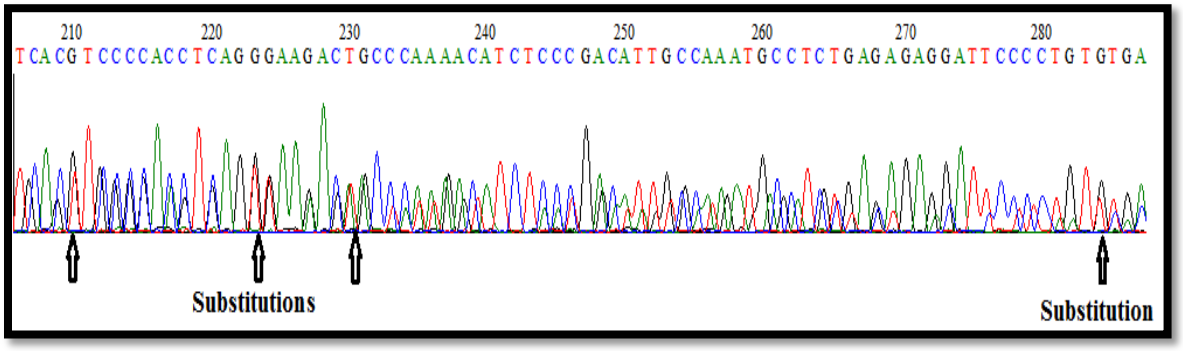
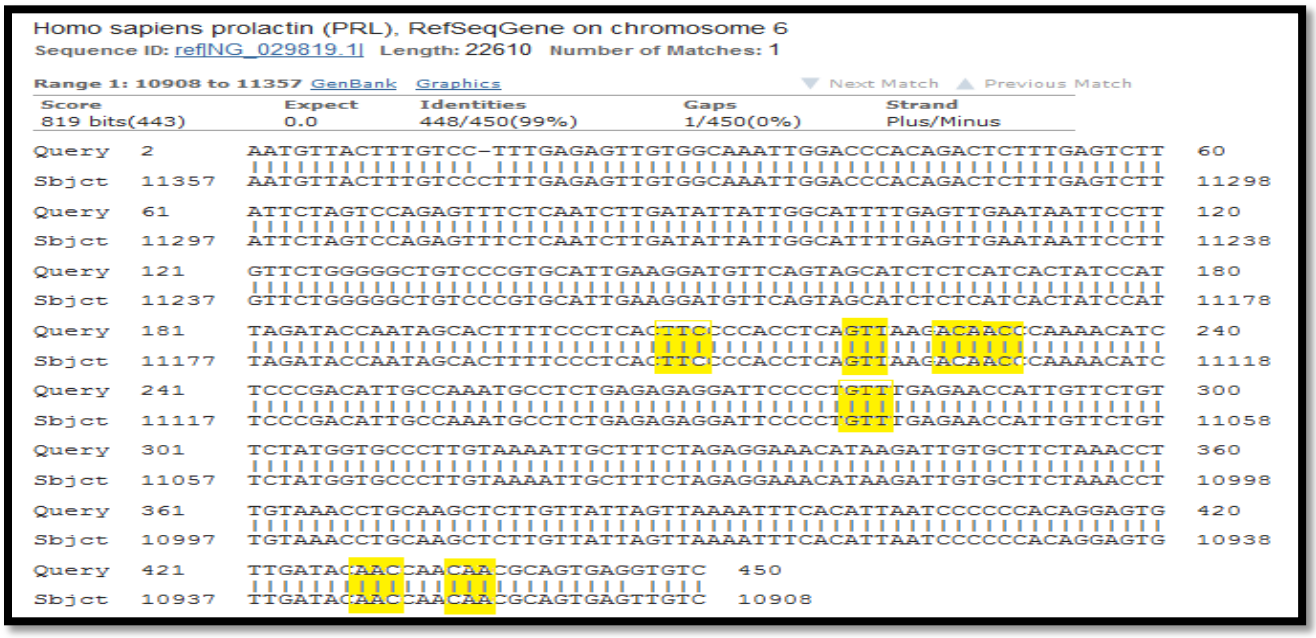


Figure (3-16) a and b. Achromatogram comparision between (a) control, (b) substitutions SNPs for Exon 2.

a:



b:

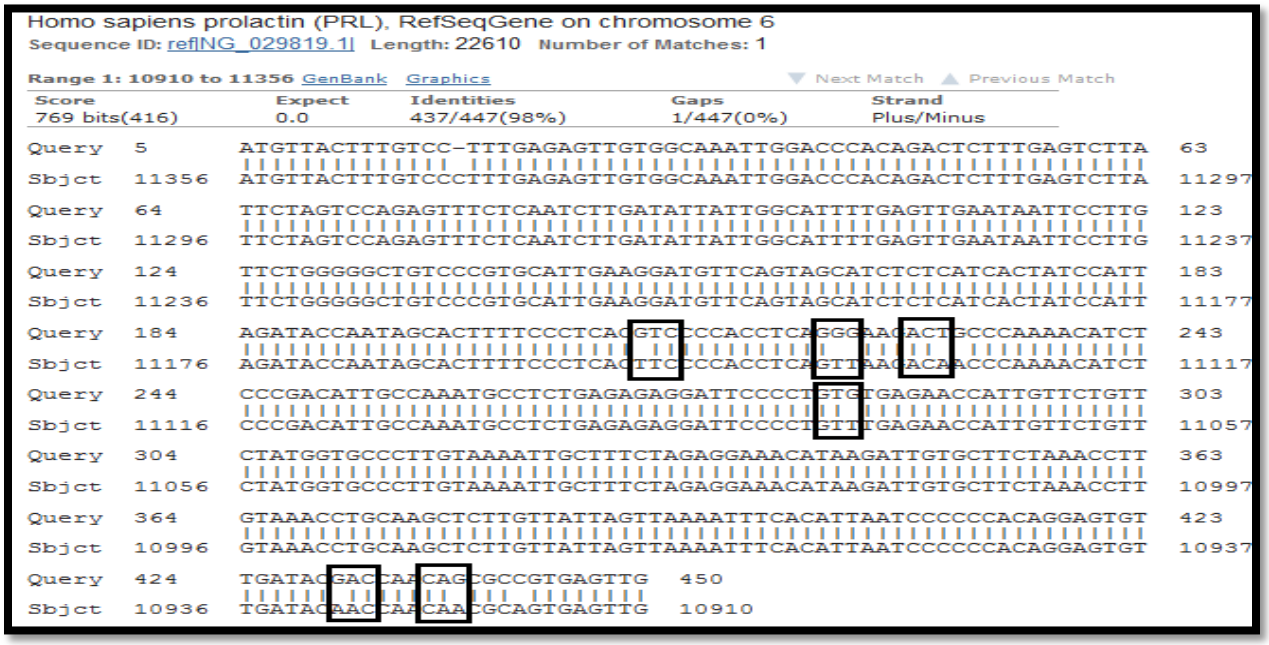


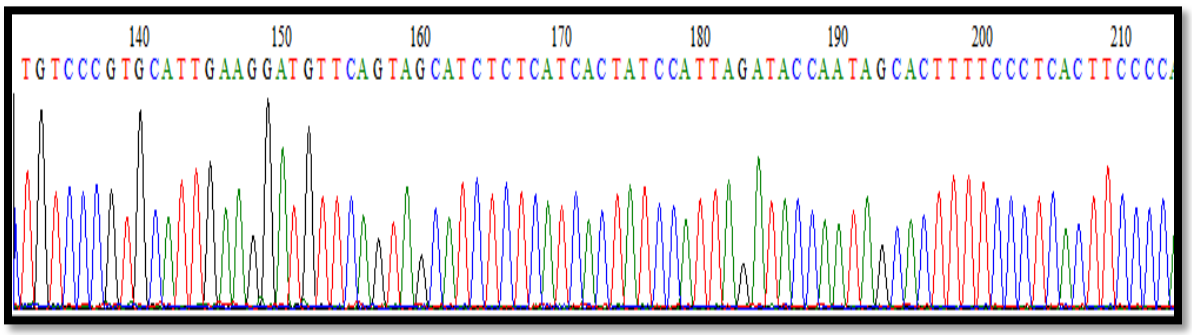
Figure (3-17) a and b: The automated sequencing of the exon 2 of PRL gene a: of healthy samples, b: for hyperprolactinemic patient.

Representation of the sample by query and the subject representation of database of the National Center for Biotechnology Information.

The region of the gene of hyperprolactemic patients, there are many SNPs, 7 SNPs in sample 1 which are TTC/GTC, in position 210 that convert a.a Phe to Val, the other GTT/GGG in position 223 and 224 that convert a.a Val to Gly, also in position 230, the ACA/ ACT which convert a.a Thr to Thr, but in position 231 the ACC changed to GCC that convert a.a Thr/Ala. In 284 the GTT converts to GTG which convert a.a Val/ Val. The last two SNPs in this sample that is common with sample 4 are , AAC/GAC in position 438, that converts a.a Asn/Asn, and the other common SNP between two samples is CAA/CAG in position 464 which converts a.a Gin/Gin. In the same region of exon , also in sample 4 of hyperprolactemic patients there are 2 SNPs, the first in position 393 in which ATT convert to GTT, that converts Ile/ Val, and the other one in position 400 TAA/TGA that converts Stop/Stop, the blast clear in (Appendix 7).

There are many heterozygous SNPs in patient samples, the five heterozygous SNPs are in hyperprolactinemic patient (sample 8) and in (sample 14). In the same region in the gene T, C, T, T and A, are found which are in positions (179, 180, 183, 194 and 195), as the peaks in figure (3-18) a and b show, in which they are harboring both wild and mutated allels.

(a)



(b)

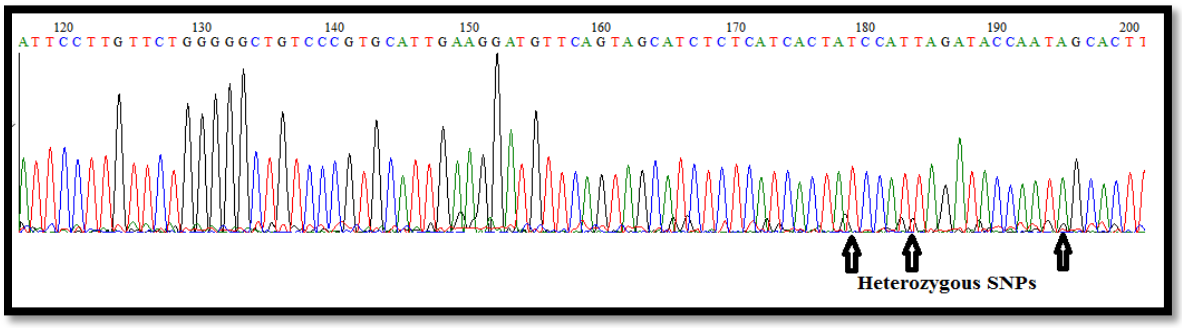


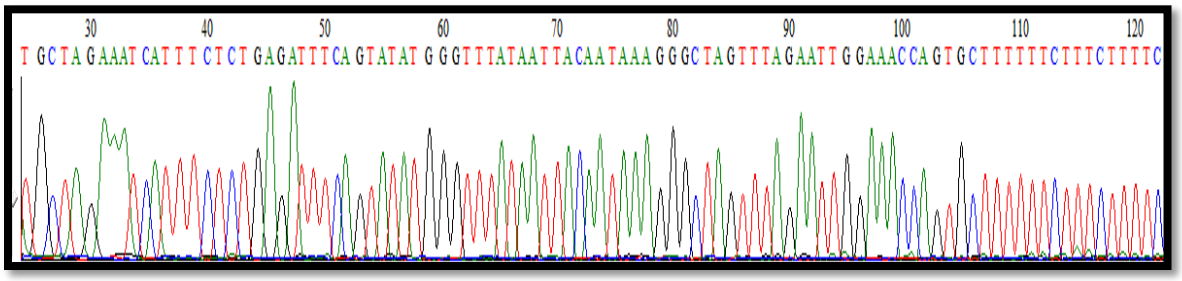
Figure (3-18): a and b. Achromatogram comparison between a: control, b: heterozygous SNPs for Exon 2.

Besides, there are other mutations that are detected in PRL gene in exon 3 using primer 5 that amplifies the region from 13878 to 14410. The polymorphisms observed within the exon 3 are shown in figure (3-19).

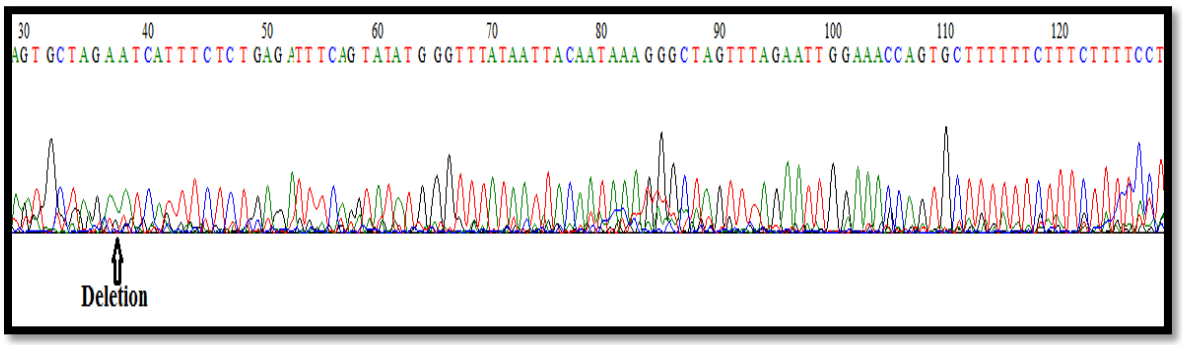
Figure(3-19): PRL gene (Exon 3) point mutations as illustrated by Mega 6. Arrows indicate the region of point mutations in hyperprolactemic patients in comparision with control.

The peaks that appear the mutations are clear in figure (3-20) a, b and c. The homology of this region of exon with the blast of NCBI was obvious in figure (3-21) a and b.

(a)



(b)



(c)

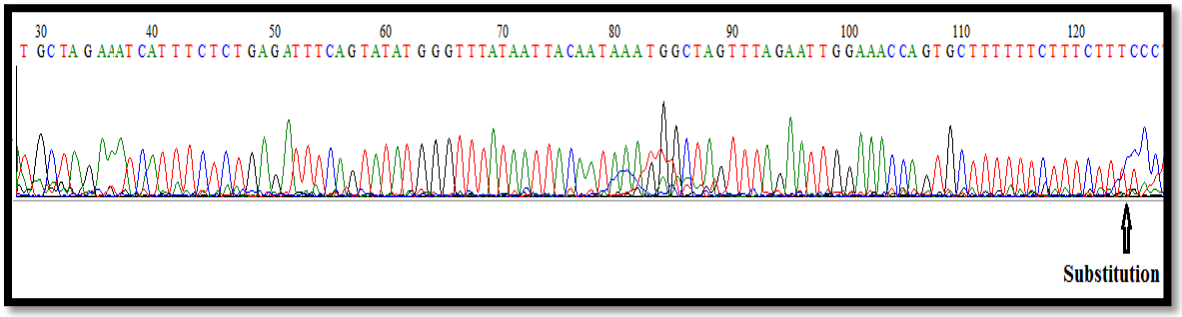
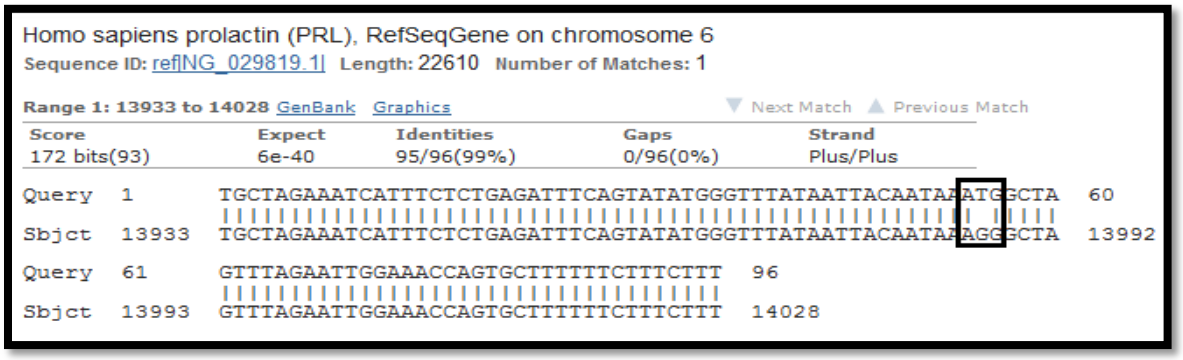


Figure (3-20) a, b and c: Achromatogram of (a) control, (b) and (c) hyperprolactemic patient, of exon 3 amplified by using primer 5.

(a)



(b)

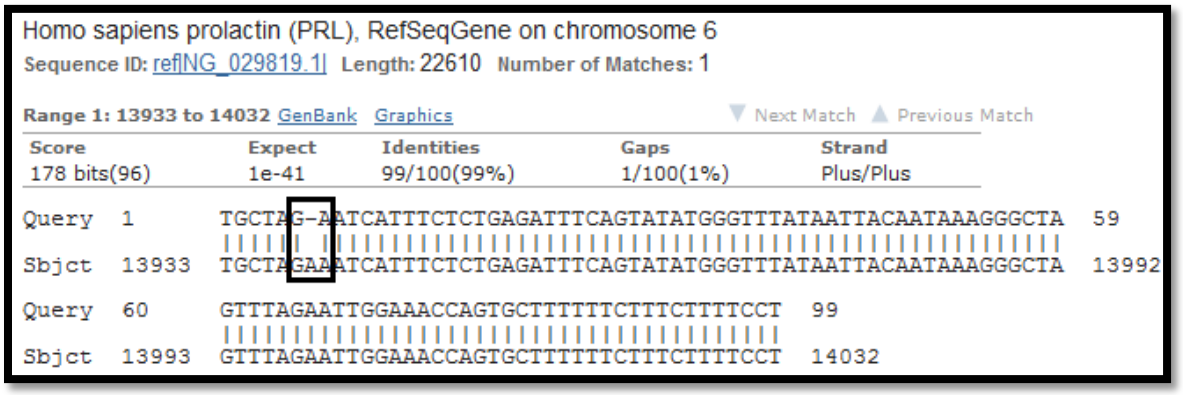
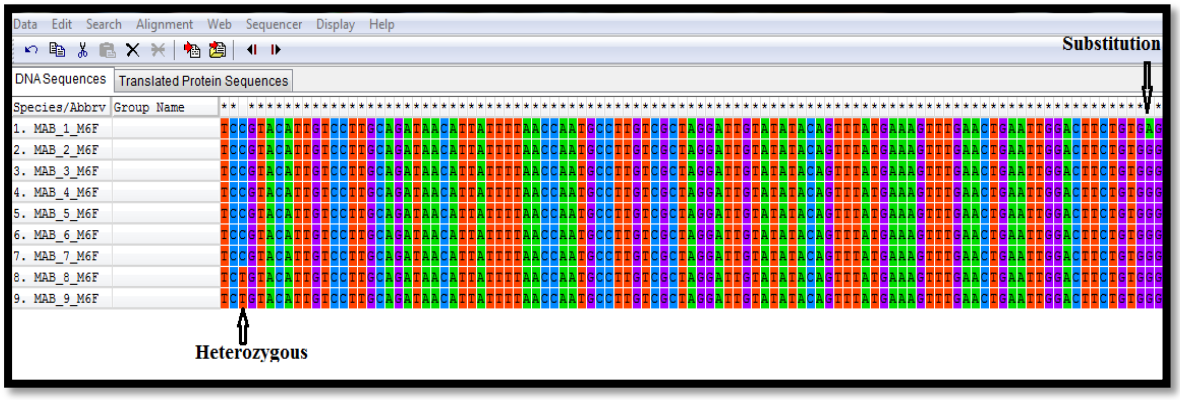


Figure (3-21) a and b :Sequencing of exon 3 of PRL gene A: of hyperprolactinemic patient

The two SNPs in this region of the gene are AGG/ATG in position 125 of hyperprolactemic patient which convert Arg to Met, and GAA which converts to G-A in position 36 that converts a.a Glu to deletion.

The other exon examined is exon 4 by using primer 6(PRL6), and it is shown that there is heterozygous SNPs (substitution) in (sample 8,9) that convert CCG to CTG (Pro/Leu), and also there are a substitution SNPs in all samples hyperprolactemic and in breast cancer in position 389, TGA to

TGG that converts a.a Stop/Trp. The SNPs are clear in figure (3-22), and the peaks are shown in figure(3-23) NCBI is shown in figure (3-24). The nucleotide sequence profile of exon 4 was in appendix (4).



Figure(3-22):PRL gene (Exon 4) point mutations as illustrated by Mega 6. Arrows indicate the region of point mutation.

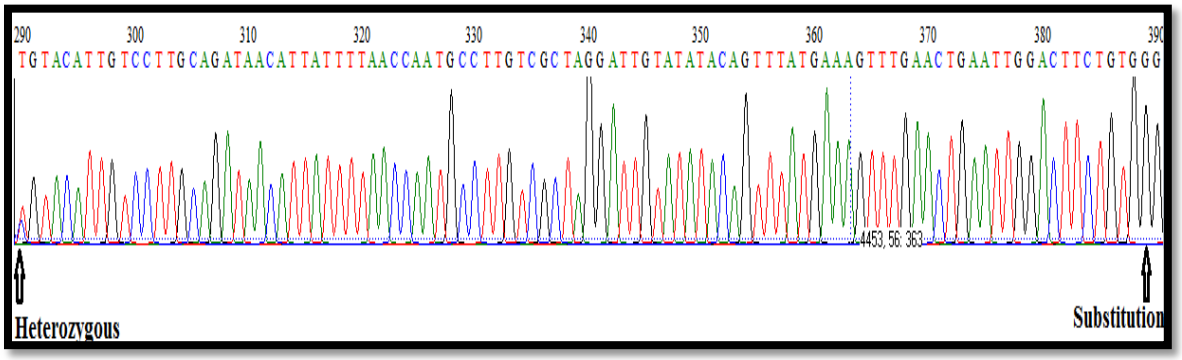


Figure (3-23). Heterozygous and substitution SNPs patient .

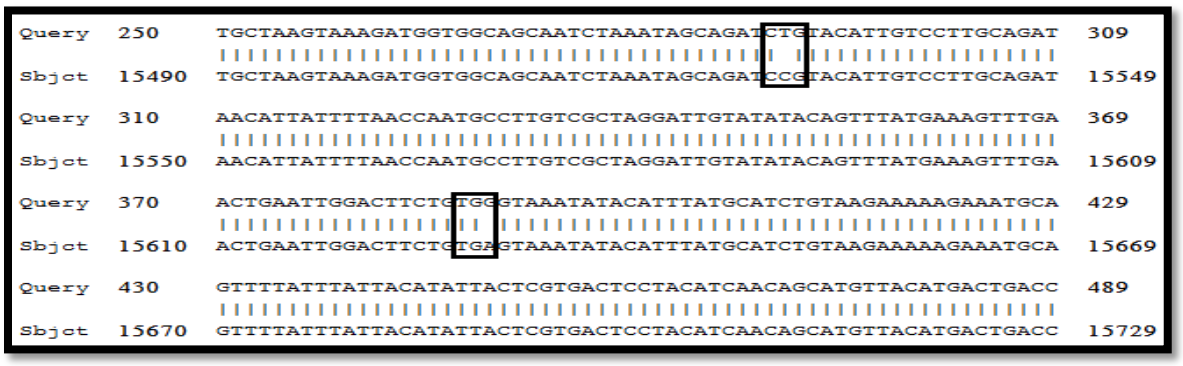


Figure (3-24): Sequencing of exon 4 of PRL gene for hyperprolactinemicr patient illustrating the substitution and heterozygous mutations.

The last exon examined in the PRL gene for both hyperprolactemic and breast cancer patients is exon 5 by using primer 7 that amplifies the region from 17687 to 18161, and the product size is 475. But it was noted that no mutations or SNPs were detected in this exon in all hyperprolactemic patients (Appendix 5).

The first intron region of PRL region was detected and examined for the presence of any mutations or SNPs and the effect of this alteration in the function of the gene. This region was amplified using three primers, the first one amplifies the PRL at the gene region from 6169 to 6489 with product size 312bp. The second primer also amplifies the same region from 6556 to 7173, with the product size 618bp, the third primer amplified the region from 8737 to 9152, with the product size 416bp. The first and third primers were selected for gene sequencing. And it was shown that there were too many SNPs in the beginning of the gene region or in the end, so it was not considered.The nucleotide sequence profile of intron 1 using primer1 was in appendix(1). But there were four SNPs in some samples (3-25),

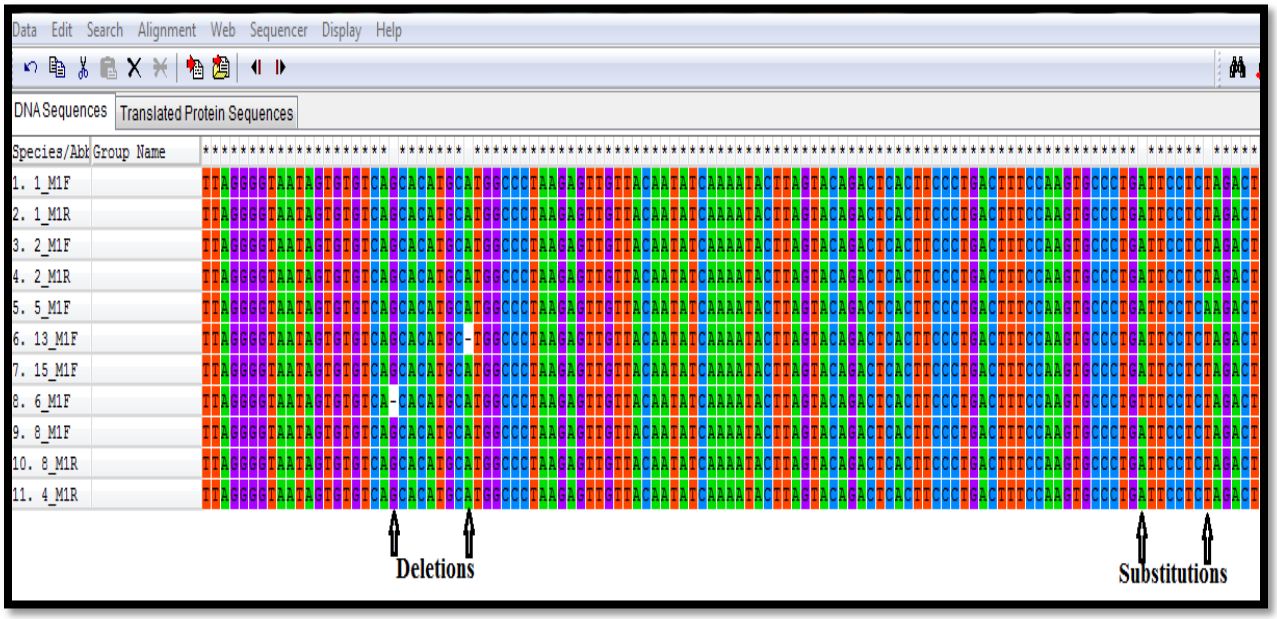
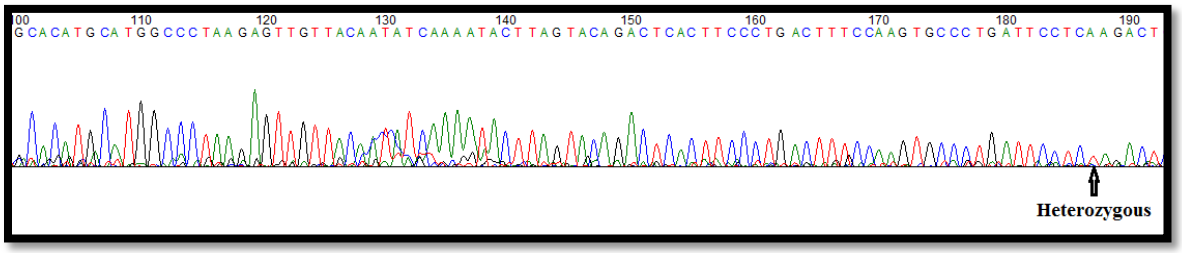


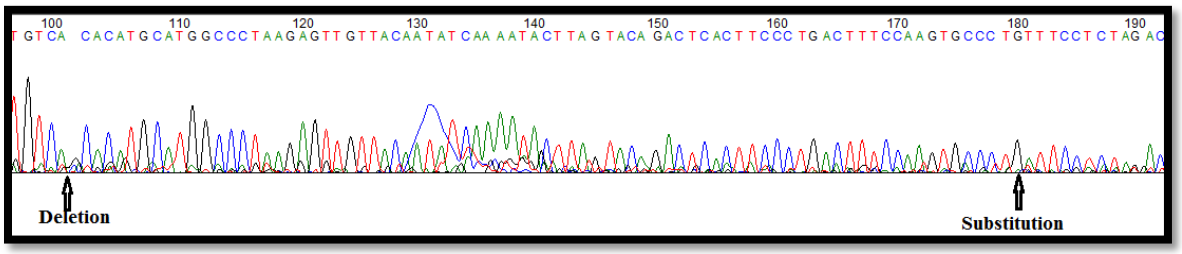
Figure (3-25): SNPs in intron one of hyperprolactemic and breast cancer patients by using primer 1, product size 312bp.

The peaks of SNPs in these samples are obvious in figure (3-26) a, b and c. The details about these mutations which appear in intron 1 in hyperprolactemic patients when compared to NCBI are shown in figures (3-27) a, b and c.

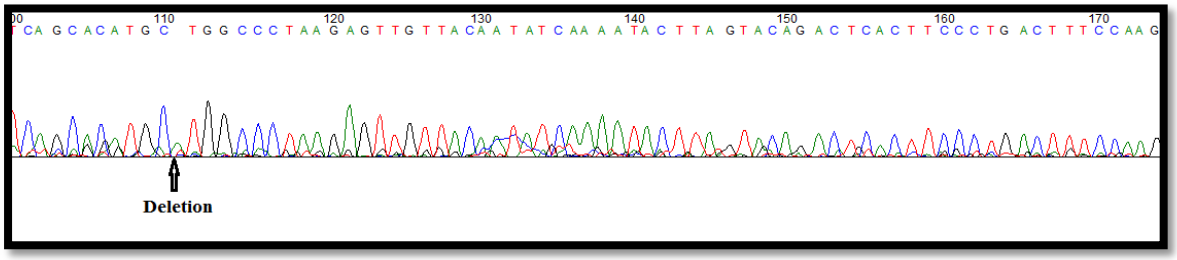
(a)



(b)



(c)



(3-26): Peaks of (a): hyperprolactemic patient (Sample 5) shows the heterozygous SNP, (b): hyperprolactemic patient (sample 6) shows the deletion and substitution mutations. (c): (sample 13) shows the deletion mutation.

(a)

Homo sapiens prolactin (PRL), RefSeqGene on chromosome 6

Sequence ID: ref|NG_029819.1| Length: 22610 Number of Matches: 1

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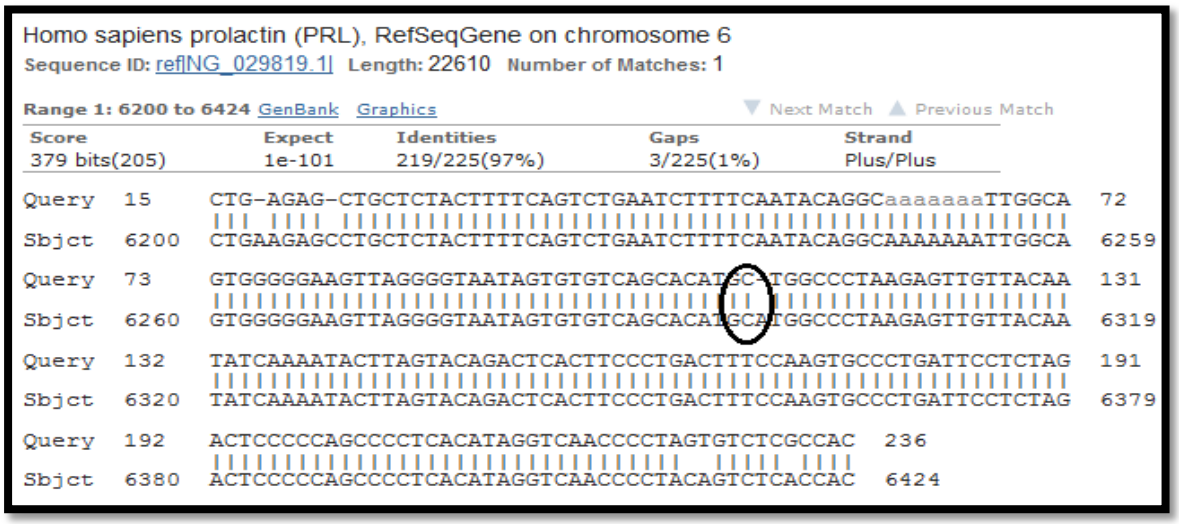
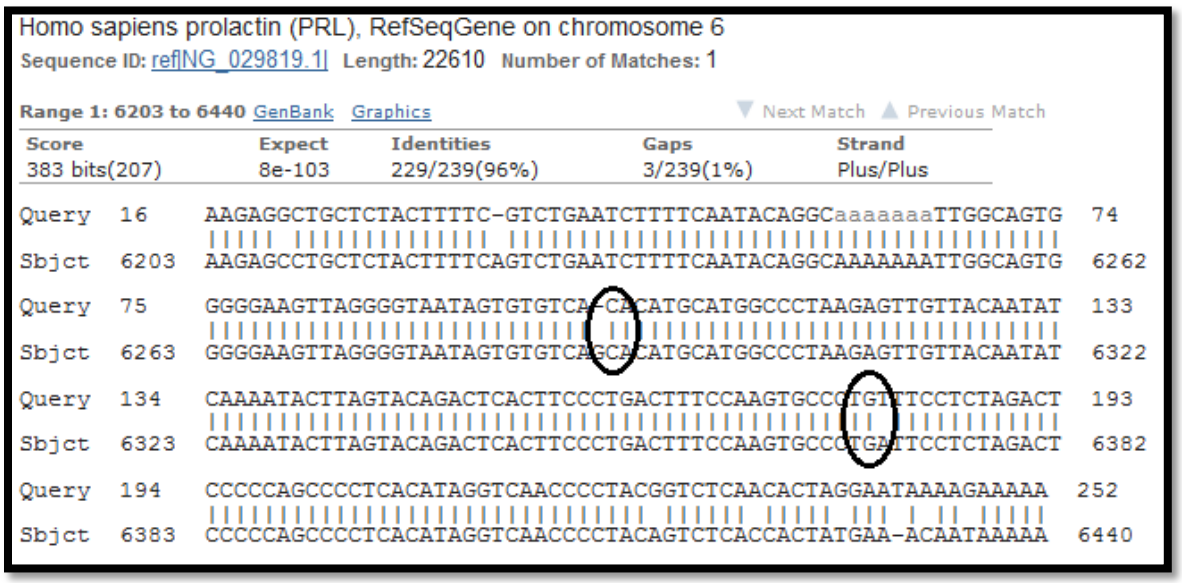
(b)

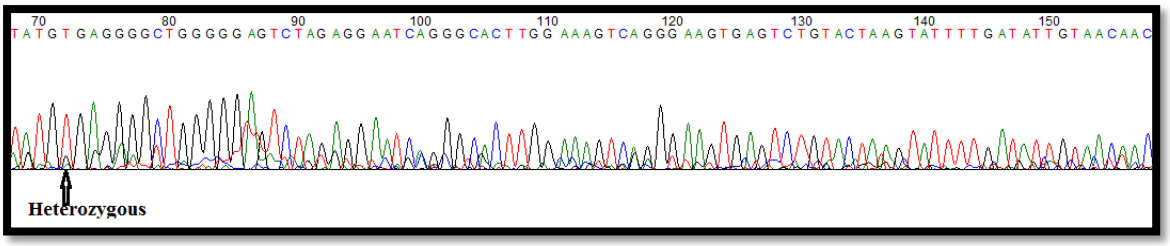


(c)



(3-27) a, b and c: NCBI of intron1 of prolacten gene by using primer 1 (a): hyperprolactemic patient(sample 5), b: (sample 13), c: hyperprolactemic patient (sample 6).

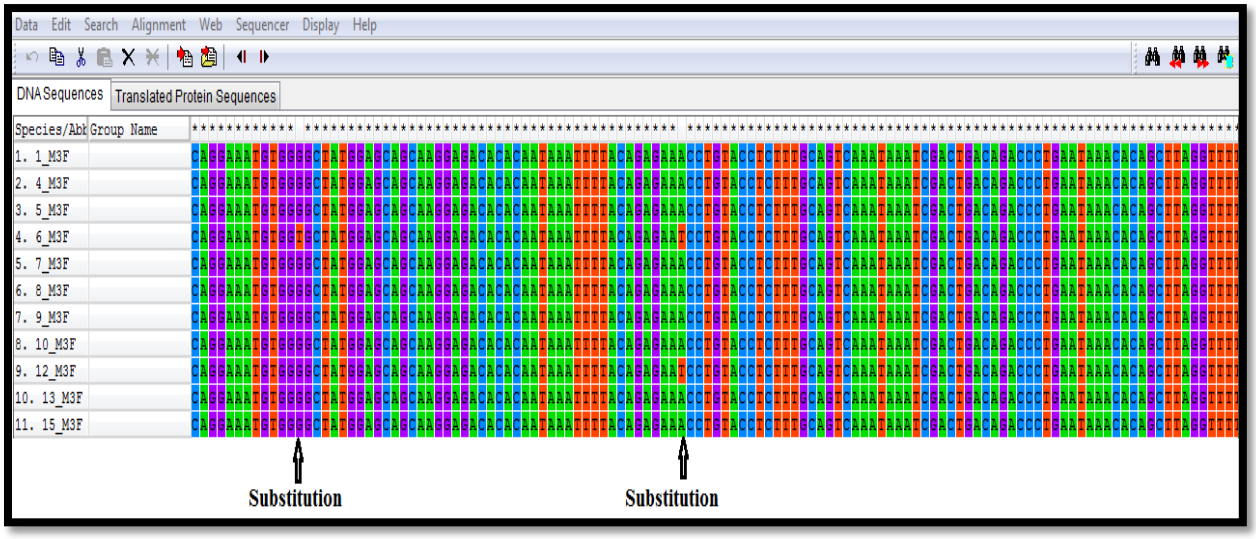
The four SNPs of hyperprolactemic patients are as follows: two in sample 6 where one is substitution TGA/TGT that converts stop codon to Cys at position 147, the other is deletion mutation in which GCA/ -CA in position 69. In the same region of the gene of hyperprolactemic patients in sample 5, there is a substitution mutation in position 156 that converts TAG/AAG that convert Stop codon to Lys. The deletion mutation is in the same region of the gene, but in breast cancer patient it is in position 77 of sample 13 that converts GCA to GC-,also in other sample but in same region which is intron 1 of the gene , there is heterozygous SNP as is clear in the peaks Fig (3-28).



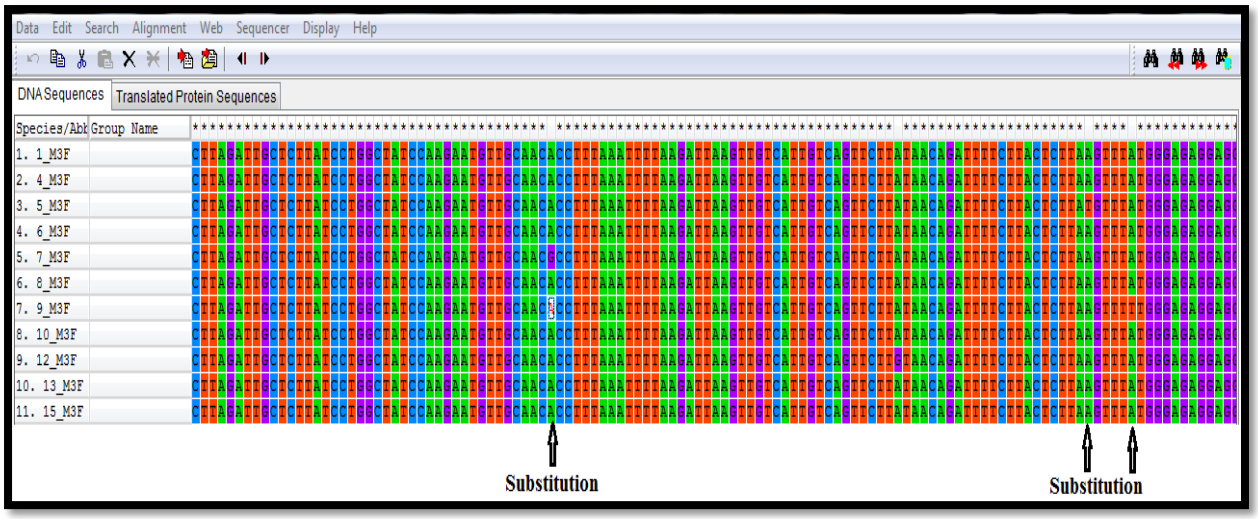
(3-28): Peaks of intron 1 (sample 4) show the heterozygous SNP.

Intron one was amplified also by using primer 3 product size 416bp (appendix 2)and it was found that there are many substitution SNPs. In figure (3-29) a, the SNPs that are common between hyperprolatemic patient are clear in which AAA convert to AAT is in sample 6 and in sample 12 (breast cancer patient), in position 104 in which Lys/Asn. The other substitution mutation is in sample 6 in which GCC is converted to TGC. In the same region, but in other samples of hyperprolactemic patients, sample 5 TAA/TAT in position 272 conver stop codon to Tyr. But in 210 position of sample 7, the ACC/GCC converts Thr/Ala. The last one is sample 9 in 277 in which TAT/TTT that converts Tyr to Phe as its clear in figure (3-29) b

(a)



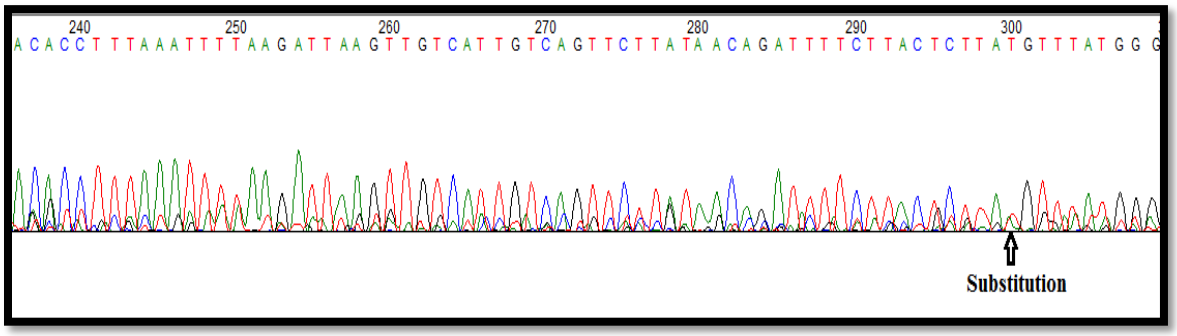
(b)



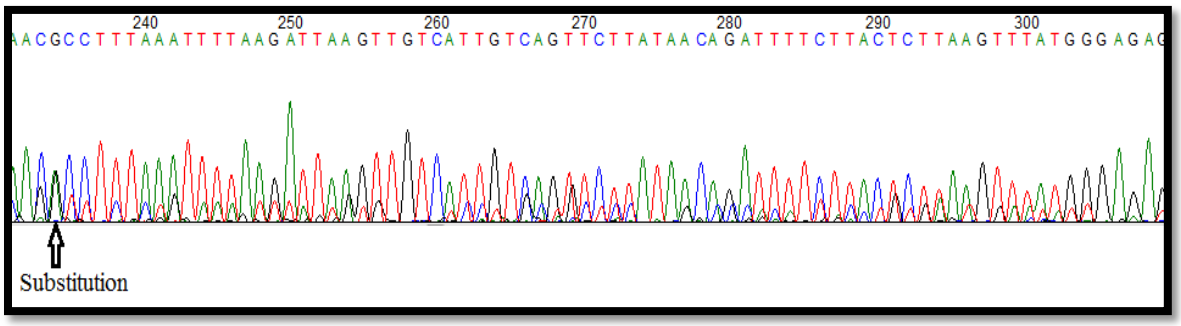
(3-29) a and b: Substitution mutations of intron 1 by using primer 3 hyperprolactemic patient

The peaks for mutation samples that have mutation are clear in figure (3-30), a, b, c, d and e. The NCBI results of the mutations that are detected in intron one using primer (3) are obvious in figure(3-31), a, b, c, d, e and f.

(a)


240
250
260
270
280
290
300
Substitution

(b)


240
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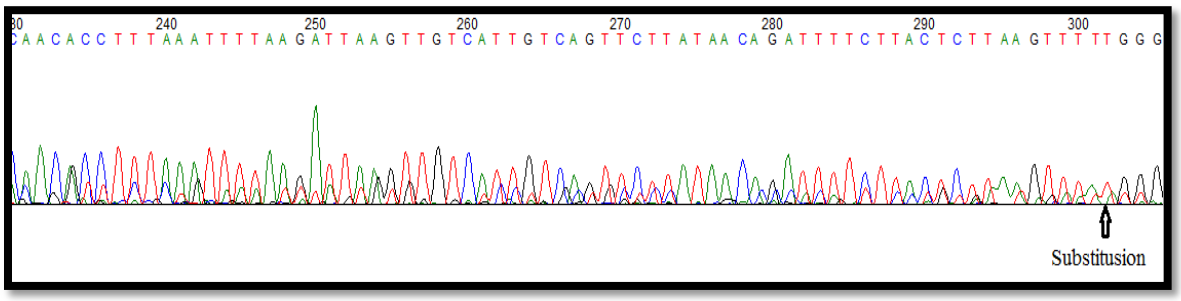
300
Substitution

(c)


240
250
260
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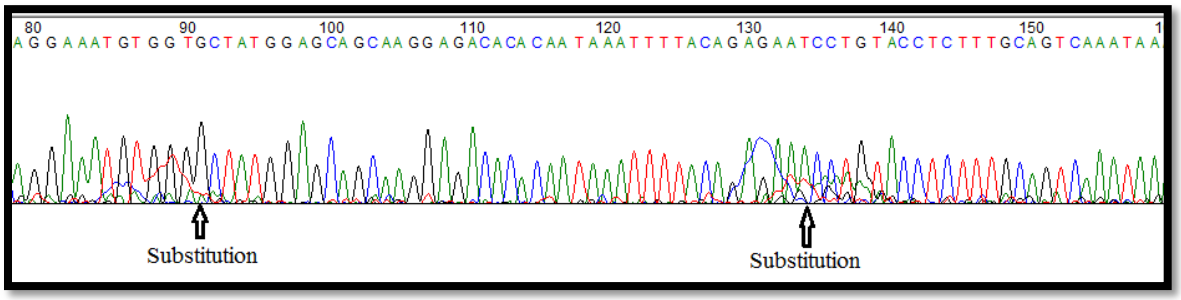
280
290
300
Substitusion

(d)



(e)

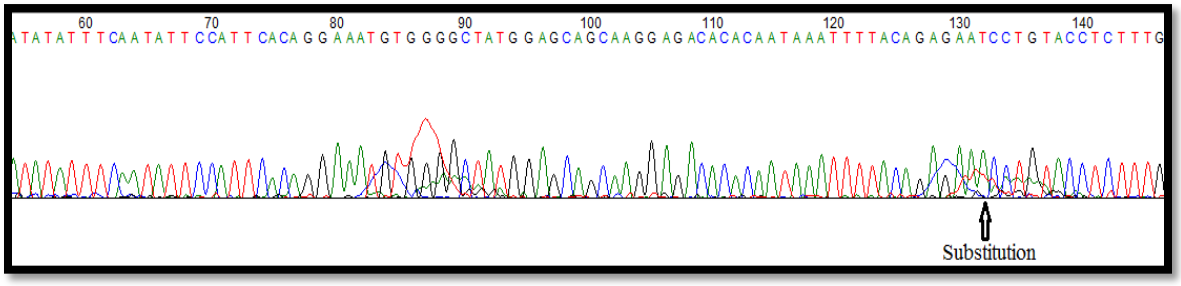
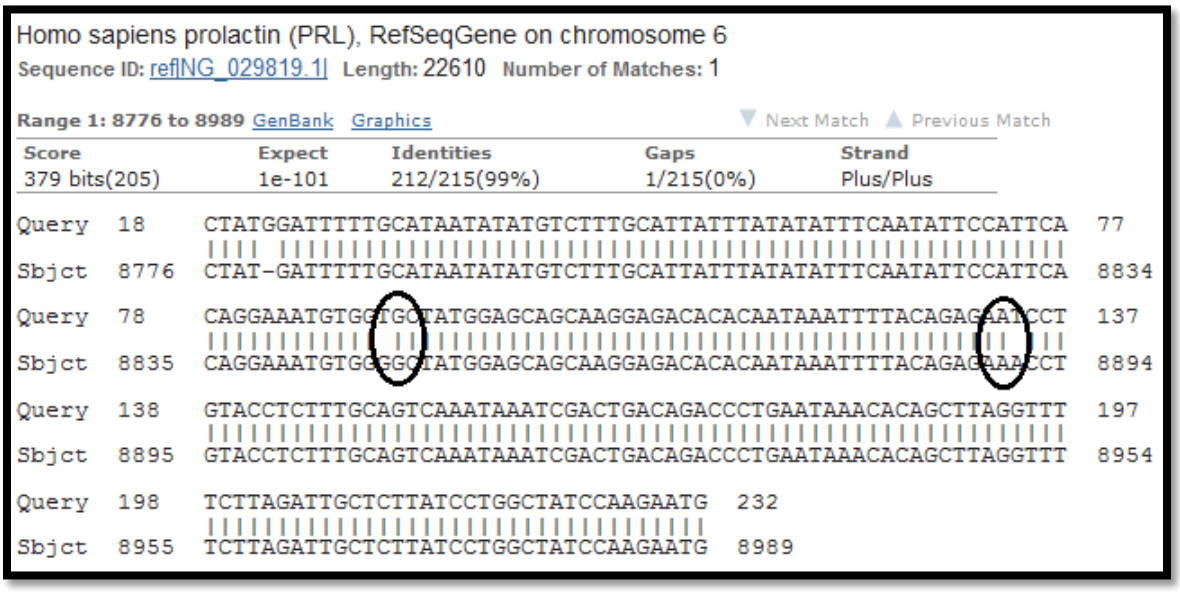


Figure (3-30): a, b, c, d and e: Peaks of intron 1 by using primer 3 for Hyperprolactemic patient a, (Sample 5), b: Sample 7, c: Sample 9, d: Sample 6, e: Sample 12.

(a)



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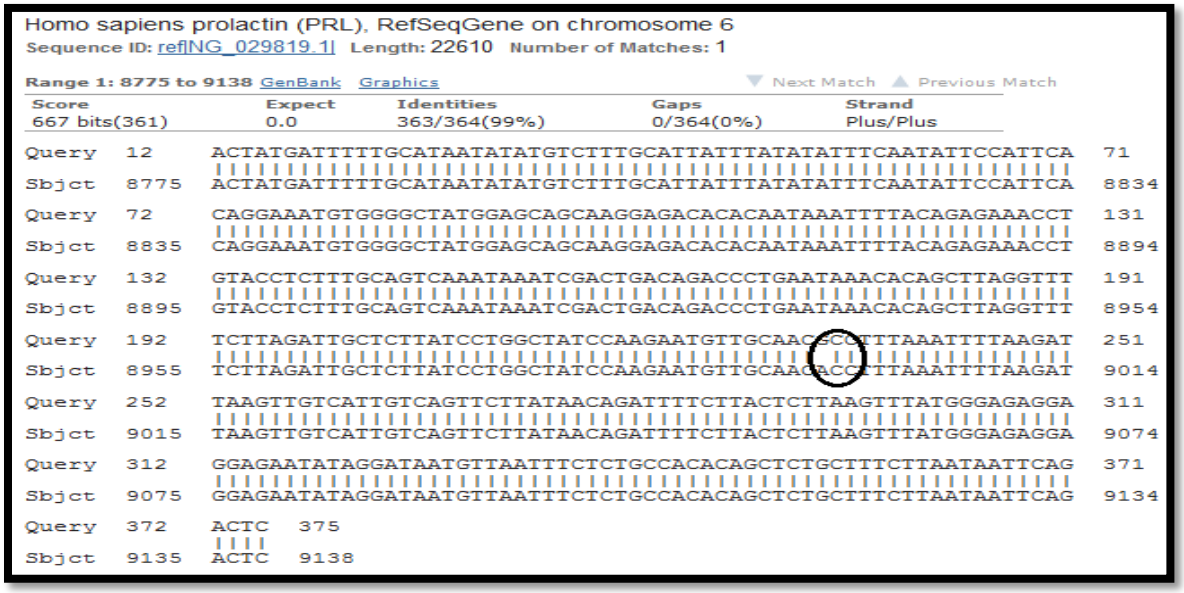
(b)



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Query  192   TCTTAGATTGCTCTTATCCTGGCTATCCAAGAATGTTGCAAGACCTTTAAATTTTAAGAT  251
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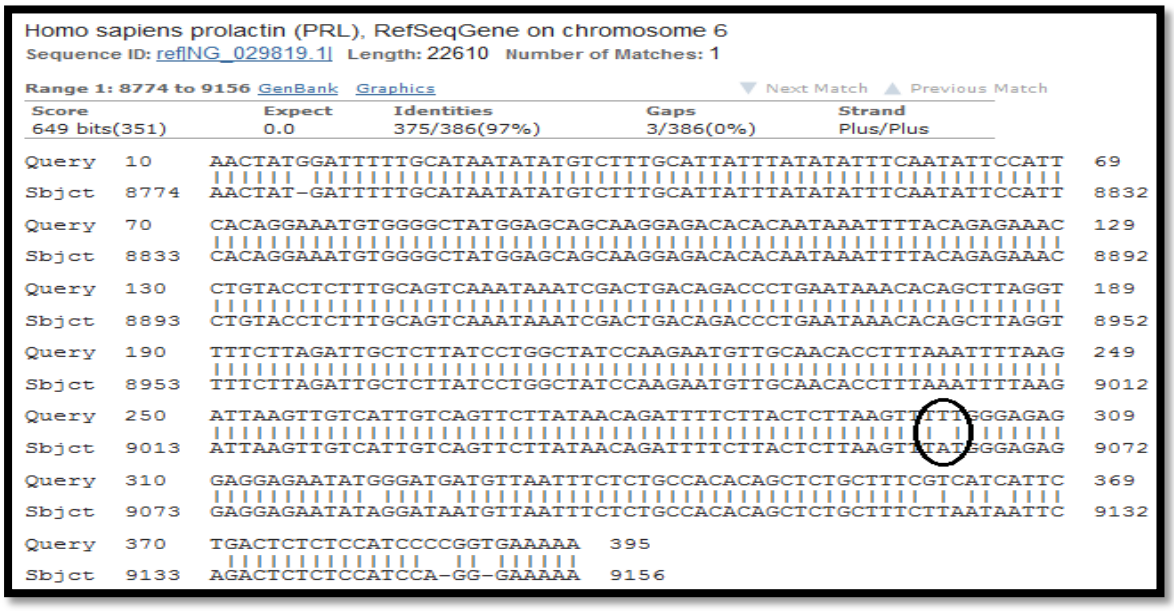
(c)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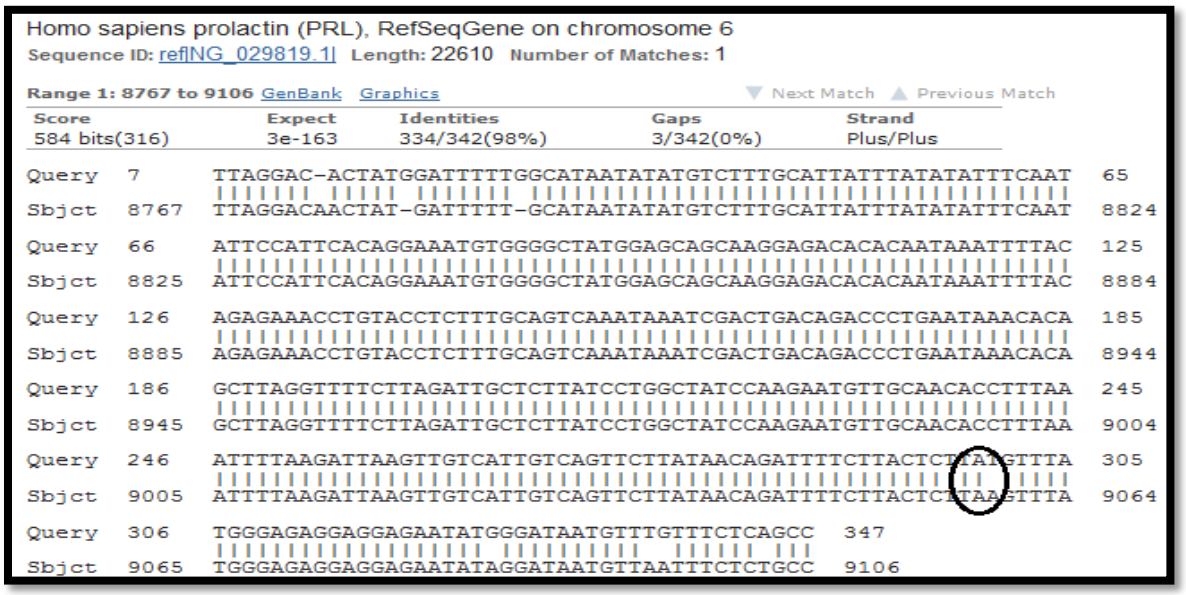
(d)


Homo sapiens prolactin (PRL), RefSeqGene on chromosome 6
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Next Match Previous Match
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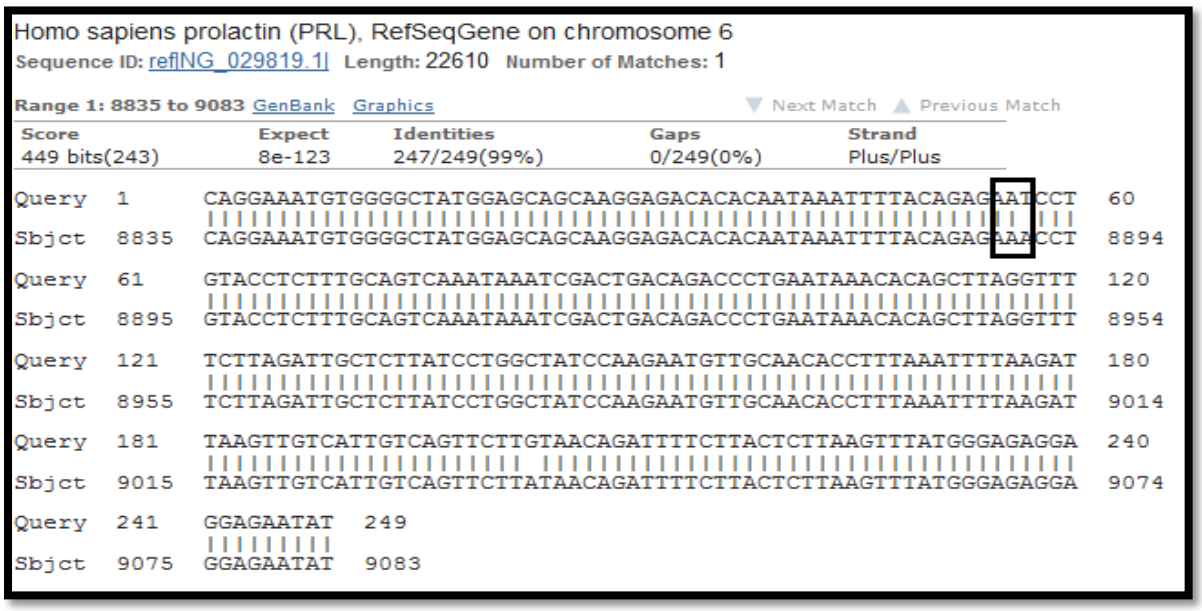
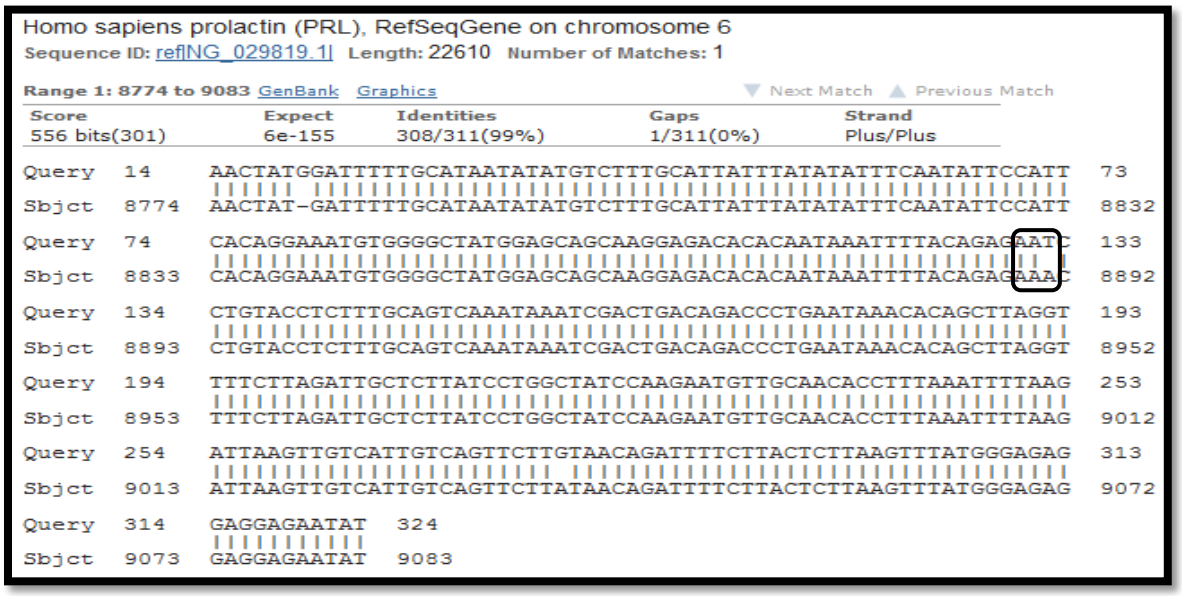


(f)



(3-31) a, b, c, d, e and f: NCBI appear mutation of intron of PRL gene for hyperprolactemic patient. a:(sample 6), that appear the substitution mutations b: (sample 7). c: (Sample 9), as it appears the substitution mutation. d: (sample 5). e sample 12 , f: sample 6 forward.

Table (3-9) shows differnt mutations of one or more than located gene region. However point mutation , substitution , deletion affected the PRL gene in Iraqi patients.

Table (3-9): Mutation types of human PRL gene in hyperprolactemic patients

Site on gene	Wild type	Mutant type	Change in a.a	Site on nucleic acid	Type of mutation
Exon 2	TTC	GTC	Phe/Val	210	Substitution
	GTT	GGG	Val/Gly	223,224	Substitution
	ACA	ACT	Thr/Thr	230	Substitution
	ACC	GCC	Thr/Ala	231	Substitution
	GTT	GTG	Val/Val	284	Substitution
	ATT	GTT	Ile/Val	363	Substitution
	TAA	TGA	Stop/Stop	400	Substitution
	AAC	GAC	Asp/Asp	438	Substitution
	CAA	CAG	Gin/Gin	446	Substitution
Exon 3	GAA	G-A	Glu-Deletion	36	Deletion
	AGG	ATG	Arg/Met	125	Substitution
Exon 4	TGA	TGG	Stop/Trp	379	Substitution
	TGA	TGG	Stop/Trp	379	
	TGA	TGG	Stop/Trp	379	
	TGA	TGG	Stop/Trp	379	
	TGA	TGG	Stop/Trp	379	
	TGA	TGG	Stop/Trp	379	
	TGA	TGG	Stop/Trp	379	
	TGA	TGG	Stop/Trp	379	
	TGA	TGG	Stop/Trp	379	
	TGA	TGG	Stop/Trp	379	
	CCG	CTG	Pro/Leu	٢٩٠	Substitution
	TAG	AAG	Stop/Lys	156	

Intron 1.p1	TGA	TGT	Stop/Cys	147	
	GCA	-CA	Ala-Deletion	69	Deletion
	GCA	GC-	Ala-Deletion	77	Deletion
Intron 1. P.3	TAA	TAT	Stop/Tyr	272	Substitution
	ACC	GCC	Thr/Ala	210	Substitution
	AAA	AAT	Lys/Asn	104	Substitution
	GGC	TGC	Gly/Cys	59	Substitution
	AAA	AAT	Lys/Asn	104	Heterozygous
	TAT	TTT	Tyr/Phe	277	Substitution

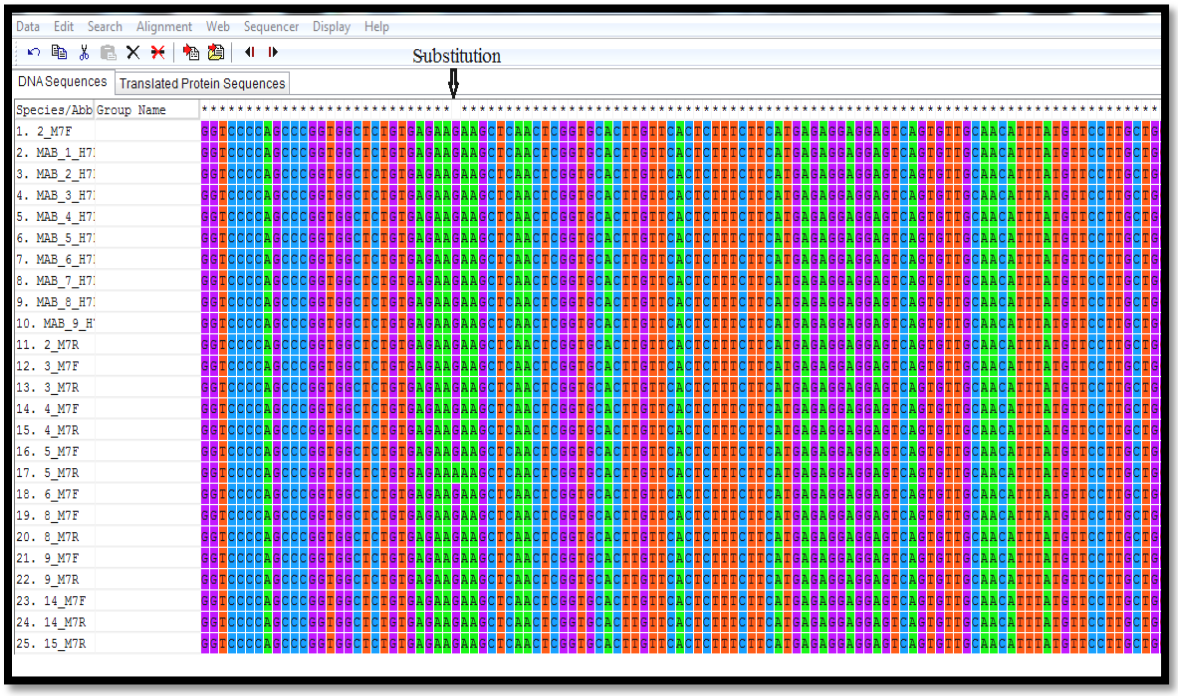
Genetic factors are important for the disease in many samples of patients, but its not clear which region of the gene contributes to disease. The exons and intron region of the PRL gene show mutations and some region of the gene shows a common mutation in some bases in some patient samples. The mutations detected in exons region of PRL gene of hyperprolactemic patients give evidence that these mutations play a part in this hyperprolactinemia.

As there is increasing evidence that PRL gene is involved in the hyperprolactinemia, it was found that SNPs in the exons and introns of the PRL gene were detected and these polymorphisms alter the expression attributable to altered transcription factor gene binding.

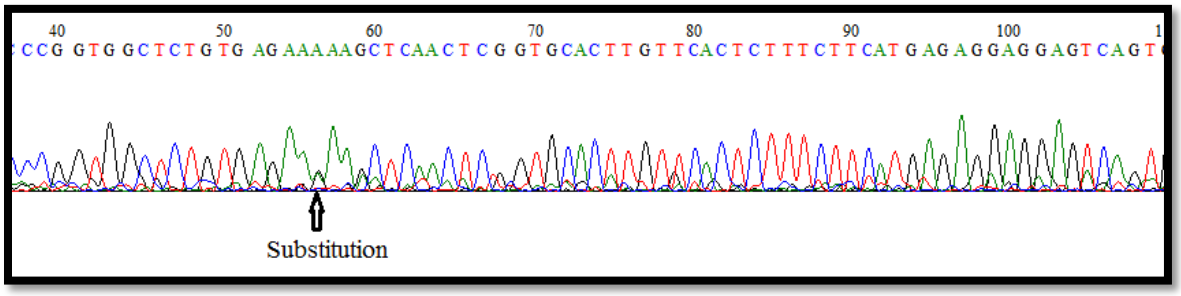
In present study, there are SNPs in hyperprolactinemic patients may be because patients with hyperprolactinemia might be exposed to higher levels of PRL for several years either because of delay of diagnosis or treatment failure (Delgrange *et al*., 1997). It is agrees with Lee et al., 2007, who discovered a low frequency synonymous SNP in exon 3 (A/G) also in exon 5, but not in exon 2, and also a missense SNP in exon 4 when he made a comprehensive analysis of common genetic variation in PRL and PRLR genes in relation to plasma prolactin levels. Hormone PRL physiologically influences the mammary gland in several ways during development, growth and stimulation of milk protein gene transcription (Wennbo *et al*., 1997). Besides PRL is important in pathological conditions such as mammary tumor growth in which PRLR has been formed in 40-70% of human breast tumors and PRL stimulate growth of several human breast cancer cell lines *invitro* indicating a possible auto/paracrine function of PRL in many cases of tumor growth. In the present study, it was found that in addition to mutations that are detected in exon region of the PRL gene of infertile hyperprolactemic patients, there are also many mutations in intron region of the gene as its clear in the above figures concerving the mutations in intron 1. This agree with the result obtained by the Iraqi study which reported the mutations in intron 1 and 2 of prolactin gene of infertile hyperprolactinemic women. Nore *et al.* (2013) reported the mutations in hyperprolactinemic patients in intron region of the PRL gene, and thus they considered them as genetic markers for high prolactin level causing infertility in Iraqi women.

3.3.2. Detection of mutation in PRLR gene.

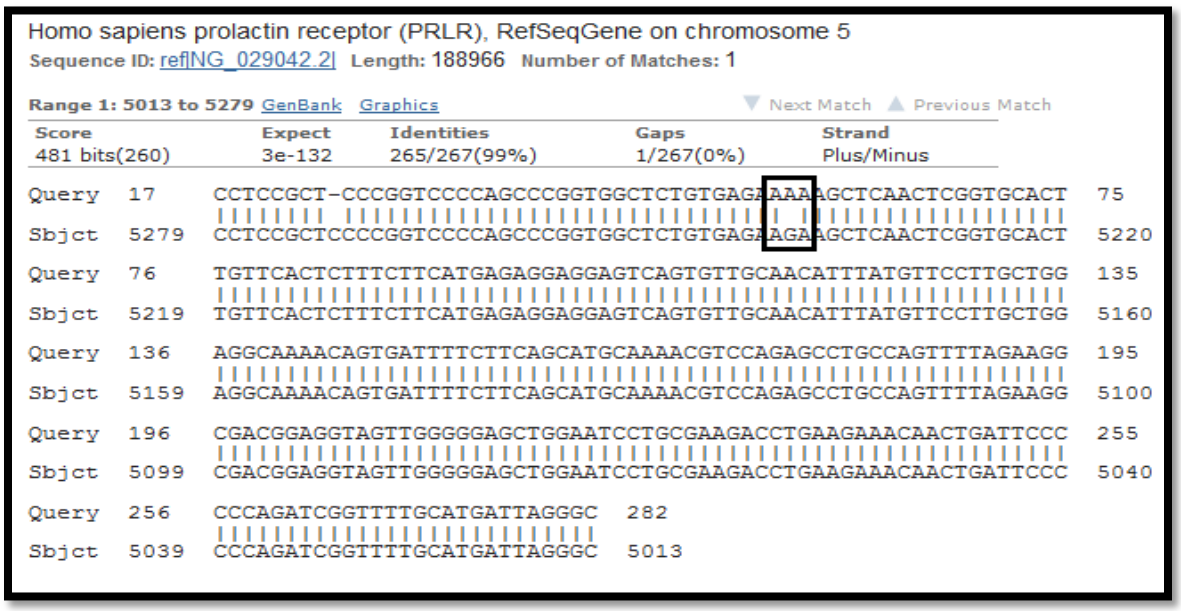
The prolactin gene receptor was also sequenced by sending PCR product of primer 2(PRLR 2) of product size 306bp which amplify the region from 5013 to 5318 and primer 4 (PRLR4) of product size 436 bp, which amplify the region from 5020 to 5455. Homology search was conducted between the sequancses of standard gene blast program which is available at the National Center for Biotechnology Information(NCBI), and Mega6 program. Results of sequencing of the coding regions of the amplified product (exon 1) for these samples were collected for seeking of any mutations within these sequences related to hyperprolactinemic patients. Its clear that the product of primer 2 has one mutation in sample 5 reverse in hyperprolactemic patient site (61), which is obvious in the figure (3-32). Table (3-10) shows the type of mutation and predicted effect in which it changes the codon from AGA to AAA that changes the amino acid from Arg to Lys. The peaks are obvious in figure (3-33), while the blast of it in NCBI is clear in figure (3-34). Nucleotide sequence profile of exon 1 of PRLR gene was in appendix (6).



(3-32): Comparison between hyperprolactemic patients and control by using primer 2, product size 306bp.



(3-33): Peaks of sample 5, that indicate the substitution mutation.



(3-34): NCBI of hyperprlactemic patient (sample 5), of PRLR gene.

Table (3-10): Mutations of human PRLR gene of hyperprolactemic patients

No. of patient sample	Site on gene	Wild type	Mutant type	Change in a.a	Site on nucleic acid	Type of mutation
5	Exon 1	AGA	AAA	Arg/Lys	61	Substitution

In this study there is an association between the PRLR gene mutation in sample 5 of hyperprolactemic patient, and hyperprolactinemia. This result agrees with that of Newey *et al*., (2013) who showed that there was a heterozygous mutation in the prolactin receptor gene resulting in an amino acid change from histidine to arginine at codon 188, and this substitution disrupted the high affininty ligand-binding interface of the prolactin receptor resulting in a loss of down stream signaling by Janus Kinase2 (JAK2) and signal tranducer and activator of transcription 5(STAT5). This in turn,

resulted in a loss of function which led to hyperprlactinemia. This result coincided with the study of Vaclavicek *et al*.(2006), who first screened the promoter regions and one SNP in each exon 5 and exon 6 which were supposed to change a.a , but the SNPs did not exist or they were too rare they were not concidered. In this study, was choosen exon 1 as the PRLR gene region which is not equally well characterized in the public database NCBI as PRL gene. Besides, the exon 1 that exists in several variant forms and in non coding region (Hu, 2002).

As a human, PRLR is a single transmembrane spanning protein belonging to a superfamily of growth hormone/prolactin/cytokine receptors and alternative splicing results in different isoforms of PRLR (Binart, 2010) The isoforms of PRLR have different effects on intracellular signaling that may be stimulatory or inhibitory.

These results suggest that the SNPs are located within non coding regions of PRLR genes based on location, the most likely mechanisms responsible for the increased risk is through effect on transcriptional regulation (Le *et al*., 2013). Furthermore, a PRLR variant causes a change from Isoleucine to Leucine at codon 146 in the extracellular domain of the mature protein and results in an increased basal JAK2-STAT5 signaling invitro as Bogorad *et al*., 2008, reported that in women with breast fibroadenomas, as the exracellular doman is common location for mutations affecting cytokine receptors (David *et al*., 2011).

Conclusions and Recommendations

Conclusions and Recommendations

It was concluded from this study that:

1- The statistical analysis of fertility hormone measurements showed that the levels of the LH, FSH and PRL were significantly changed in hyperprolactemic patient when compared to the control, when PRL showed a high elevation while LH and FSH decreased.

2- The LH and FSH were found to decrease more in secondary infertility women and when patient were divided into three age groups, the more decrease in FSH and LH was with the age group (31-40) years old.

3- The mutations in the PRL gene were successfully detected in exons 2 to 5 and in intron 1 of the same gene, and it seems that the mutated codon for an Iraqi patient is not same of that reported by world wide researchers.

4- Mutation was detected in PRLR gene of hyperprolactinemic patient.

5- For some patients there are more than one mutation in more than one exon of PRL gene detected, including deletion a substitutions.

Recommendations:

1-Further studies with a large further size and long follow-up are necessary to validate the variation in prolactin levels in these two groups and to clarify the etiology of the higher prevalence of hyperprolactinemia in secondary infertility than in primary infertility for better management of infertility cases

2- Detection of other types of mutations by amplifying another regions of the gene by using other primers.

3- Amplifiy other introns regions of the PRL gene to detect if there are any variations.

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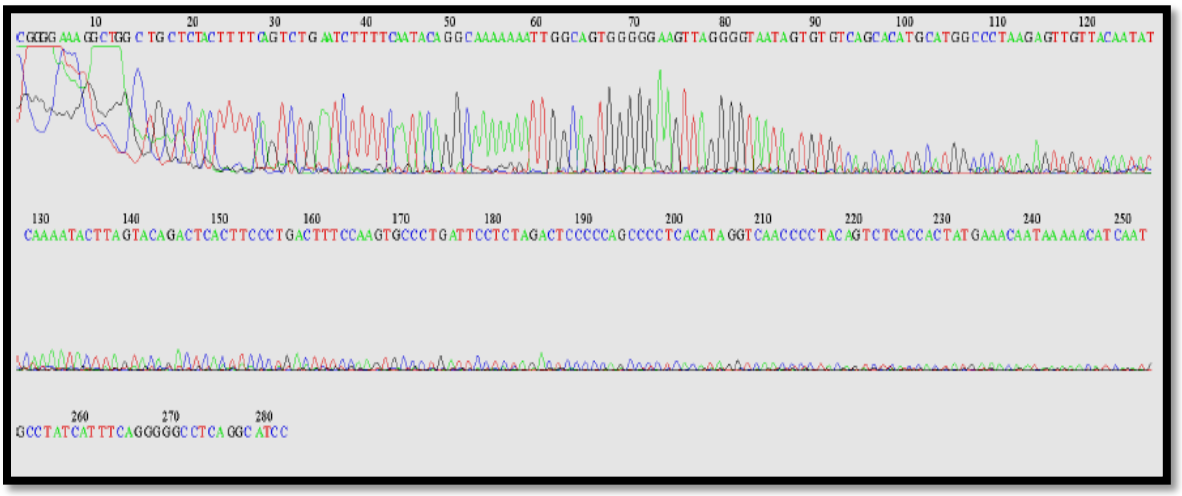
Appendices

Appendex(1).

A. Nucleotide sequance profile of intron 1 of PRL gene using primer 1



B.Nucleotide sequence of intron 1

CGGGGAAAGGCTGGCTGCTCTACTTTTCAGTCTGAATCTTTTCAATACAGGCA
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GGCCCTAAGAGTTGTTACAATATCAAAATACTTAGTACAGACTCACTTCCCTG
ACTTTCCAAGTGCCCTGATTCCTCTAGACTCCCCCAGCCCCTCACATAGGTCA
ACCCCTACAGTCTCACCACTATGAAACAATAAAAACATCAATGCCTATCATTT
CAGGGGGCCTCAGGCATCC

C.Sequence alignment of intron 1 using BLAST from NCBI.

Homo sapiens prolactin (PRL), RefSeqGene on chromosome 6
Sequence ID: refNG_029819.1| Length: 22610 Number of Matches: 1

Range 1: 6209 to 6474 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
492 bits(266)	1e-135	266/266(100%)	0/266(0%)	Plus/Plus

```
Query  15    CTGCTCTACTTTTCAGTCTGAATCTTTTCAATACAGGCaaaaaaaTTGGCAGTGGGGGAA  74
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Sbjct  6209  CTGCTCTACTTTTCAGTCTGAATCTTTTCAATACAGGCAAAAAAAATTGGCAGTGGGGGAA  6268

Query  75    GTTAGGGGTAATAGTGTGTCAGCACATGCATGGCCCTAAGAGTTGTTACAATATCAAAAT  134
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  6269  GTTAGGGGTAATAGTGTGTCAGCACATGCATGGCCCTAAGAGTTGTTACAATATCAAAAT  6328

Query  135   ACTTAGTACAGACTCACTTCCCTGACTTTCCAAGTGCCCTGATTCCTCTAGACTCCCCCA  194
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  6329  ACTTAGTACAGACTCACTTCCCTGACTTTCCAAGTGCCCTGATTCCTCTAGACTCCCCCA  6388

Query  195   GCCCCTCACATAGGTCAACCCCTACAGTCTCACCACTATGAAACAATAAAAACATCAATG  254
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Sbjct  6389  GCCCCTCACATAGGTCAACCCCTACAGTCTCACCACTATGAAACAATAAAAACATCAATG  6448

Query  255   CCTATCATTTCAGGGGGCCTCAGGCA  280
             |||||||||||||||||||||||||
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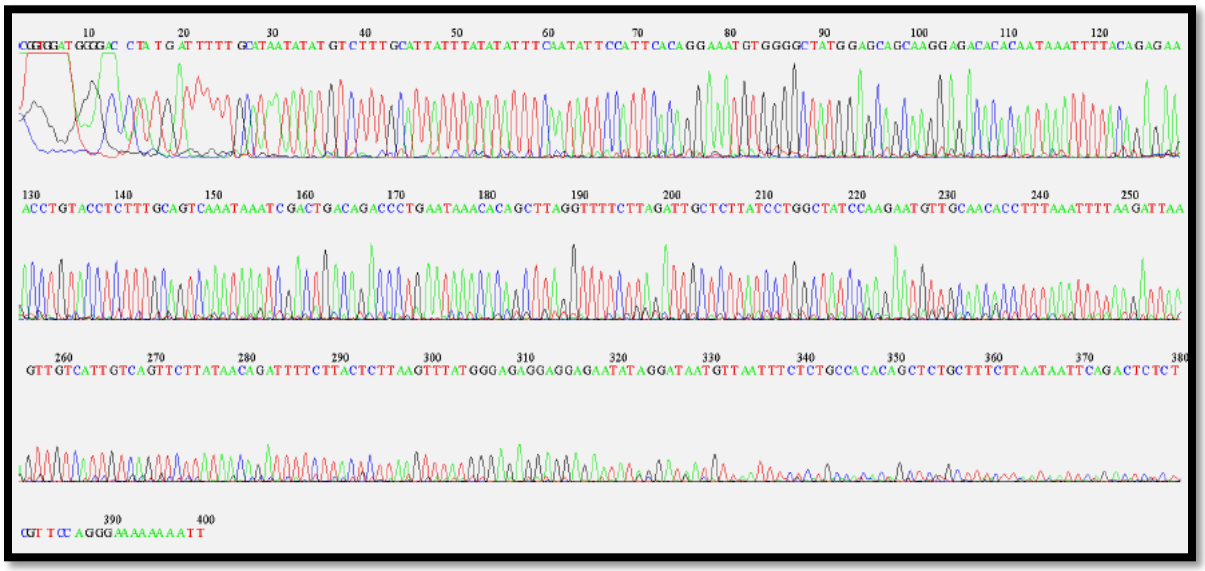
```

Appendix (2).

A: Nucleotide sequence profile of intron 1 of PRL gene using primer 3



A. Nucleotide sequence of intron 1

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GACACACAATAAATTTTACAGAGAAACCTGTACCTCTTTGCAGTCAAATAAA
TCGACTGACAGACCCTGAATAAACACAGCTTAGGTTTTCTTAGATTGCTCTT
ATCCTGGCTATCCAAGAATGTTGCAACACCTTTAAATTTTAAGATTAAGTTG
TCATTGTCAGTTCTTATAACAGATTTTCTTACTCTTAAGTTTATGGGAGAGGA
GGAGAATATAGGATAATGTTAATTTCTCTGCCACACAGCTCTGCTTTCTTAAT

C. Sequence alignment of intron 1 using BLAST from NCBI.

Homo sapiens prolactin (PRL), RefSeqGene on chromosome 6

Sequence ID: ref|NG_029819.1| Length: 22610 Number of Matches: 1

Range 1: 8776 to 9157 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
695 bits(376)	0.0	380/382(99%)	0/382(0%)	Plus/Plus

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Query  15    CTATGATTTTTGCATAATATATGTCTTTGCATTATTTATATATTTCAATATTCCATTCAC  74
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Query  75    AGGAAATGTGGGGCTATGGAGCAGCAAGGAGACACACAATAAATTTTACAGAGAAACCTG  134
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Query  195   CTTAGATTGCTCTTATCCTGGCTATCCAAGAATGTTGCAACACCTTTAAATTTTAAGATT  254
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Sbjct  8956  CTTAGATTGCTCTTATCCTGGCTATCCAAGAATGTTGCAACACCTTTAAATTTTAAGATT  9015

Query  255   AAGTTGTCATTGTCAGTTCTTATAACAGATTTTCTTACTCTTAAGTTTATGGGAGAGGAG  314
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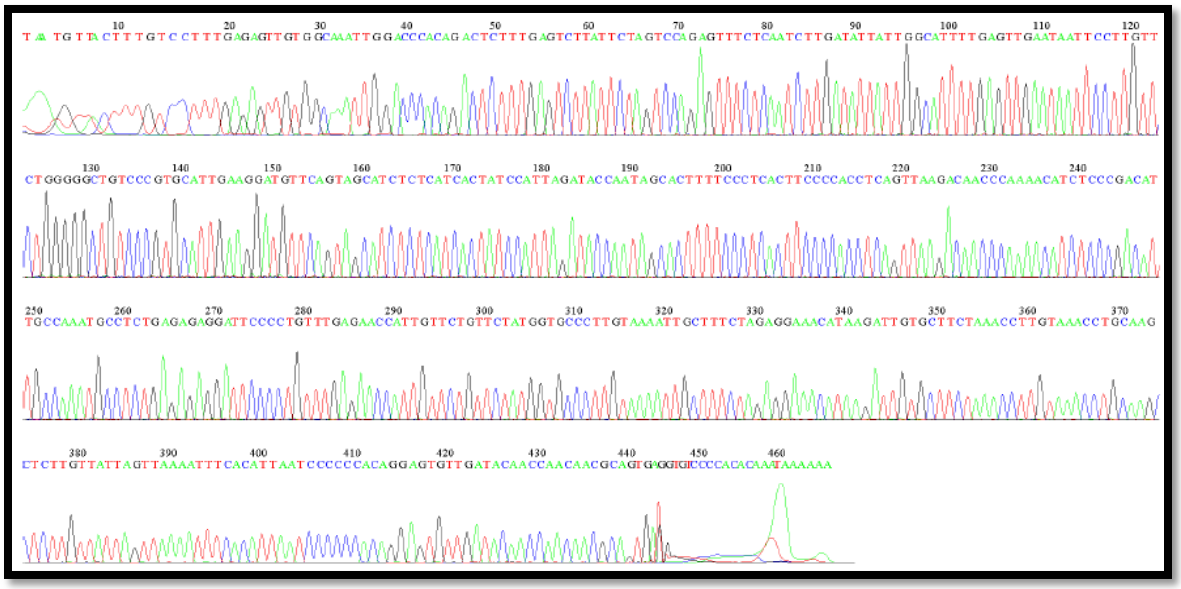
Appendix(3).

A: Nucleotide sequence profile of Exon 2 of PRL gene using primer 4.



B. Nucleotide sequence of Exon 2

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TGAATAATTCCTTGTTCTGGGGGCTGTCCCGTGCATTGAAGGATGTTCAGTAG
CATCTCTCATCACTATCCATTAGATACCAATAGCACTTTTCCCTCACTTCCCCA
CCTCAGTTAAGACAACCCAAAACATCTCCCGACATTGCCAAATGCCTCTGAG
AGAGGATTCCCCTGTTTGAGAACCATTGTTCTGTTCTATGGTGCCCTTGTAAA
ATTGCTTTCTAGAGGAAACATAAGATTGTGCTTCTAAACCTTGTAAACCTGCA

C. Sequence alignment of Exon 2 using BLAST from NCBI.

Homo sapiens prolactin (PRL), RefSeqGene on chromosome 6
Sequence ID: refING_029819.1| Length: 22610 Number of Matches: 1

Range 1: 10908 to 11350 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
806 bits(436)	0.0	441/443(99%)	1/443(0%)	Plus/Minus

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Query  180    CAATAGCACTTTTCCCTCACTTCCCCACCTCAGTTAAGACAACCCAAAACATCTCCCGAC  239
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Sbjct  10990  CTGCAAGCTCTTGTTATTAGTTAAAATTTCACATTAATCCCCCCACAGGAGTGTTGATAC  10931

Query  420    AACCAACAACGCAGTGAGGTGTC  442
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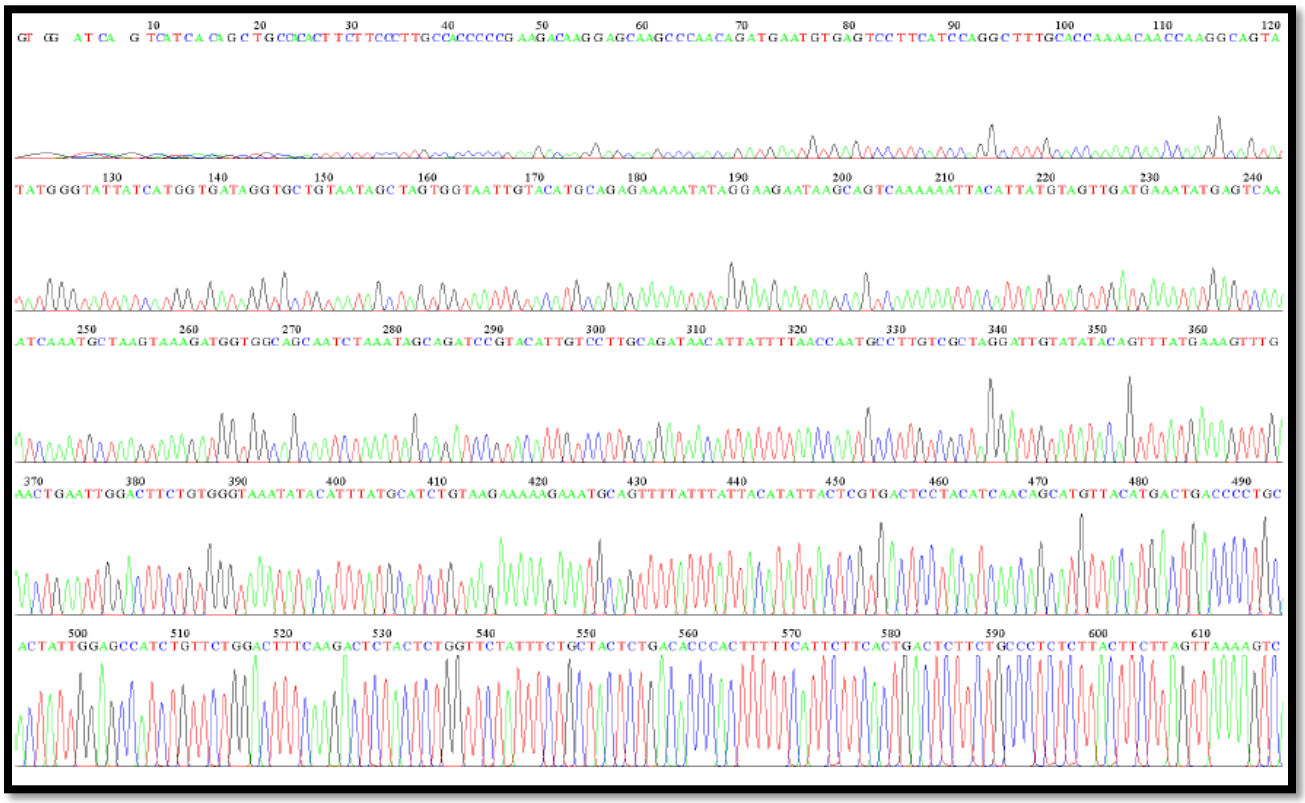
Appendix(4).

A. Nucleotide sequence profile of Exon 4 of PRL gene using primer 6



B.Nucleotide sequence of Exon 4

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AAATTACATTATGTAGTTGATGAAATATGAGTCAAATCAAATGCTAAGTAAA
GATGGTGGCAGCAATCTAAATAGCAGATCCGTACATTGTCCTTGCAGATAAC
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CCCAAAAAAAGTATAAATTTTACGGGCCCCTTTTAACAGGGATAATACCCAT
ATCCGGCCTTGGTGGTTTTGGGTGCTAAGGCTGGGATGAAGGGACTCACATT
CATCTTGTTGGGGTTGGCTCCCTGGTCTTCGGGGGGTGGCAAGGGGAAGAAG
TGTGGCATCCTGTTTGATTGCCTTGGTTAATGAACCCCCGGGCCATGGGTCTC
CCGGGTTAACTA

C. Sequence alignment of Exon 4 using BLAST from NCBI

Homo sapiens prolactin (PRL), RefSeqGene on chromosome 6

Sequence ID: ref|NG_029819.1| Length: 22610 Number of Matches: 1

Range 1: 15276 to 15924 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1173 bits(635)	0.0	645/649(99%)	3/649(0%)	Plus/Plus

```
Query  1      TTGCCACCCCCGAAGACAAGGAGCAAGCCCAACAGATGAATGTGAGTCCTTCATCCAGGC  60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  15276  TTGCCACCCCCGAAGACAAGGAGCAAGCCCAACAGATGAATGTGAGTCCTTCATCCAGGC  15335

Query  61     TTTGCACCAAAACAACCAAGGCAGTATATGGGTATTATCATGGTGATAGGTGCTGTAATA  120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  15336  TTTGCACCAAAACAACCAAGGCAGTATATGGGTATTATCATGGTGATAGGTGCTGTAATA  15395

Query  121    GCTAGTGGTAATTGTACATGCAGAGAAAAATATAGGAAGAATAAGCAGTCAAAAAATTAC  180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  15396  GCTAGTGGTAATTGTACATGCAGAGAAAAATATAGGAAGAATAAGCAGTCAAAAAATTAC  15455

Query  181    ATTATGTAGTTGATGAAATATGAGTCAAATCAAATGCTAAGTAAAGATGGTGGCAGCAAT  240
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  15456  ATTATGTAGTTGATGAAATATGAGTCAAATCAAATGCTAAGTAAAGATGGTGGCAGCAAT  15515

Query  241    CTAAATAGCAGATCCGTACATTGTCCTTGCAGATAACATTATTTTAACCAATGCCTTGTC  300
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  15516  CTAAATAGCAGATCCGTACATTGTCCTTGCAGATAACATTATTTTAACCAATGCCTTGTC  15575

Query  301    GCTAGGATTGTATATACAGTTTATGAAAGTTTGAACTGAATTGGACTTCTGTGGGTAAAT  360
              |||||||||||||||||||||||||||||||||||||||||||||||||||| |||||||
Sbjct  15576  GCTAGGATTGTATATACAGTTTATGAAAGTTTGAACTGAATTGGACTTCTGTGAGTAAAT  15635

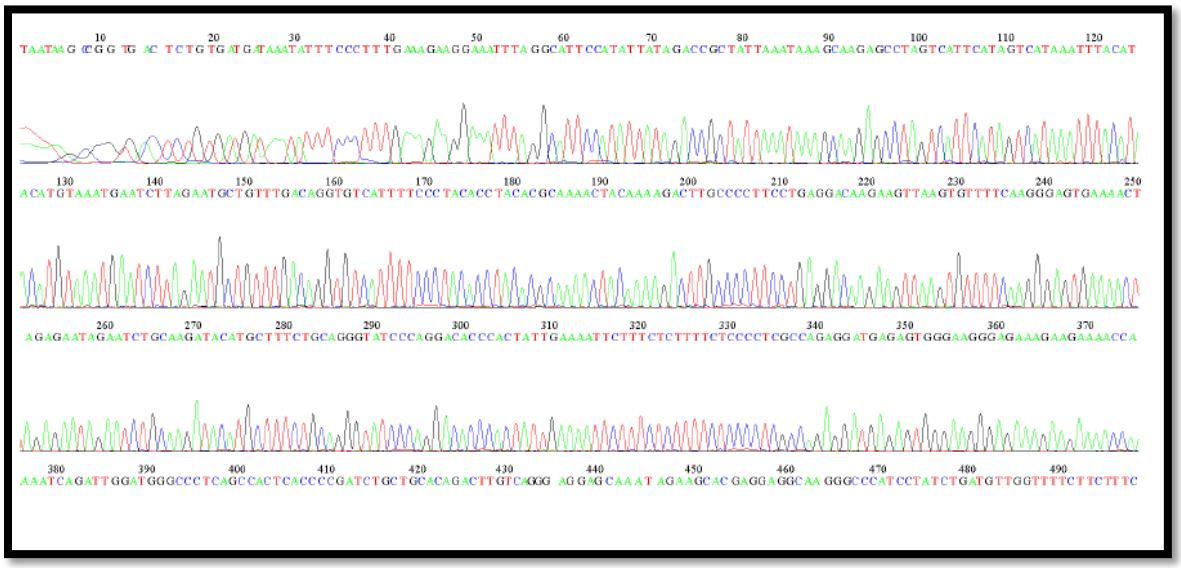
Query  361    ATACATTTATGCATCTGTAAGAAAAAGAAATGCAGTTTTATTTATTACATATTACTCGTG  420
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  15636  ATACATTTATGCATCTGTAAGAAAAAGAAATGCAGTTTTATTTATTACATATTACTCGTG  15695

Query  421    ACTCCTACATCAACAGCATGTTACATGACTGACCCCTGCACTATTGGAGCCATCTGTTCT  480
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  15696  ACTCCTACATCAACAGCATGTTACATGACTGACCCCTGCACTATTGGAGCCATCTGTTCT  15755

Query  481    GGACTTTCAAGACTCTACTCTGGTTCTATTTCTGCTACTCTGACACCCACTTTTTCATTC  540
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  15756  GGACTTTCAAGACTCTACTCTGGTTCTATTTCTGCTACTCTGACACCCACTTTTTCATTC  15815
```

Appendix(5).

A. Nucleotide sequance profile of Exon 5 of PRL gene using primer 7



B. Nucleotide sequence of Exon 5

TAATAAGCCGGTGACTCTGTGATGATAAATATTTCCCTTTGAAAGAAGGAAA
TTTAGGCATTCCATATTATAGACCGCTATTAAATAAAGCAAGAGCCTAGTCAT
TCATAGTCATAAATTTACATACATGTAAATGAATCTTAGAATGCTGTTTGACA
GGTGTCATTTTCCCTACACCTACACGCAAAACTACAAAAGACTTGCCCCTTCC
TGAGGACAAGAAGTTAAGTGTTTTCAAGGGAGTGAAAACTAGAGAATAGAA
TCTGCAAGATACATGCTTTCTGCAGGGTATCCCAGGACACCCACTATTGAAA
ATTCTTTCTCTTTTCTCCCCTCGCCAGAGGATGAGAGTGGGAAGGGAGAAAG
AAGAAAACCAAAATCAGATTGGATGGGCCCTCAGCCACTCACCCCGATCTGC
TGCACAGACTTGTCAGGGAGGAGCAAATAGAAGCACGAGGAGGCAAGGGCC
CATCCTATCTGATGTTGGTTTTCTTCTTTCTCCCTTCCCTCTAACCACTGGCGA
GGGGAGAAAAGAGAAAGAAATTTTCAATAGTGGGGTGTCCTGGGATACCCTG
CAAAAGCAGTTATTTGCCATCTATTCTTGTTGTCCTCTTTGAAACACTTAATTC
TGTCTCAGGAAGGGGCAGTCTTTGTAGTTTTGCGTGTAGGCTATAGGGAAAA
TGACACCGGTCAACACACTCTAGATCATTTACTGTATTAATTATACTATTAAG
ACAGCTCTGCTTATTAAAGCGGTTTTAAATGGAATGCCAATTCCCTCTTTCAA
GGGAATATTATCACCACAGTGTCTTCTATTTAATGAGAAAACAAAAGAACCA
CGGTGCTGACTAAATTCT

C. Sequence alignment of Exon 5 of PRL gene using BLAST from NCBI

Homo sapiens prolactin (PRL), RefSeqGene on chromosome 6

Sequence ID: ref|NG_029819.1| Length: 22610 Number of Matches: 1

Range 1: 17756 to 18165 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
752 bits(407)	0.0	409/410(99%)	0/410(0%)	Plus/Plus

```
Query  1      TTTGAAAGAAGGAAATTTAGGCATTCCATATTATAGACCGCTATTAAATAAAGCAAGAGC  60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  17756  TTTGAAAGAAGGAAATTTAGGCATTCCATATTATAGACCGCTATTAAATAAAGCAAGAGC  17815

Query  61     CTAGTCATTCATAGTCATAAATTTACATACATGTAAATGAATCTTAGAATGCTGTTTGAC  120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  17816  CTAGTCATTCATAGTCATAAATTTACATACATGTAAATGAATCTTAGAATGCTGTTTGAC  17875

Query  121    AGGTGTCATTTTCCCTACACCTACACGCAAAACTACAAAAGACTTGCCCCTTCCTGAGGA  180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  17876  AGGTGTCATTTTCCCTACACCTACACGCAAAACTACAAAAGACTTGCCCCTTCCTGAGGA  17935

Query  181    CAAGAAGTTAAGTGTTTTCAAGGGAGTGAAAACTAGAGAATAGAATCTGCAAGATACATG  240
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  17936  CAAGAAGTTAAGTGTTTTCAAGGGAGTGAAAACTAGAGAATAGAATCTGCAAGATACATG  17995

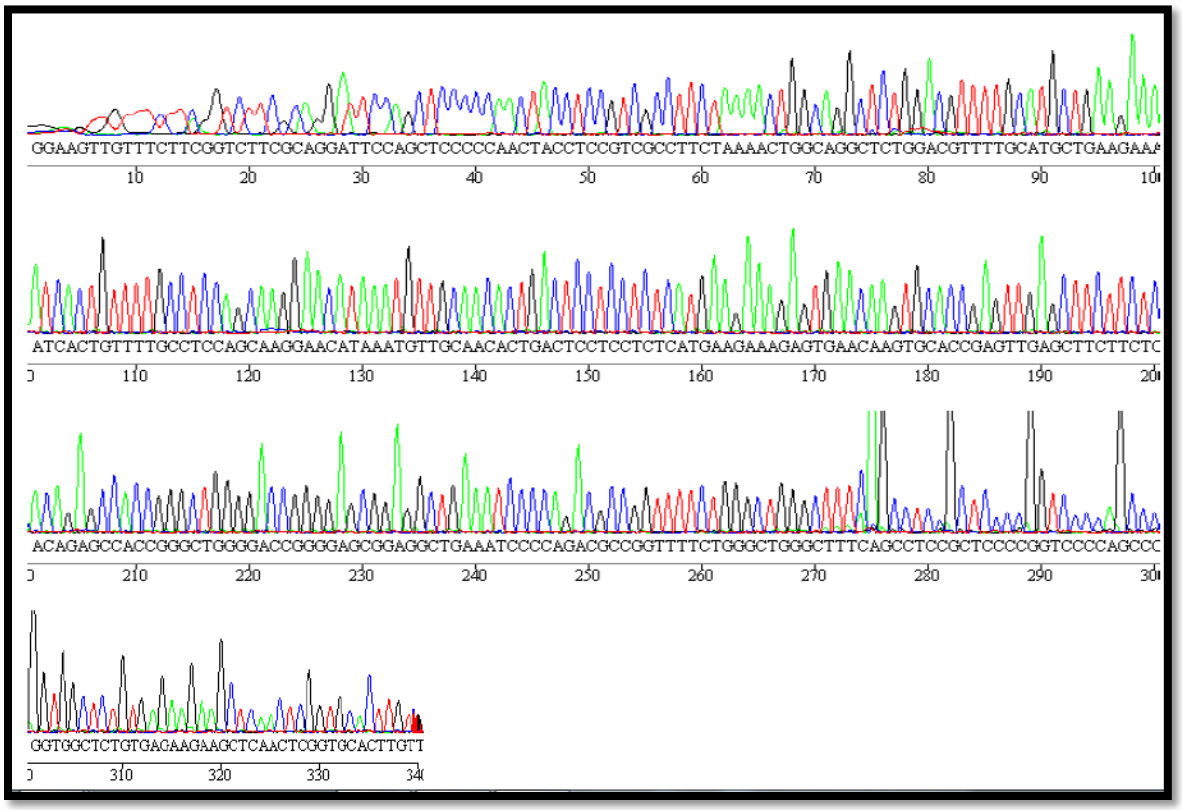
Query  241    CTTTCTGCAGGGTATCCCAGGACACCCACTATTGAAAATTCTTTCTCTTTTCTCCCCTCG  300
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  17996  CTTTCTGCAGGGTATCCCAGGACACCCACTATTGAAAATTCTTTCTCTTTTCTCCCCTCG  18055

Query  301    CCAGAGGATGAGAGTGGGAAGGGAGAAAGAAGAAAACCAAAATCAGATTGGATGGGCCCT  360
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  18056  CCAGAGGATGAGAGTGGGAAGGGAGAAAGAAGAAAACCAAAATCAGATTGGATGGGCCCT  18115

Query  361    CAGCCACTCACCCCGATCTGCTGCACAGACTTGTCAGGGAGGAGCAAATA  410
              |||||||||||||||||||||||||||||||||||||||||||||||||  |||
Sbjct  18116  CAGCCACTCACCCCGATCTGCTGCACAGACTTGTCAGGGAGGAGCAGATA  18165
```

Appendix(6).

A. Nucleotide sequance profile of Exon 1 of PRLR gene using primer 1



B.Nucleotide sequence of Exon 1 of PRLR gene

GTCTTCGCAGGATTCCAGCTCCCCCAACTACCTCCGTCGCCTTCTAAAACTGG
CAGGCTCTGGACGTTTTGCATGCTGAAGAAAATCACTGTTTTGCCTCCAGCAA
GGAACATAAATGTTGCAACACTGACTCCTCCTCTCATGAAGAAAGAGTGAAC
AAGTGCACCGAGTTGAGCTTCTTCTCACAGAGCCACCGGGCTGGGGACCGGG
GAGCGGAGGCTGAAATCCCCAGACGCCGGTTTTCTGGGCTGGGCTTTCCAAG
CCTCCGCTCCCCGGTCCCCGCGGGGGCCCCTTGAAAAAAAACTCCAACGGGG
GAACTTGTTCACCCCTCCTTCTCGAAAAAGAGGAGACCCGGTTGCTCCATTTA
TTTTTCCCTGTGGGAGGGAAAACACCGCTTTTTTTTTGAAAGGAAAAAATCCA
CACTGCTCGGTTTTTAAAAAAAAAGAAAGGAGTTGGGGGGAGGGAGAAAAA
ATCCTGCAAATACATAAAACAAACAACTGCCCCCCCCCAGATTTTTTTTGCAT
GAAAAAAAAAA

C. Sequence alignment of Exon 1 of PRLR gene using BLAST from NCBI

Homo sapiens prolactin receptor (PRLR), RefSeqGene on chromosome 5

Sequence ID: ref|NG_029042.2| Length: 188966 Number of Matches: 1

Range 1: 5059 to 5318 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
481 bits(260)	6e-132	260/260(100%)	0/260(0%)	Plus/Plus

```
Query  3     AGGTCTTCGCAGGATTCCAGCTCCCCCAACTACCTCCGTCGCCTTCTAAAACTGGCAGGC  62
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  5059  AGGTCTTCGCAGGATTCCAGCTCCCCCAACTACCTCCGTCGCCTTCTAAAACTGGCAGGC  5118

Query  63    TCTGGACGTTTTGCATGCTGAAGAAAATCACTGTTTTGCCTCCAGCAAGGAACATAAATG  122
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  5119  TCTGGACGTTTTGCATGCTGAAGAAAATCACTGTTTTGCCTCCAGCAAGGAACATAAATG  5178

Query  123   TTGCAACACTGACTCCTCCTCTCATGAAGAAAGAGTGAACAAGTGCACCGAGTTGAGCTT  182
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  5179  TTGCAACACTGACTCCTCCTCTCATGAAGAAAGAGTGAACAAGTGCACCGAGTTGAGCTT  5238

Query  183   CTTCTCACAGAGCCACCGGGCTGGGGACCGGGGAGCGGAGGCTGAAATCCCCAGACGCCG  242
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  5239  CTTCTCACAGAGCCACCGGGCTGGGGACCGGGGAGCGGAGGCTGAAATCCCCAGACGCCG  5298

Query  243   GTTTTCTGGGCTGGGCTTTC  262
             ||||||||||||||||||||
Sbjct  5299  GTTTTCTGGGCTGGGCTTTC  5318
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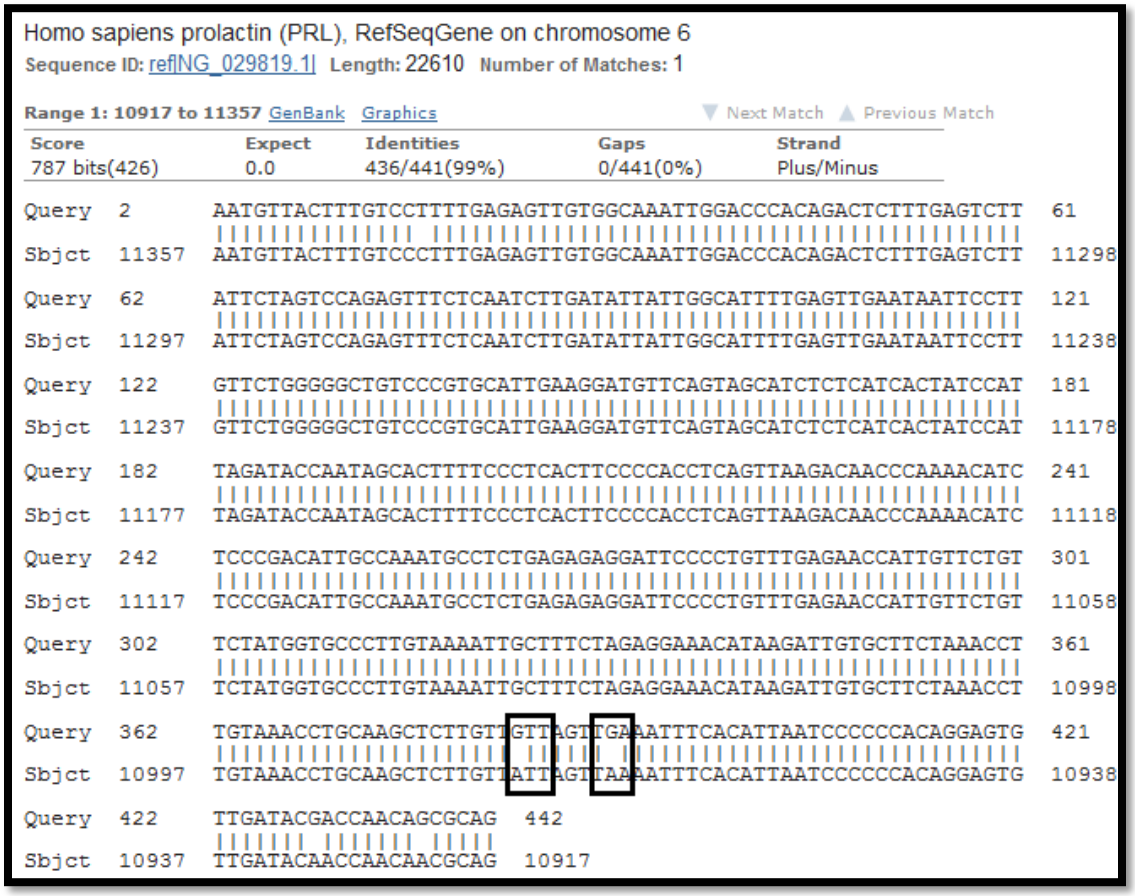
Appendix(7).



Homo sapiens prolactin (PRL), RefSeqGene on chromosome 6

Sequence ID: refING_029819.1| Length: 22610 Number of Matches: 1

Range 1: 10917 to 11357 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
787 bits(426)	0.0	436/441(99%)	0/441(0%)	Plus/Minus

```
Query  2      AATGTTACTTTGTCCTTTTGAGAGTTGTGGCAAATTGGACCCACAGACTCTTTGAGTCTT  61
              ||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  11357  AATGTTACTTTGTCCCTTTGAGAGTTGTGGCAAATTGGACCCACAGACTCTTTGAGTCTT  11298

Query  62     ATTCTAGTCCAGAGTTTCTCAATCTTGATATTATTGGCATTTTGAGTTGAATAATTCCTT  121
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  11297  ATTCTAGTCCAGAGTTTCTCAATCTTGATATTATTGGCATTTTGAGTTGAATAATTCCTT  11238

Query  122    GTTCTGGGGGCTGTCCCGTGCATTGAAGGATGTTCAGTAGCATCTCTCATCACTATCCAT  181
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  11237  GTTCTGGGGGCTGTCCCGTGCATTGAAGGATGTTCAGTAGCATCTCTCATCACTATCCAT  11178

Query  182    TAGATACCAATAGCACTTTTCCCTCACTTCCCCACCTCAGTTAAGACAACCCAAAACATC  241
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  11177  TAGATACCAATAGCACTTTTCCCTCACTTCCCCACCTCAGTTAAGACAACCCAAAACATC  11118

Query  242    TCCCGACATTGCCAAATGCCTCTGAGAGAGGATTCCCCTGTTTGAGAACCATTGTTCTGT  301
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  11117  TCCCGACATTGCCAAATGCCTCTGAGAGAGGATTCCCCTGTTTGAGAACCATTGTTCTGT  11058

Query  302    TCTATGGTGCCCTTGTAAAATTGCTTTCTAGAGGAAACATAAGATTGTGCTTCTAAACCT  361
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  11057  TCTATGGTGCCCTTGTAAAATTGCTTTCTAGAGGAAACATAAGATTGTGCTTCTAAACCT  10998

Query  362    TGTAAACCTGCAAGCTCTTGTTGTTAGTTGAAATTTCACATTAATCCCCCCACAGGAGTG  421
              |||||||||||||||||||||| ||||||| ||||||||||||||||||||||||||||
Sbjct  10997  TGTAAACCTGCAAGCTCTTGTTATTAGTTAAAATTTCACATTAATCCCCCCACAGGAGTG  10938

Query  422    TTGATACGACCAACAGCGCAG  442
              ||||||| ||||||| |||||
Sbjct  10937  TTGATACAACCAACAACGCAG  10917
```

Appendix (8).

Medical Report

Date of blood taken:

No. of blood sample:

Information:

Patient name: Age:

Years of marriage: No. of kids:

Infertility type: any miscarriage?

Is period come monthly in date? Any medication taken?

Is there a history of breast cancer in family?

Region:

Results

Prolactin :

LH:

FSH:

Notes

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الملخص

هدفت هذة الدراسة لدراسة موضوع يعود ألى حالات عدم الخصوبة ألناتجة عن فرط هرمون البرولاكتين واستقصاء العلاقة بين فرط البرولاكتين لدى النساء غير الحوامل وعدم الخصوبة وتأثير ذلك في النساء ذوات العقم الاولي والثانوي وذلك بواسطة التحري عن الاساس الكيميائي الحيوي لهذا المرض فضلا عن ايجاد العلاقة بين الطفرات بجين هرمون البرولاكتين وجين مستقبل هرمون البرولاكتين وبين ألارتفاع المرضي لهذا الهرمون المسبب للعقم . جمعت عينات دم من ١٥٠ امرأة مصابة بمرض فرط البرولاكتين من مستشفى كمال السامرائي ومستشفى العلوية وجمعت ٥٠ عينة دم من نساء صحيحات أستخدمت مجوعة سيطرة. لدراسة الكيمياء الحيوية لهذا المرض. قسمت مجموعة المريضات (١٥٠مريضة) الى مجموعتين، مجموعة ذوات ألعقم الاولي ومجموعة ذوات ألعقم الثانوي لمعرفة تاثير فرط البرولاكتين على العقم، ثم لدراسة تأثير فرط البرولاكتين في الفئات العمرية (تم تقسيم المرضى ألى ثلاث مجاميع عمرية وهي ٢٠-٣٠، ٣١-٤٠ و ٤١-٥٠ سنة).

عينات المصل لمريضات أرتفاع الحليب تم أستخلاصها وكذلك قياس هرومونات الخصوبة العائدة لها وهما الهرمون اللوتيني والهرمون المحفز للجريبات، ولقد وجد ان هناك اختلاف معنوي في تركيز هرمون البرولاكتين لدى النساء المصابات بأرتفاع هرمون البرولاكتين بالمقارنة مع الصحيحات حيث كان مرتفع عند المريضات بينما سجل كل من هرمون اللوتيني والهرمون المحفز للجريبات أنخفاضا محلوضا مما يدل على تاثير المرض وقد كان أرتفاع هرمون البرولاكتين أكثر في النساء ذوات العقم الثانوي ولكن الفرق بين المجموعتين لم يكن معنويا كذلك في المجموعة العمرية ٣١-٤٠ سنة كان الاكثر أرتفاعا لهرمون البرولاكتين والأكثر أنخفاضا للهرمون اللوتيني والمحفز للجريبات المبيضية ولكن الفرق لم يكن معنويا.

لدراسة الجانب الجزيئي لمرض أرتفاع هرمون البرولاكتين ولدراسة التغيرات الحاصلة على جين المسؤل على تصنيع هرمون البرولاكتين، لقد تم أستخلاص المادة الوراثية (الحامض النووي) الديوكسي رايبوزي من عينات المريضات المصابات بأرتفاع البرولاكتين كذلك ولقد تم تضخيم جين هرمون البرولاكتين بجميع العينات كذلك تم تضخيم مستقبل هرمون جين البرولاكتين بواسطة تفاعل سلسلة البلمرة البي سي ار، على الحامض النووي المستخلص بأستخدام مجموعة من البادئات (٨ ازواج لجين هرمون البرولاكتين و٤ أزواج لمستقبل جين هرمون البرولاكتين). أرسلت نواتج تفاعل سلسلة البلمرة لجين البرولاكتين الى شركة من أجل أجراء فحص ودراسة تتابع تسلسل القواعد

النتروجينية على قطعة الجين موضع البحث. تم تقدير العلاقة بين التركيب الجيني،أظهرت النتائج أن بعض مرضى أرتفاع البرولاكتين قد ظهرت فيهم طفرات نقطية من حذف واستبدال بينما لم تظهر طفرات بباقي العينات فظلا عن وجود طفرات مشتركة بين مرضى أرتفاع البرولاكتين. كان نسبة طفرات الاستبدال ٨٨.٤٦% بينما بلغت نسبة طفرات الحذف ١١.٥٤%، ومن هذا نستنتج أن وجود طفرات عديدة في المناطق المشفرة للجين والمناطق غير المشفرة بجين هرمون البرولاكتين لمريضات أرتفاع البرولاكتين وجين هرمون البرولاكتين وجود طفرات مشتركة بين المرضى هذا يوضح علاقة هذة الطفرات بحدوث المرض.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعةالنهرين
كلية العلوم

المظاهر الجزيئية والكيموحيوية لفرط البرولاكتين في الدم في النساء العقيمات

أطروحة

مقدمة الى مجلس كلية العلوم- جامعة النهرين كجزء من متطلبات نيل

درجة دكتوراه فلسفة في العلوم- تقانة الاحيائية

من قبل

مروة عباس عبد الرزاق

بكالوريوس علوم/ تقانة احيائية/ جامعة النهرين/ ٢٠٠٤

ماجستير علوم/ تقانة احيائية/ جامعة النهرين/ ٢٠٠٧

بإشراف

د. عبد الواحد شمخي جابر استاذ مساعد

د.رحاب صبحي رمضان استاذ مساعد

أذار ٢٠١٦

جمادي الثاني١٤٣٧