Genetic Analysis of Growth Hormone Releasing Hormone Receptor Gene in Individuals with Dwarfism in District Karak of KPK

By

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Chromosomal disorders.

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"In the name of ALLAH The Most Gracious and The Most Beneficial"

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FINAL APPROVAL

It is certified that we have read the final project report submitted by **Mr. Amir Atlas** and it is our Judgment that this project is of sufficient standard to warrant its acceptance by the International Islamic University, Islamabad for the MS Degree in Biotechnology.

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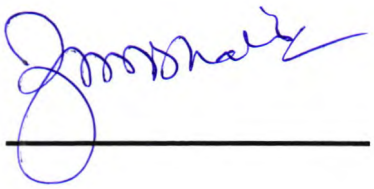

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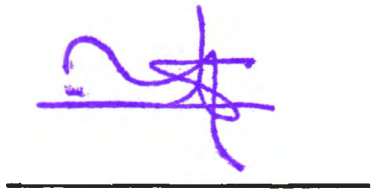

A thesis submitted to Department of Bioinformatics and Biotechnology,

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(MSBT)

This humble effort is

Dedicated

To
My Sweet mother (Addey), Brothers and Sisters

DECLARATION

I hereby solemnly declare that the work entitled "Genetic Analysis of Growth Hormone Releasing Hormone Receptor Gene in Individuals with Dwarfism in District Karak of KPK" presented in the following thesis is my own effort, except where otherwise acknowledged and that the thesis is my own composition. No part of the thesis has been previously presented for any other degree.

Dated: _____

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"Universe is full of resources now it is your duty to explore it"

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AMIR ATLAS

ABBREVIATIONS

Abbreviation	Definition
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
tRNA	Transfer Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SED	Spondyloepiphyseal Dysplasia
cGMP	Cyclic guanosine monophosphate
cGTP	Cyclic guanosine triphosphate
FGHR3	Fibroblast growth hormone receptor 3
NPR2	Natriuretic peptide receptor 2
GHRHR	Growth hormone releasing hormone receptor
GH	Growth hormone
GTP	Guanosine triphosphate
PCNT	Pericentrin
GHRH	Growth hormone releasing hormone
KPK	Khyber pakhtunkhwa
IGHD	Isolated growth hormone deficiency
GD	Growth deficiency
DraIII	Deinococcusradiophilus III
cM	Centi Morgan
IB II	Infectious bronchitis II
MRI	Magnetic resonance imaging
SOX	Supper oxides
LIG3	Ligase 3
NHEJ	Non homologous end joining

BD	Becton, Dickinson
EDTA	Ethylene diamine tetra acetic acid
UK	United kingdom
mM	Mili molar
M	Molar
SDS	Sodium dodecyl sulphate
TEDTA	Tris ethylene diamine tetra acetic acid
DPA	Diphenylamine
PCR	Polymerase chain reaction
dNTPs	Deoxyribo nucleoside triphosphates
PAGE	Polyacrylamide gel electrophoresis
APS	Ammonium persulphate
TBE	Tris borate EDTA
UV	Ultra violet

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ABSTRACT

Dwarfism is the genetic disorder that is associated with anomalous body growth in which body remains short as compared to the normal level. There are many types of dwarfism on the bases of physiological condition like achondroplasia, hypoachondroplasia, skeletal dysplasia and simple dysplasia. In achondroplasia, there is a problem with cartilage formation in the body due to which hyper extensive type of joints are produced. Hypoachondroplasia is similar to that of achondroplasia but difference is only of its penetrance. Skeletal dysplasia is not only concerned with body physiology but it relates to all those illnesses which cause abnormalities of body stature associated with bone, cartilage and muscles.

There are many reasons for dwarfism like environmental factor, hormones and genetic factors. In hormonal factors, numbers of hormones are involved in body growth regulation, due to disruption in such hormones the body remain short. The disruption in these hormones is either because of the interruption in pathways producing it or in genes coding for it. Some of the other factors are environmental conditions including nutrition. Food and nutrition have prominent role in body growth and normal function. A body may remain short, weak and undeveloped because of malnutrition. The most important is the genetic disruption of different genes which are related with growth directly or indirectly contributes to body growth in one way or other. Approximately 5-6 genes are identified as a genetic cause of dwarfism like *GH*, *FGHR3*, *PNCT* and *GHRHR* gene.

In the current study, two consanguineous families were genetically investigated. Both of the families were having autosomal recessive type of inheritance. Clinical features of the disease showed shortening of fore and hind limbs and a truck too in some cases.

The clinical record of individuals of both families showed that although the individuals were having a short stature but exhibited normal physiology. Family B was clinically diagnosed as a hypo-pituatrized with protruding eyes. The genotyping results of both the families show that family A and family B did not show linkage for the candidate gene through microsatellite markers which were synthesized for the defective gene locus for both normal and affected individuals of the families. Hence the results indicate an exclusion of both families.

INTRODUCTION

1. INTRODUCTION

1.1 Genetics:

Genetics is one of the most important branch of science specifically a branch of biology that identify how a particular characteristics will transfer from parents to offspring because human contain a genetic material that is DNA which are further containing genes and each and every gene have specific structural and functional importance that actually determine how the traits will be shifted from parents to their young ones. Disruption in these genes may lead to some unusual abnormal functioning of a body part which produces disorder so called genetic disorder. Till now approximately 4000 of different genetic disorder have been identified. These disorders have certain harsh impacts on individuals' health and also on society. These disorders are inborn and it can be a cause of an extreme illness and ultimately death (Kaku & Maia, 1992). Approximately, 3 in 100 of pregnant mothers give birth to an abnormal individual having a genetic disorder. Humans have an approximately 35000-40000 genes and each gene transcribed into a functional RNA and this functional RNA encodes for protein production or for other RNA types that is rRNA and tRNA which helps in different cellular process at protein levels.

1.1.1 Human Molecular Genetics:

Human molecular genetics is the branch of genetics which is related to the studies of all characteristics including mental and physical both which can be transmitted from parents to their kids. Human molecular genetics includes studies of genetic disorder such as autosomal dominant, autosomal recessive and X-linked (Tomlinson and Bodmer., 1995).

1.2 Genetic Disorder Classification:

Disorders at gene level occur because of many reasons such as mutation in particular gene or disruption in the pathways of these genes which then lead into truncated product productions (Sertié *et al* ., 2000). Genetic disorders are classified into different types on the bases of their functions and origin such as chromosomal, single gene disorder and polygenic.

1.2.1 Chromosomal Disorder:

Chromosomal abnormalities are because of change in chromosomal structure and sometime in chromosome numbers during cell division. During cell division chromosomes changes their chromatid during crossing over in which some mismatch parts of chromosomes are attached to one another that causes abnormal functioning of chromosomes and sometime it is

because of chromosomes numbers one cell may get one chromosome extra or less such as trisomy and monosomy which results in loss of normal function and this loss or gain of the chromosome lead to incompetence to survival or production of disorder (Scriver *et al.*,1973).

1.2.2 Single Gene Disorder:

This is another important type of disorder which caused due to defect in a gene of the cell. Single gene disorder is because of gene product truncation which usually because of single base pair mutation in gene or sometimes because of deletion of insertion of certain portion of gene due to which gene could not perform its normal function. These single gene disorders have further three types autosomal dominant, autosomal recessive and X-linked which are named because of its mode of inheritance (Sriram *et al.*, 2005).

1.2.3 Enzymatic Defects:

Mutation in genes of enzymes that are controlling certain cellular pathways can also be a cause of genetic illness although it is indirectly regulating cellular activities but have very worst effects on total cell functions, these defect results in metabolic pathways irregulation and also unusual conversion of substrate into product that causes accumulation of substrate.

1.3 Transmission Pattern of Genetic disorders:

Mode of inheritance of genetic disorder is different like autosomal dominant, autosomal recessive and X-linked which are discussed below.

1.3.1 Autosomal Dominant Disorder:

Each individual carries two pair of the genetic trait that is one from father and one from mother, if the disease mode is dominant then you need only one copy of gene to be effected with that particular disease (Hong *et al.*, 2000).

1.3.2 Autosomal Recessive Disorder:

In autosomal recessive disorder there is always two copies of an affected gene is required for a person to be diseased that is one copy from each parent one from father and one from mother (Bonati *et al* ., 2003).

1.3.3 X-Linked Dominant:

X linked dominant condition is that type of inheritance in which a disease is caused by alteration in those genetic traits that is present on X chromosome. It is most rare case that the

individuals will need two copies of the gene that is mutated so all the females will be affected having mutated gene while male be carrier.

1.3.4 X-linked Recessive:

X-linked recessive is that mode of inheritance in which mutation occurs in those genes which are present on X chromosome. The physiological characteristics can only be observed in those individuals which carries altered paired of that gene.

1.4 Polygenic Disorder:

The word polygenic is combination of two words that is poly and genic, poly means many or more in number while genic refers to genes so polygenic are those disorder which are control by many genes and which are multifactorial disorders (Purcell *et al.*, 2009).

1.5 Somatic Cell Genetic Disorders:

Somatic cell disorders are those which are caused in mutation in other than germ cells because it is caused due to mutation in gene which are present in other than germ cells (Park *et al* .,2008). In cases of cancer their occur mutation in the somatic cell of body in which cell division become uncontrolled.

1.6 Multifactorial Disorders:

Multifactorial are two disorder in which certain and environmental conditions interact with each other and contribute to disorder as a cause. Neural tube and heart disease are some example of multifactorial diseases (Neal & Kendler., 1995).

1.6.1 Mitochondrial Disorders:

Disorders are generally divided on the bases of where does it occur or because of gene due to mutation in that gene it is caused. Mitochondrial disorders are two disorders which are caused due to non-genomic DNA that is mutation in mitochondrial (Schaefer *et al.*, 2004).

1.7 Causes of Genetics Disorders:

1.7.1 Mutation:

Mutation is change in structure and function of gene that is caused by single base pair change which is either deleted or inserted resulting in inappropriate functioning of that gene and it is transmitted from generation to generation (Bamford *et al.*, 2004). Most information on

human mutation rate comes from studies of rare autosomal dominant traits for which it is much easier to estimate mutation other than to estimate this for recessive train (Edwards, 1992).

1.7.2 Small Population:

In human population are divided on the bases of religion and geography etc. Population is sub-divided which form genetic isolates. This population may be a carrier for the mutant gene, so it becomes a cause of genetic disorder. This genetic disorder varies in size means in large population the percentage of disorder will be high and vice versa (Lichtenstein *et al.*, 2009).

1.8 Dwarfism:

Dwarfism is that medical condition in which living organism either plant, animals or human being having under developed growth of the body as compare to the person of the same age. There are approximately 200 types of dwarfism on the bases of morphology and their percentage affecting people of the world (Brancati *et al.*, 2005). Dwarfism is a genetic condition that is present early at the time of birth (Backstrom 2012). If the person who grow under five feet that is upto 4 feet are characterized as a dwarf (Kruse & Robert, 2003). There are more than 200 types which approximately repeats 1/10000 birth irrespective of race (Kruse & Robert, 2003). Dwarfism is a medical condition in which a person having a short stature (Guse & Harvey, 2010).

Dwarfism have many types such is achondroplasia, skeletal dysplasia and hypoachondroplasia but the most frequently occurring is achondroplasia which is about 70% of all types. The frequency of achondroplasia is from1/26000-40,000 birth which imparts physiology with below normal height (Nicholson *et al.*, 2008).

1.8.1 Types of Dwarfism on Bases Physiology:

1.8.1.1 Achondroplasia:

Achondroplasia is mostly occurring form of dwarfism which is characterized by a short stature and its frequency is 1/15000 and person with achondroplasia having short limb and microcephaly sometime as an additional characteristics, the individuals having the ability reproducing and transmitted as non sex chromosal disorder (Rutter *et al.*,1990).

1.8.1.2 Spondyloepiphyseal Dysplasia (SED):

Spondyloepiphyseal dysplasia is another form of short stature which is characterized as heterogeneously inherited group of disease. It is caused due to mutation in collagen type II gene and it involves pleotropic skeletal and ocular system (Lee *et al.*, 1989).

1.8.1.3 Hypoachondroplasia:

Hypoachondroplasia is a developmental disorder that is associated with body growth normally its mode of inheritance is autosomal dominant which caused due to error in FGHR3 gene. It also results in short stature which has additional properties of micromelia and usually with large head size when compared with developed part of a body (Hecht & Francomanolz, 1995). The body remains short because of disturbance in a particular process called ossification in which conversion of cartilage into long bone of Arms and Legs.

1.8.1.4 Skeletal Dysplasia:

Skeletal dysplasia is a condition in which abnormal differences occurs in body size due to under development of bone and cartilage due to which legs, arms and trunk remains undeveloped so the body size remains below normal height (Pollock *et al.*, 2007).

1.8.1.5 Primordial Dwarfism:

This type of dwarfism can begin and may be characterized at early stages of life that just before birth even during embryonic stages of the fetus and these individuals are extremely small for their age. The effected person can be analyzed and diagnosed for the respective illness when they attains the three years of age. The diagnosis process starts after gestation period and they may be detected with intrauterine disability when diagnosed through ultrasound.

1.9 Genes involved in Dwarfism:

There are certain elements that are involved in controlling body growth, some of these are involved directly controlling the growth of the body and some are involved indirectly in other words they have no actual concerned with body growth but they catalysis certain biochemical pathways that in turn produces certain product that help in growth of the body. Those elements which are directly involved in growth are called cis acting elements and those which are indirectly involved are called trans acting elements.

In cis acting elements there includes promoter, enhancer and transcriptional unit of genes while in trans acting elements co transcription factors. Some genes that are involved in dwarfism are FGHR3, GHRHR, NPR2 GH, and PCNT. Mutation in all of these genes results in different types of dwarfism.

1.9.1 NPR2 gene:

NPR2 stands for natriuretic peptide receptor 2 and its cytogenetic location is 9p13.3. The end product of the gene transcript is guanylate cyclase that help in formation of cyclic guanosine monophosphate (cGMP) from its intracellular precursor guanosine triphosphate (GTP) (Miura *et al.*, 2014).

1.9.1.1 Gene Structure:

Structure of this *NPR2* can be described by 22 coding portions having size of approximately 16.5kb. The 5′ overhangs is further having active sites for approximately 10 different SPI, TATA box are absent and the noncoding region contains CA/GT some VNTs repeats that is variable.

1.9.1.2 Function:

The experiments carried out on ANPB receptor showed that these receptors makes wide range of active site for radiolabel human-ANP and bovine brain ANP which increases radiolabeled ANP to membrane that adds to the production of cGMP in contrast to control.

1.9.1.3 Short Stature with Nonspecific Skeletal Abnormalities:

Population that was consisting of many individuals were inspected with some mutation having role in short stature in which body growth is retarded and remain below average height. Sixteen patients were having the mutation in a single base pair change due to which frame was shifted (Olney *et al.*, 2006).

1.10 FGHR3 Gene:

FGHR3 gene is another most important gene that is having key role in controlling activities and it work is a factor that are involved in number of body control mechanism like wound healing of when their some cut or disruption of muscles take place.

1.10.1 Gene Structure:

This gene is having enormously large size because comprises of 13 codding regions and it does not carries some of the important structure that other genes does carry (Perez *et al.*, 1997).

1.10.2 Gene Function:

This gene function was studied when it was incorporated to some special types of cell which play an important role in body defense. So on the bases of these studies certain other isoform were identified like *FGFR* 2 and 4 which show little response provoked to B cell (Shimizu *et al.*, 2001). Some of the isoform requires other activating molecules like heparin for *FGR3* due to which the mouse were made chondro and were rounded (Shimizu *et al.*, 2002).

These features were clearly different from those seen in thanatophoric dysplasia or other skeletal dysplasias. Due to alteration *FGR3* there was an individual analyzed for feature like shorting of trunk and up and down limbs (Hyland *et al.*, 2003).

1.11 PCNT Gene:

PCNT is abbreviated as pericentrin and its cytogenetic location on the human genome is 21q22.3, mutation in this gene causes many phenotypic conditions like microcephalic osteodysplastic primordial dwarfism, type II.

1.11.1 Function:

Number of individuals were characterized and examined with genetic alteration in *PNCT* gene which were having clear indications of the disease of under developed stature (Rauch et al., 2008) and some other were having an uncharacterized genetic disorder that was similar in low height but was having some extra features (Griffith *et al.*, 2008). After this microarray analysis was done for all these 25 patients' samples so for observed with non-described alteration but physiologically all were suffering with dysplasia.

1.11.2 Mutation in *PCNT* gene a cause of Primordial dwarfism, type II:

Some other positions are also identified in the same gene which is causing all types of disorder that may be related to body growth in one or other way. The clinical analysis of this patient showed that she presented minor skeletal changes and clinical features compatible with

the diagnosis of type II microcephalic osteodysplastic primordial dwarfism (Willems *et al.*, 2009).

1.12 GHR Gene:

This is the gene which a direct role in human body performance in term of height because this is the receptor gene for *GH* which releases hormone for body growth activation and it is an abbreviated name of growth hormone receptor *GHR* is an abbreviation of growth hormone receptor gene and its cytogenetic position in genome is *5p13-p12.*

Nuclear level changes in this gene are a reason of growth failure which produces abnormal products that causing body low growth. Mutation in these genes always results in truncated product and as results causes different genetic disorders. All disorders that are associated with growth of the body can be caused due to this gene (Pantel *et al.*, 2003).

1.12.1 Structure of *GHR* gene:

This gene have a complex structure because it is composed up of several domains that intracellular and extracellular domains of nearly 246 amino acid chain. These domains are decided on the bases of exon from exon 3-7 is the outer cellular domain and different types of human *GHRH* gene are available because of some exon missing, one is a running isoform having exon 3 and the other isoform have no exon 3 of the *GHRHR* gene so it results in two type of transcripts one having exon 3 and the other with no exon 3 (Pantel *et al.*, 2003).

1.12.2 Laron Syndrome:

Laron syndrome is that type of disorder in which mutation occur in different growth controlling genes and in this syndrome body growth is stopped due to failure of growth related hormone or their respective genes. A girls was identified (Shevah *et al.*,2005) with two different types of alteration in the gene one case was deletion in of 1 bp and other change was that of substitution of one base pair by another of the different group that is C-A due to which termination of chain occurred before of maturation at the position of cysteine -83.

1.13 GHRHR Gene:

GHRHR is abbreviation for growth hormone releasing hormone receptor gene. This gene is indirectly related with growth of the body means it is the receptor for growth hormone releasing gene which activate growth hormone and its Cytogenetic location: 7p14.3.

1.13.1 Gene Structure:

The structure of the genes are highly variable and GHRH gene is having the full gene size of twelve kilo base pairs and it contain 5′ overhanging region and this gene have approximately 10 different transcribing exons. (Petersenn *et al.*, 1998).

1.13.2 Gene Function:

GHRH and some other variant of these are studied and identified in some additional role that in controlling adenocarcinomas that ate related to humans because of mutation in the binding site of these GHRH become causative agent for the diseases. (Martati and Salvatori., 2009). The alteration in this and related genes were studied in Brazilian families which having mutant copies of these gene in which there occurred a transition of G-A base pairs so manipulating the normal gene function. (Oliveira *et al.*, 2007).

1.14 OBJECTIVES OF THE PROPOSED RESEARCH

Research regarding dwarfism in Pakistan showed a very little knowledge and it was previously studied in Sindh in 1997 although it is affecting a large population worldwide. Secondly and mostly importantly the reason for selecting this topic is, KPK is having the most dwarfism-affected individuals in Pakistan.

- Identification of families with dwarfism in district Karak of KPK.
- Screening of selected gene in collected families.
- Analysis of identified mutation and its impact on the gene.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Human body is a complex structure and it is dependent upon certain body mechanism which is activated by body controlling traits that are called the gene. So science that facilitate the analysis of structure and function of these traits is known is molecular biology.

In this field of science we study and analyze molecular characterization of gene, their structure and the dependency of functions on the particular gene structure. This also provides knowledge how individuals traits are transferred from generation to generation so one of the milestone achievement that was made in this field is the identification and sketching of the whole genome of human being that took approximately ten years for its completion. Genome contains all the possible information that can be related to particular persons (Hultman et al., 1989).

Alteration in these gene is a cause of inability of doing normal function of the body and these alterations may be because of several reasons some times because of wrong place of a base at nonspecific position that may lead to diseases which are then decided to subclasses on the base of their transmission. To study familial disorders it is necessary to have their generation to generation history etc, (Slamon *et al.*, 1989).

There are many genetic disorders such is Dwarfism (Achondroplasia), Down syndrome, hereditary hemochromatosis, microcephaly etc. All these genetic disorder are caused due to single base pair mutation (Amselem *et al.*,1989).

Dwarfism is the genetic disorder that is caused due to mutation in those genes, which play role in growth control directly or indirectly. There are more than 200 types of dwarfism worldwide but the most common and highly understood type is achondroplasia and it is reported that mutation in many genes such FGHR3 gene, GH-R gene and the mostly reported gene is that of GRHR-R gene believe to be linked to a single point mutation in FGHR3 gene (Amselem *et al.*,1989).

Godowski *et al.*,(1989) was analyzing different families individuals who were suffering with low height disorder called the dwarfism. This type of disorder was because of the alteration in the gene that was related to non sex chromosome. This disorder after experimentation was identified due to the disruption in the gene that produces GH factor I and such patients were identified for low insulin level and their GH were performing a normal function. GH factor I is a receptor for GH due to disruption in the receptor gene causes this particular syndrome which was

named Laron syndrome after the researcher name. The structure of the gene showed some unbelievable results because it having 9 coding exons but in spite of these are some other additional exon in the non-transcribe region too. The size of these coding sequences is 97kbs. GH was intensively studied in upto hundred patients who were identified for deletion of the receptor site of the gene. Second astonishing feature is that the deletion is random and nonspecific at several places in the gene which causes the abnormal function of the gene that result in nonspecific binding to the receptor sites disturbing the total pathways.

Amselem *et al.*, 1989 concluded from the experiment he conducted on dwarfism and he identified a novel mutation in the human GHRHR gene which was then named after his name the Laron type of dwarfism. He identified this mutation by using indirect approach of study that is first tried to establish linkage of the defective gene and then sequencing of this mutated gene. For his experiment he used approximately 35 patients to study their dwarfism at genetic level that is it because of mutation in GHRHR gene or not. In this study he discovered that at least one family disorder was because of thiamine to Cytosine substitution in the affected individuals which produces truncated protein product in which serine type of amino acid is replaced with phenylalanine at position 96 in the extracellular coding domain of the mature protein. Due to this defect the binging activity of this receptor is adversely affected due to which the growth hormone releasing pathway is enormously affected which results in low height which is well below normal.

Maheshwari *et al.*, (1998) reported different reasons of dwarfism as he was studying different aspects of dwarfism which were included of hormonal effect on the body growth, its impact on resulting phenotype and also the genetic changes that occurs during this phenotype. This study was designed when he noticed an article in dawn newspaper which highlighted that some villages in Sindh are having in individuals with height well below the normal. This study was comprises of two steps in first step all the patients were clinically diagnosed and then their genetic bases of disorder was studied. In his experiment he used a sample size of and 18 patients in which there were 15 male and 3 females included respectively. The physiological properties of these patients were also studied such as height, weight and body mass index. In his experiment he identified a mutation in GHRHR gene which was a missense type of mutation in which glu-stop codon at position number 503 in the extra cellular domain of this gene. In this way because of this mutation the release of GH is affected by pituitary gland due to interruption in the binding

mechanism of GH to the receptor site of GHRHR gene. This study was similar to the previously reported researches that mutation in little mice and also mutation identified by Maheshwari in the dwarfism of Sindh.

Savatori *et al.*, (1999) recognized GHRHR gene mutation in some patients who were affect with most frequent type of disorder in which a body growth remains under develop and the ultimate result is the patients with dwarfism which is clinically identified as isolated growth hormone deficiency (IGHD). Salvatori was working on population genetics in his experiment he maintained the sample size of his research upto 105 patients which were collected from indian subcontinent. In which the mutation of growth hormone releasing hormone receptor (GHRHR) gene was reported. In this research 22 numbers of patients were identified with failure of GH which was because of the previously studied gene mutation that in mutation the receptor of GHRH gene. All the patients of this research were having autosomal recessive type of dwarfism. A newly unreported mutation was investigated which was a splice site mutation in the GHRH receptor gene. In sixty four clinically identified patients who were having isolated growth hormone deficiency only thirty patients were having this mutation.

Baumann., (1999) illustrated short stature in patients who were suffering from growth failure and were clinically identified for growth deficiency (GD). When these patients were subjected to genetic level research they were investigated for all those gene mutation which have links in the growth of the body either directly or indirectly controlling the body growth.

This research worked concluded an inactivating mutation in GHRHR gene. A similar mutation which results in the formation of stop codon replaces a functional codon that truncates the whole gene product. There are number of mutations of the same locus is identified and they are named on the bases of how much peptide they are affecting which was investigated in different families of different origin some were from Pakistan other were from india and Sarilanka. Some of the unexpected mutation that is in the intron region but having effect on the gene performance is also reported in Brazilian families at donor splice site at intron number 1. The mode of inheritance in all these was autosomal recessive. This new syndrome corresponds to the human homologue of the previously identified 'little mouse.

Hayashida *et al.*, (2000) analyzed syndrome in Brazilian families which was having autosomal recessive mode of inheritance and in clinical features it was similar to that of growth hormone deficiency GHD syndrome in which a body of human or other animals faces the problem of growth retardation. This disease was due the novel mutation in the GHRHR gene. Some other hormonal and clinical test were performed of these individuals in which they showed a variation in results because some of them were homozygous for the GHRH-R mutation while some were heterozygous for GHRHR gene mutation. All the patients were having consistent in the feature of growth hormone deficiency. This type of disease was also having autosomal recessive disorder which caused to due mutation in both the alleles of the GHRHR gene. The truncation of this gene product due to mutation leads to remarkable mutation which becomes a causative agent of the many type of dwarfism which is highly variable in phenotypic and hormonal feature variations. Those children who were having the mutation alleles of this were also reported for the partial defect of GH gene as well.

Savatori *et al.*, (2002) explored the function of genes which are of prime importance in the maintenance of the body growth such as GHRHR gene. Some other dwarfism related diseases are also reported which are also familial and are classified as isolated growth hormone deficiency type IB. in this research samples size all those mutation were found which were previously reported by some other researchers. All these individuals were sharing some common ancestors.

Some new mutation were expected in which transversion of T-A took place which substituted the histidine amino acid for leucine in codon number 144 due to which restriction site DraIII is created. This mutation is also reported in some other countries which were having different ethnic backgrounds and does not share any common ancestors. They also determined this mutation in some other continent that of Africa and identified that they were homozygous of L144H. They used indirect method of screening using linkage analysis and analyzed that one out of these 3 selected families do not having the intergenic polymorphism as compared to the other two families' members.

Aviezer *et al.*, (2003) elucidated that short stature is having number of types and most common among these types is achondroplasia which is observed in approximately 1/20,000 individuals those who are having dwarfism. In this type of genetic disorder a person have to face bone growth and craniofacial anomalies. There are many genetic reasons behind this but the

most known is the mutation in fibroblast growth hormone receptor gene 3. This mutation in the gene which is reported is single point mutation. Treatment for this defect is available to some extant but these are not having long lasting advantages. Majority of patients are having glycine to arginine substitutions at position number 380 of the gene. Due to alteration in FGR3 gene lead to an inappropriate development of bones and cartilage. This also affects the signaling mechanism of the cell with help in the growth plate's formation. Some attempts are made to synthesis drugs that my block the extracellular domain of the ligand binding or the checkpoints of this cascade can be a milestone achievement in the treatment of achondroplasia. this research study was designed to study and elaborate the all the disorders that are either related to body skeletal and either to cartilage and bone developments.

Wajnrajch *et al.*, (2003) emphasizes the role of growth hormone releasing hormone receptor gene that how it help out the pituitary gland to release growth hormone in the body and to contribute to developmental stages. Some individuals were studied those who were having dwarfism in the indian subcontinent and they were identified for the three unrelated mutation because they were not sharing any ancestral backgrounds. Study was aimed to identify and investigate the relationship of these mutations in the indian subcontinent among all the three different families which were identified for dwarfism. These individuals were analysed for the mutation at cytogenetic location 15.5 cM in the GHRHR gene and its chromosomal location was 7p band 15 and sub band 5 that was coined as a causative agent of dwarfism.

Alba *et al.*, (2005) explored the same gene mutation in some mice species that were having a dwarfism. He elucidated that there is a big relationship between growth hormone releasing hormone receptor gene and release of growth hormone rom the pituitary glands. Activation of growth hormone is done by growth hormone releasing hormone gene which is further switched on by growth hormone releasing hormone receptor gene and so as for the cascade works. The growth of the body is not directly attached to mutation of GHRHR gene but it work is a trans-acting elements which activates some other gene to express and give their products. There are number of diseases which are identified as a reason of missense mutation in the GHRHR gene. The study clarified that the disorder was because of loss of function of GHRH to bind to the GHRHR which was disrupted because of other mutation.

Haskin *et al.*, (2006) demonstrated certain types of short stature and also it was correlated with dwarfism and isolated growth hormone deficiency was considered as a major cause of it. It was also worked out that is these because of disruption in growth hormone related gene of some other factors are also involved. Haskin worked on two families who were sharing common ancestors and were from Arab origin. To instantiate all the coding portions of the gene which is approximately having 13 exons are amplified and were sequenced to know whether there is alteration or not. When these samples were observed on gradient gel electrophoreses it was notice that all the individuals were having mutation in which cytosine is replaced by arginine. The penetrance of this gene mutation among those Arab families was upto 2%. All the patients were having a small but detectable value of short stature and though the mutation is not frequently penetrant but still it cannot be ignored as a cause of dwarfism.

Godi *et al.*, (2009) summarized number of mutation analysis he worked out that were related to growth in some way or in other. Mutation in GHRHR gene was notice in some familial type IB IGHD syndrome which was diagnosed is an autosomal recessive disorder. This study was proposed to find out the causative mutation due to which shorting of the stature do occur. This study was having a population size of about 1084 patients and all of them were clinically identified for GHD which in turn is a major cause for dwarfism and other related short stature disorders. In this experiment only hundred and thirty four patients were not having any previous record of the disease and were found for some novel variation that the substitution of valine to glycine at position number 10 with in some peptide which are working in signalling pathways that might be the reason of the disease.

Alatzoglouet *et al.*, (2009) scrutinized some gene for the respective mutations that may have its role in body growth and all that mechanism which are considered is an important in cellular and genome level changes and expression of certain product of such genes. 41 patient were studied in this research from 21 different pedigrees. All such individuals were identified with previously reported new alterations in GHRHR gene. The record of this study shows that 11.1% of these were the familial cases of the diseases it that this particular percentages of patients were having the family history with the disease. When these patients were subjected to hormonal studies and MRI than it was concluded that all were having no differences in normal and mutant as well. The study suggests that the patients were having extreme growth failure which was because of the mutation GHRHR gene that causes dwarfism among them.

Wang *et al.*, (2009) workout Chinese family identified with special type of dwarfism called the pituitary dwarfism which is an autosomal recessive type of inherited disorder. Some necessary clinical tests were done as a pre diagnosis process such is MRI and bone, cartilage tests. The MRI reports revealed that all patients were having a decrease pituitary gland size at their anterior side which was 2.2mm. All the possible genes were screened for linkage analysis and the families established linkage at GHRHR gene. Direct sequencing was done of all the coding exon and their intron-exon boundaries and was identified a novel mutation that was transition from G-A at the splice site at the start of intron number 8. When some other normal individuals were studied no such mutation was noticed. Furthermore in vitro splicing was carried out using hela cells line using expression vector having normal GHRHR gene showed this the G-A transition is cause of truncated protein product of GHRHR gene that is a cause of dwarfism.

Legendre *et al.*, (2010) demonstrated some patients with growth hormone releasing hormone receptor gene (GHRHR) mutation which gives a dwarf phenotype. Two of the sibling were having isolated growth hormone feature and two of the females were having severe short stature as compare to the individual of the same age. Genetic analysis of these two genes G-T transversion in the coding region of exon 3. The patient with taller size was 153 cm. Two patient in this study was due to loss of function of the gene due to mutation in it which were clear indication of growth failure.

Franca *et al.*, (2011) illustrated number of mutations in the GH and GHRHR gene in some families who were suffering from isolated growth hormones deficiency. Study was proposed to identify mutations in short stature individuals. The exons and intron-exon boundaries were amplified by PCR with sequence specific primers. But all individual were not having mutation of GHR, GHRH gene so the other strongest candidate for the screening.

Inoue *et al.*, (2011) scrutinized mutation in GHRHR gene in growth hormone deficient in the families of Japan which was later on identified in some other Japanese. When the mutated gene was sequenced for the mutation in approximately 125 individuals they were identified with G-A mutation that was reported by many researchers previously. After the mutation analysis the gene was amplified which showed that the abnormality occurs due the skipping of exon 12 of the gene that is the second last exon of the gene. Some other patient were identified for T-C substitution in the pit I binding site of the gene. Due to the mutation in pit binding site of promoter the activity of promoter was highly affected, which results in the truncated product of

GHRHR gene. This mutation studied is very much rare and some further study of this promoter region is suggested by the scientist to identify the real cause of the disease and to get some new breakthrough.

Alatzoglou and Dattani., (2012) evaluated some short stature patients which were congenital in mode inheritance which is either because of mutation and may be acquired because some carcinogenic radiations. The gene which is kept on priority were GH and GHRHR gene. Some mutations are reported that have taken place in the transcription factors such SOX3, OTX2 etc. there are number of ways in which gene function is affected sometime because truncation in its transcription factors or either promoter sequence. But the mutation reported in this research is mutation in transcription factor that activates GHRHR gene to carry out its normal function.

Demirbilek *et al.*, (2014) scrutinized the mutation of growth hormone releasing hormone receptor (GHRHR) in individual with growth hormone deficient individuals. In this disease some other additional clinical properties were studied which were including of high level of hypoglycaemia in the all cases. After this the gene was sequenced by Sanger method of sequencing was used for all individuals. The clinical diagnosis of the patients indicates that all of them were having mutated GHRHR. MRI test indicate that they were having anterior pituitary hypoplasia not in all but in some individuals. The homozygosity mapping showed that anterior pituitary hypoplasia due to G-C missense mutation. It is the only case in which patients were having an additional feature of hypoglycaemia.

Kale *et al.*, (2014) analyzed that all patients were identified with GHRHR gene mutation which is not reported before by any other scientist is known as indel and the word indel represent insertion and deletion in the AGAGATCCA sequence. This mutation was observed in two unrelated families. This indel type of mutation is reported in the 5′ regulatory sequence and also in exon 1 of this gene. No patient of the study was identified for GH mutation and the entire patient were still having short stature as compared to those who were having normal functions.

Murray *et al.*, (2015) worked out many gene that were identified which were involved indirectly in the maintenance of body growth through channelizing the pathways. Mutation in the gene is reported which considered due to the joining of non-homologous ends. Several mutations in different component are reported as a result of recombination which bring about diversity.

Gene like LIG3 is also reported as a causative agent of special dwarfism that is called the primordial dwarfism. The alterations in these NHEJ lead to decrease in body protein. In contrast some other group of genes that is LIG4 is reported for bone marrow formation and mutation in the gene lead to bone marrow failure.

MATERIALS AND METHODS

3. MATERIALS AND METHOD

3.1 Investigation of Families:

Investigation of the families is the most difficult step while you are working on genetic disorders. In the present studies two non-related families. To study this genetic disorder two non-relevant families A,B with autosomal recessive dwarfism from different region of distract Karak. Family A was collected from village Ganderi Khattak of distract Karak and family B was collected from Ahmad Abad, distract Karak, Khyber Pakhtunkhwa, Pakistan.

Both the families A and B have autosomal recessive dwarfism, their pedigree shows that there are no ancestors having dwarfism in both the families. Both the families were asked and inquired about the necessary questions regarding this disease. The those information and queries that were thought to be beneficial regarding the diseases were taken like X-rays, Medical reports and picture were obtained with the consent from the elder of the families.

The medical reports were showing of the family that they were categorized as hypo-pituatrized by the medical board of police line hospital, Peshawar.

The aims and objectives of this research were explained to the elders of the families and informed consent was signed and was taken from the elders and responsible persons of these families.

Digital pictures of the individuals with the abnormalities were taken which clearly showing the phenotype of the individuals to compare that with previous literature that may be available about different types of dwarfism.

For proper understanding of the abnormalities the environmental condition and daily routine food and other observation were also carried out. All though the condition were harsh saline and were having the traces of heavy isotopes of uranium but it was not directly considered as a reason for this disorder.

Further genetic analysis is required to study gene mutation of the defective gene that may be a cause of the diseases. This distracts having approximately more than 40 families with dwarfism affected individuals which is really alarming.

3.2 Pedigree construction:

After investigation of the families with a particular disease condition the information given by the elders of the families were used for constructing pedigree chart of the effected families according to the standard method of genetics.

It is necessary to show the relationship between different individuals in a family symbolically and there is a special representation called pedigree. Through this symbolic representation we can get knowledge about a specific family and can easily interpret their inheritance from their ancestors. To determine the mendalian inheritance of genetic traits, especially familial diseases, across several generations of a family this phylogenetic pedigree is prerequisite.

Pedigree chart contain certain specific symbols including circles, squares, filled squares and circles, double lines, single line and straight lines. Each and every symbol has its own genetic meaning.

Male and female are represented by squares and circles respectively and those who were abnormal were shown by filled circles and squares for both male and females respectively.

To show those individuals who or died is represented by fully black circle/square for male/female. Number in roman style indicates generation of the respective family. Family or cousin marriage was showed double line.

3.3 Blood sample collection:

After pedigree construction and all possible and relevant information that were necessary were taken and then the blood from all affected individuals and their other family members were taken up to by disposable syringes.

3.4 Storage of Blood:

The collected blood was stored in BD vacutainer K2E (EDTA), Plymouth, PL6 78B. UK and was stored for isolation of DNA at freezing temperature to bring its maximum density so place at 4 degree Celsius.

3.5 DNA Isolation from whole Blood:

Extraction of genomic DNA is required for further work either you are working on plants or animals. For plants DNA isolation is usually from leaves and in animals it is usually from blood but rarely from tissues and some time from hair cells.

There are different methods of DNA isolation like organic method (phenol-chloroform) and other one is inorganic method. Organic method (phenol-chloroform) through which DNA can be isolated in many steps which are divided into three days.

3.6 Day First:

3.6.1 Thawing of Stored Blood:

The store blood was liquefied by thawing it and tube was rubbed in hands for several times to speed up the liquefaction process and the blood was kept at room temperature for several mints and was shacked slowly to mix the blood homogeneously.

750 µl of blood is placed in eppendorf tube which having capacity of 1.5 milliliter and was placed for few mints.

3.6.2 Addition of Solution A Lysis Step:

An equal amount of Solution A added that is 750 µl for Lysis of cells and mixture was than centrifuged for 1 mint at 13000 rpm to get the two layers separate that is supernatant and pallets, and then supernatant obtained was discarded up to pellet.

3.6.3 Solution A Component and their Function:

3M Sucrose, 10mM Tris, 5mM MgCl2 and 1% triton X-100.

3.6.3.1 Tris:

Tris (tris hydroxymethyl Animomethane) is used is a buffer which keep the pH for DNA constant. Tris is given preference over other buffers because it have the ability to interact with outer layer which is of lipopolysaccharides and Tris enhances its permeability, this action is boosted with the addition of EDTA because it is chelating agent and capture metal ions.

3.6.3.2 $MgCl_2$:

When membrane are busted by TRIS, there is no compartmentalization in solution anymore then $MgCl_2$ is added which protect DNA from DNA degrading enzymes by binding to DNA which are now in easy contact with DNA because of lacking of membrane.

3.6.3.3 Sucrose:

Sucrose actually act as an exo-osmotic reagent which absorb all cell contains water and help in cell shrinkage to make the cell Lysis process easy.

400 µl of solution A is again added to complete Lysis if there is any remaining cell debris and centrifuged again for 1 minute at 13000rpm.

3.6.4 Addition of solution B, Proteinase K and 20 %SDS:

3.6.4.1 Solution B components:

After first two steps upper layer was collected and was jettisoned and Solution B was added which contains 10mM Tris, 2mM EDTA and 400mM NaCl.

3.6.4.2 Tris:

Tris is used to maintain the pH of DNA though out the experiment.

3.6.4.3 EDTA:

EDTA is used to bind to MgCl2 to make it unavailable for certain DNAs that uses MgCl2 as a cofactor; EDTA is a chelator that forms complexes with metal ions.

400 mM of table salt that is NaCl is added and by taking the phosphate sides of DNA nearer to make the process of DNA sleeting and gave it proper form.

3.6.4.4 Proteinase K:

08-12 µl of proteinase K is added to dissolve are blood protein usually hemoglobin.

3.6.4.5 20 % SDS:

17 µl of SDS is added which is also a detergent that further dissolves cell component and than draw off DNA associated proteins.

3.7 Incubation period:

After this the DNA samples were than place for overnight at 37° C.

3.8 Day 2nd:

250 ml of phenol and 250ml of chloroform and isoamyl alcohol was added. Solution C is saturated phenol and function of phenol is to remove nonpolar proteins, lipid residue and solution D contain mixture of Chloroform and isoamyl alcohol. Chloroform removes extra phenol from DNA isoamyl alcohol removes nonpolar proteins, lipid residue and after addition of sol C and D and proper mixing of this mixture is necessary so spin it for ten minutes at 13000 revolutions per second.

Two phases were obtained and the upper phase was taken into fresh eppendorf tube by 200 µl micropipette. 500 µl of solution D was added again to remove the traces of protein if present there to the sample and was centrifuged for 10 minutes at 13000 revolutions per minutes.

The upper layer was again migrated to new tube using micropipette which have the capacity upto 200 ml which was having pH and was molarity of 3 and the function of sodium acetate is to precipitate the cellular protein and to make the precipitation process speedy super cooled isopropanol was added because it causes the phosphate backbone junks and attraction of these junks results in DNA precipitation to end up with good DNA form samples was taken into freezer to proceed excellent precipitate for several minutes.

DNA formation usually requires some other additional steps like proper slowly mixing of these samples with solution for this purpose tube was shacked gently. When the DNA was observed it was spun for proper settlement at maximum centrifugation speed that is 13000 rpm. After this process two layers were formed the upper layer was carefully discarded without disturbing DNA hard lower pallet portion.

3.9 Purification of DNA:

The DNA that was formed was not pure because it case having some other chemicals and traces of proteins. Two hundred microliter of super cooled ethanol was added which mostly used for sterilization purposes to clean up the DNA from debris. Spin was given at 13000 revolutions per minutes; washing was done with ethanol having concentration of 70%. This process are carried out number of times and after this DNA stuck to the eppendorf tube bottom.

3.10 Drying:

To make the DNA available for use it was then subjected to drying process in incubator for several minutes approximately to 30 mints at temperature was maintained upto 37^0C.

DNA was dissolved in T.E and upto 150-200 microlitre and was place for 24 hours at temperature of 37^0C and this whole process was carried out in incubator.

3.11 Day 3rd steps:

3.11.1 DNA Conformation:

To study a specific disorder certain genetic marker are required to link for that particular gene that are mutated and a cause of this disease.

There are many methods to conform DNA like DPA indicator, Spectrophotometry and 1 % agarose Gel.

3.11.1.1 Diphenylamine (DPA) indicator:

There are some chemical which work is an indicator for identification of several chemical compound through interaction between these molecules. This whole chemical process is based upon one main procedure that is ionization in the presence of water ions at when they are exposed to temperature of (e.g. ≥95 °C) and the reaction is deoxyribose sugar dependent and imparts some specific color as an indication of the presence of genomic molecules.

3.11.1.2 Spectrophotometry:

Each biomolecule have it specific value of its ability to absorb light when it fall on it and the differences between them is on structural composition of these molecules and spectrophotometer is one of the mostly using method to identify molecules and for their conformation too and DNA can be determined at specific wavelength of 600nm and the wavelength curve is calculated comparing to standard.

3.11.1.3 1% Agarose Gel:

The mostly used and favored method for DNA conformation is the gel electrophoresis method in which is mostly used to separate DNA fragments on the bases of size (Sambrook, 2001). It is extract of an algae called *Gelidium* and *Gracilaria* which is having a red color that extract chemical composition is repeats of (L- and D-galactose) subunits (Kirkpatrick ,1991). These molecules form a jelly like structure and bind to one another through covelent bonding and it behave like sieve like for DNA/RNA molecules and separate them when they are provided an electricity through supply. As the DNA molecules are charged due to sugar and phosphate. Separation of these molecules is fragmented on the bases of charge to mas/ ratio. There is negative or inverse relationship between molecules charges and their masses (Helling *et al.*, 1974).

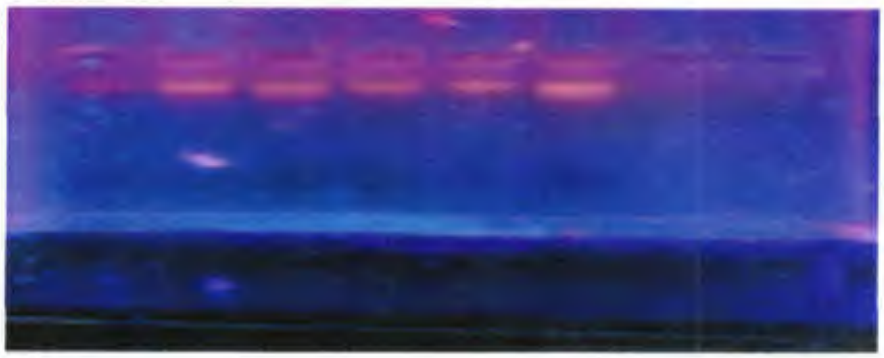


Fig: 3.1 Confirmation of DNA of the family by 1% agarose gel

3.12 Genotyping and linkage analysis:

To study a specific disorder certain genetic marker are required to link for that particular gene that are mutated and a cause of this disease. These genetic markers are diluted to make it ready for use, each marker have its own dilution concentration number to which TE is added in 3 times. Means marker to T.E ratio is 1:3.

3.12.1 Micro-satellite Markers:

Markers were synthesized using bioinformatics tool UCSC genome browser and were ordered for suspected selected gene that is GHRH-R gene and were centrifuges for 13000rpm for 1 minute and T.E was added marker in 1:3.

3.13 PCR:

Whenever researcher is working on the genetic diseases than they try to find a genetic cause of that particular disease that is either a genetic material or its part that is gene. So they need no of copies of these genes which are amplified through and reproduces using chain polymerization process carried out by a specially designed machine call thermo cycler as the name indicate that cycles are highly temperature dependent.

It is the most using equipment that is used in many diagnostic laboratories for disease identification and it was first invented by Kerry Mullis in 1991 (Bartlett and Stirling, 2003). This PCR have wide range of applications in all type of laboratories like chemistry, biology and laborites related to diagnostic (Saiki *et al.*, 1985).

This figure totally explains the whole processes that actually take place in side thermo cycler (Rychlik *et al.*, 1990).

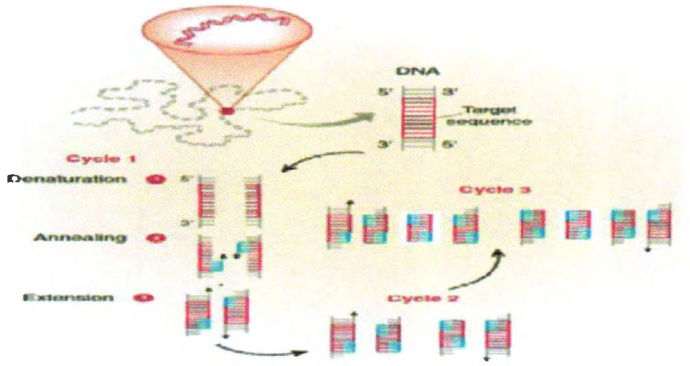


Fig.3.2. Figure showing PCR operation Cycles wise (Garibyan and Avashida., 2014)

3.13.1 Initialization step:

This step involves some pre-processing adjustment of the component of PCR master mix like enzymes, dNTPs etc. the most important is the temperature stabilization for Taq polymerase which can work more efficiently at high temperature because they are highly temperature dependent and can with stand at temperature of 94–96 °C (or 98 °C depending upon from the species it is extracted and this lasts for 1-9 minutes.

3.13.2 Denaturation step:

To increases number of copies of DNA/gene PCR is the biological method used to produces enormous copies number but it involves to open the ladder like structure of DNA biomolecule to create an active sites/ or exposing of bonding site for the precursor molecule for which the temperature is maintained upto 94–98 °C for 20–30 seconds. The reason behind rising temperature is to break the H bonging between two strands to make it sure for the next coming step of increasing chain through addition of nucleotides.

3.13.3 Annealing step:

After the first step the process proceeds for second important step in this occurs two different strands of DNA to form an attachment through hydrogen bond. The specific conditions include temperature from 50–65 °C and this step lasts for 20-40 seconds nearly. In the process there is an attachment of primer and the newly growing DNA strand to form a long chain of nucleotides and after which it form an another antiparallel strand which is running in opposite direction to that of the previous template strand. Perfect elongation can only be achieved if there is n excellent attachment of the primer to the target site of the fragment. The method for annealing temperature calculation is to calculate Tm and decrease the elongation temperature 2-3 °C. Double strand DNA can only be formed when there is an exact and highly specific binding sites in template strand to the precursor primer so that it may ensure the expedition of the process of nucleotides polymerization process and the most important and critical step is to allocate exact temperature for elongation process. The sensitivity to temperature may affect your results by irrespective biomolecules binding.

3.13.4 Extension/elongation step:

There are many steps DNA/sample to go through which it is placed in thermo cycler for amplification, in the early to steps it get adjusted to the inside conditions because the things in master mix is mostly required high optimized conditions that of temperature, time and cycles etc. The temperature at which taq polymerase can give maximum performance regarding polymerization of nucleotides is 75–80 °C (Chien *et al.*, 1976). Some changes that take place during this extension process is thee attachments of many nucleotide to the strand of the DNA that is anti-parallel to the strand which was used as precursor and dNTPs are annexed to the receiving site of DNA strand that is 5' to 3' direction.

Chemically the attachment of dNTPs involves the bonding between dNTPs and newly formed DNA strands are between OH-PO^{-4} groups and there are ten hundred nitrogenous bases combine. This whole process of extension is different component dependent such is the template DNA strand to amplify and also other conditions of temperature and activity of Taq polymerase.

Under fully optimized condition that are considered to be favorable for the DNA elongation then the DNA are twice is of the template but proper substrate and enzyme attachment which contributes to numbers of copies of DNA formed from the precursor fragments (template).

Polymerase chain reaction is not a single step process but is composed of certain steps in which every step is time and temperature dependent which are calculated using certain mathematics calculations.

3.13.5 Final elongation:

In PCR process, elongation is an important step which involves the attachment of a single nucleotide to the template one by one in the presence of chain polymerization specific enzyme called Taq polymerase which was first time extracted from bacteria which can with stand a high temperature.

The optimal temperature to be maintained upto 70–74 °C in which taq can give maximum performance. This cycle is carried out to make it sure that the template strand of DNA is processing it's from elongation and it is lasted for 5-15 minutes.

3.13.6 Final hold:

Final hold refers to the step in which end process in which the samples are given some time to complete and to end up with a reaction and temperature to be maintained at 4–15 °C.

3.14 Polyacrylamide gel electrophoresis:

There are certain methods which are used for the analysis of pcr products and these techniques are polyacrylamide gel, also 1% agarose and direct sequence analysis the samples that were amplified through PCR. But working on human molecular genetics researcher normally used polyacrylamide gel for the scrutiny of PCR samples exploration.

This type of gel work on certain principles due to which they isolate and resolve molecules. PAGE works on the principle that it separate on the bases of charge and mass ratio. There are some special components which are used in these gel pages to help the study of biomolecules easy like some coloring agents which is bromophenol blue and other components are having the denaturants.

Molecules to study on these pages are having complex three D structure which cannot move down it so the function of denaturant is to diminish their complex bonding to make it easy to move down it.

In some of the biomolecules study, SDS is the most commonly using component which have the function of linearization of the protein molecules. SDS binds to protein molecules and arrange them charge to mass ratio due to which protein molecules are fractioned into least size.

This principle is not easy to apply to some special group of protein which is hydrophobic which cannot be hydrolyzed in water. These proteins interact with membrane of the cell and also to that surfactant which are present in round about environment *(* Rath *et al.,2009).*

To study the genetic analysis of the families with disorders homozygosity, mapping is necessary which is mostly done with polyacrylamide gel. The composition of gel varies from work to work.

Polyacrylamide gel that is used to investigate samples is denaturing gel. Certain other component are including of 10X tris base EDTA. Solution is formed in the natural solvent that is distilled water is added upto 31 milliliter. Some other components such is TEMED which is added in small amount that is upto 27 µl and also the most important component APS is added upto 400 micro-litters.

The total solution is maintained upto 100 ml and overall concentration of the polyacrylamide gel is 8 percent. When the solution was made than it is poured in the gel specially minted glass plate and were then solidified for 30 minutes and the most important is the comb placement for the formation of well for sample loading.

Vertical gel is used for the sample loading which is made by Whatman Biometra (model V 16-2). The process discussed is the stationary phase and after the adjustment of the glass plate 1X TBE is added is mobile phase to facilitate the movements of molecules and charges from produced due to positive and negative terminal of the voltage given upto 120V for approximately 2:30-3:00 hours.

After completion of this process next is the observation of samples under gel documentation system and for these samples is kept in staining dye which is used because it impart glowing color to the gel bands. It is composed up of 25 µl PCR sample and also loading dye is added in small amount 5µl and gel was kept for 2-3 minutes. Finally it is observed in the gel documentation system under ultraviolet radiations.

3.14.1 Observation of PCR Product:

The PCR products that were run on polyacrylamide gel and after the completion of Gel the product were observed under gel documentation system but no linkage was observed.

Fig: 3.3 Two different markers results observed under UV in gel Doc System

Table 3.1 Microsatellite markers used for screening candidate gene (*GHRHR*)

Candidate genes	**Chromosomal location**	**markers**	**Sequence**	**cM**
(GHRHR)	**7p14.3**	D7S484 F	AACGGCTGTGTCAGGGGACAG	53.72
		D7S484 R	CAGAGGCCAGAGGGTCTCAG	
		D7S817 F	GACACCCAAATGGCTTGGCTCAT	50.85
		D7S817 R	GCCACTTCCAGATGAAAGCACCT	
		D7S2491 F	AGAGGGAAGGAGTTGTGGCTAGAG	48.65
		D7S2491 R	GTCCATGGCAGTCCTTCCTCCAA	
		D7S2252 F	TCCAAAGGCCCAGAAGCTGA	50.85
		D7S2252 R	TGGGAAATAAGGAGCCAA	
		D7S435 F	CAGAATCCCCTCTCCCTGCTT	81.86
		D7S435 R	CTGCCCAGCCCCTTCACCCATG	
		D7S2496	TGGGTGTCCCAGCTCTGAAGCAC	47.04
		D7S2496	TTTACCTCCCCATGGTGCCCAA	
		D7S510	TGGGTGTCCCAGCTCTGAAGCAC	59.75
		D7S510	TTTACCTCCCCATGGTGCCCAA	
		D7S632 F	AGATCTCAGAGTCAAGGATGCAGA	48.65
		D7S632R	TCCACTCCACACCCCATGTAGGA	
		D7S526 F	AGGTGCCTGCGTCACCACAGTGA	49.27
		D7S526 R	CAGTAGTTCCAGGGAAGTTGACT	

RESULTS

4. RESULTS

4.1 Description to Collected Families of Dwarfism:

4.1.1 Family A:

Family A of dwarfism was collected from village Ganderi Khattak, Tehsil Takht-e-Nusrati, Distract Karak, Province Khyber Pakhtunkhwa (KPK), Pakistan. Generation to generation pedigree was scripted upto four generations on the bases of information obtained from investigations and discussion with the notable of the family. Pedigree analysis suggests and clearly shows that this disorder that is dwarfism is autosomal recessive in its mode of inheritance. This family consists of seven members including two females and three affected males. All affected males were investigated for the disease but investigation show that neither it is because of environmental conditions nor because of other syndrome hence it is non syndromic. Blood was collected from all individuals. The medical reports shows that individuals were only having short stature (dwarf).

Fig 4.1: Figure 4.1 showing individual with dwarfism.

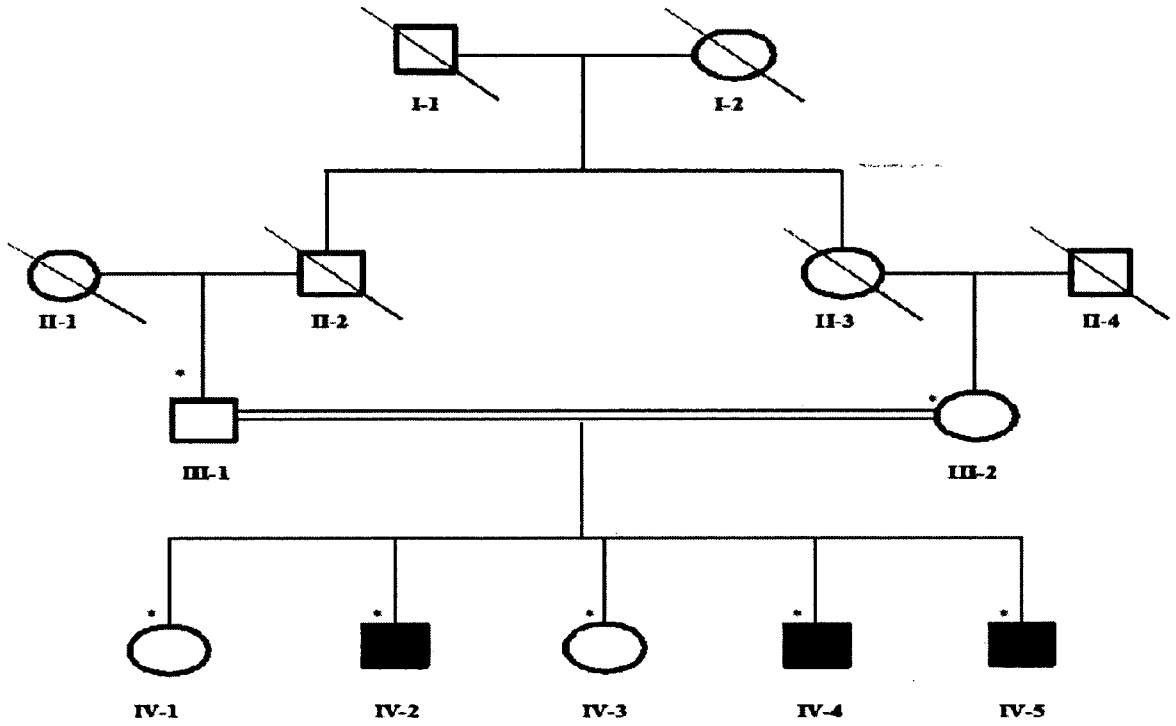


Fig 4.2: Pedigree of Family A having three dwarfism affected individuals

Family A of the collected two families show autosomal recessive genetic mode of dwarfism. Squares and circles show male and female respectively while filled squares and circles shows affected individuals. Two adjacent double lines show consanguineous marriage and cross diagonal line on circles or squares shows demised person. The roman numbers indicates the generation number of the individuals in the pedigree while that of an Arabic numbers indicates position of the individuals in the respective family.

4.1.2 Family B:

Family B of this disorder was collected from Ahmad abad, Tehsil and distracts Karak, Province of Khyber Pakhtunkhwa, Pakistan. The pedigree of the family shows that it is consanguineous marriage and this disease was not present in the first three generation and it appeared in the fourth generation of the family. This pedigree shows that this is an autosomal recessive dwarfism case because there is no affected person in the first three generations. The affected individuals were born of normal and unaffected parents. This disease is not because of harsh environmental conditions not because of any other syndrome. The medicals reports of the affected individuals' shows that they are hypo-pituatrized which means the reason for their low height may be the slow and steady function of pituitary glands. The blood samples were collected from all individuals of the generation fourth which is indicated by star about the individuals symbols.

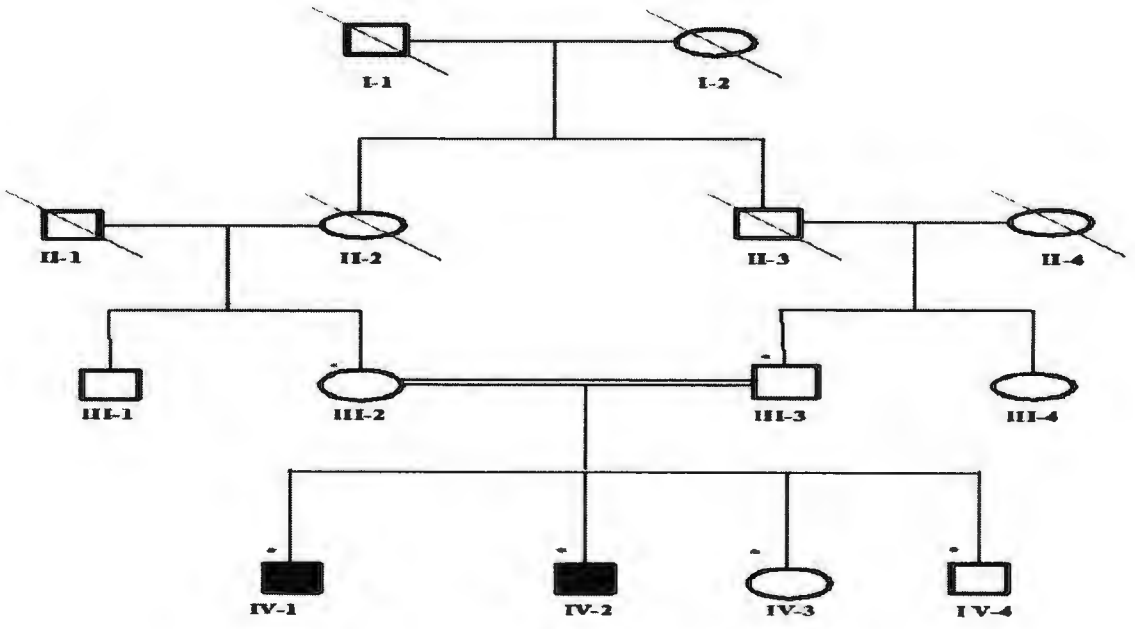


Fig 4.3: Pedigree of dwarfism affected individuals of village Ahmadabad distract Karak Khyber pakhtunkhwa, Pakistan.

4.1.3 Analysis of selected gene using micro-satellite marker for the proposed gene:

To identify mutation in the selected gene which is suggested to be a cause of dwarfism linkage analysis of that gene is necessary prior to scanning whole genome of these individuals. This study includes two families A, and B, which were examined for the linkage to the locus of the gene that is considered as a cause of this disease that is *GHRHR* gene (7p14.3). The table 3 clearly shows the summery of all the micro-satellite which were used for the known locus the particular gene. Analysis of the micro-satellite marker was carried out by using PCR reaction according to standard scientific protocol. These PCR products were observed on polyacrylamide gel having concentration up to 8%. Markers were stained with ethadium bromide having function to track the product till the end of the gel and were observed under UV documentation system and photos were taken through computerized camera attached to UV gel documentation system. To investigate mutation the proposed gene was scanned through linkage analysis and the banding pattern was studied to know wither the family is linked or excluded. If the normal individuals showing heterozygous pattern of banding and those of affected shows homozygous pattern then the particular family is considered linked to the micro-satellite markers that were used. Banding pattern of family A for the suspected gene locus was observed on micro satellite markers for the growth hormone releasing hormone receptor *(GHRHR)* gene, which shows that all the individuals of the family shows same banding pattern of all marker which were used for the gene. On the bases of all these examination of the bands of normal and affected individuals, heterozygous and homozygous respectively for micro satellite markers of the suspected loci which indicates that no linkage was established and both the families were excluded.

Table 4.1 Banding pattern of PCR product of GHRHR gene markers of family A.

Family A: *GHRHR* Gene

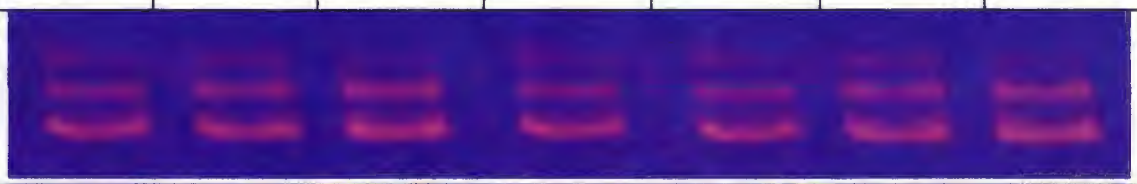
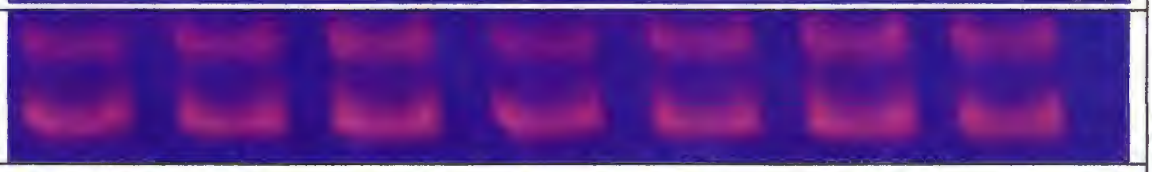
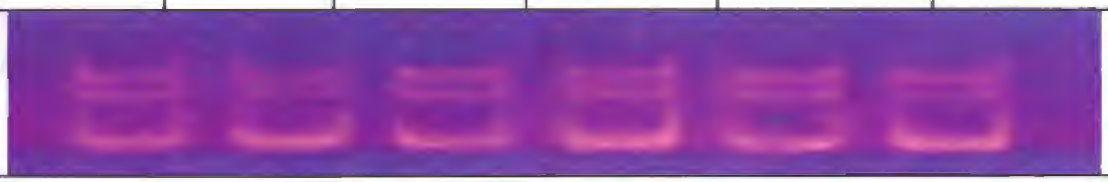
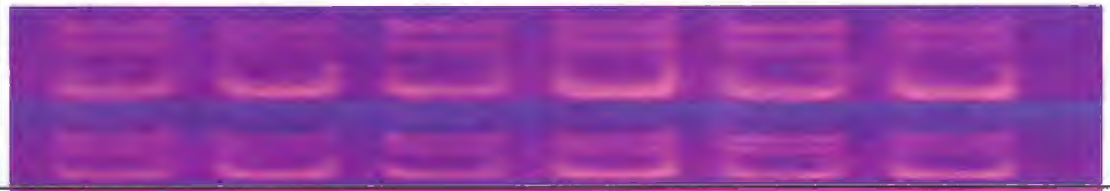
Markers	Map Unit (cM)	Normal 1	Normal 2	Normal 3	Normal 4	Affected 1	Affected 2	Affected 3
D7S2491	48.65							
D7S2496	47.04							
D7S435	46.59							
D7S484	53.72							
D7S817	50.85							
D7S2252	50.85							

Table 4.2 Banding pattern of PCR product of GHRHR gene markers of family B

Family B: *GHRHR* Gene

Markers	Map Unit (cM)	Normal 1	Normal 2	Abnormal 1	Abnormal 2	Normal 4	Normal 6
D7S2491	48.65						
D7S2496	47.04						
D7S435	46.59						
D7S484	53.72						
D7S817	50.85						
D7S2252	50.85						

DISCUSSION

5. DISCUSSIONS

Dwarfism is considered one of the most recognized congenital defects of animals and humans and can be hereditary or sporadic in cause and expression. The mode of inheritance in this genetic disorder may be autosomal recessive and autosomal dominant means it can be of both type X-linked and Y- linked. There are two general morphologic categories within this vastly diverse disease. Among humans, over 200 known distinct types of dwarfism have been reported, each with a different underlying cause. The approximate worldwide population of dwarfism numbers 190,000 in humans.

Causes of dwarfisms in humans can involve genes responsible for hormone production and/or recognition, metabolism, cartilage development, pituitary gland hormones, thyroid gland hormones, and bone growth plate development. The most common type of 9 dwarfism in humans is Achondroplasia, which affects about 1 in every 40,000 children worldwide. This number varies widely depending on the country. Eighty percent of all human dwarfisms are Achondroplasia. This disproportionate dwarfism involves mostly the shortening of the limbs with an averaged sized trunk, enlarged head with a prominent forehead with other lesser skeletal malformations. Achondroplasia is caused by a single base substitution of one of two different bases G → A or G → C, resulting in an amino acid change from arginine to glycine (Shiang *et al.*, 1994).

Stature in all organisms is the additive interaction of dozens if not hundreds of genes within an individual that determines its ultimate mature height. This polygenic trait is primarily due to genetics (80 – 90 %) and only slightly affected by other environmental factors. This mature height has been shown to be quite variable within certain isolated breeding populations of animal species including humans. Genome-wide association studies (GWAS) using SNP (Single Nucleotide Polymorphism) analyses have implicated numerous chromosomal sites involving genes that regulate to some degree mature height in humans (Allen *et al.*, 2010).

There are many genes that are involved in body growth process which are then further grouped into three different groups on the bases of their functions. These groups include skeletal development, biological processes that are segregation and mitosis and the third group is cartilage composition (Gudbjartsson *et al.*, 2008). One of the study carried out in the institute of

Korea identified other fifteen loci on the genes which are responsible height in human (Kim *et al.*, 2010).

Recent research study was designed to study an autosomal recessive dwarfism in affected families of Distract Karak belonging to consanguineous families. Blood samples were taken from both affected and normal individuals and DNA was isolated to proceed for further research study. In both the families selected gene was screened by using micro satellite marker for the GHRHR gene to check linkage analysis. Family A was screened for the gene by using 9-10 markers such as D7S2491, D7S2496, and D7S434 etc. To find out genetic defect in this family homozygosity mapping was done by genotyping micro satellite markers that were corresponding to the proposed gene involved dwarfism. Approximately 9 micro satellite markers were genotyped in all individuals of the respective families.

In family A all microsatellite markers were tested and genotyped but no linked was established for the proposed gene in all 7 individuals and the family was considered excluded for the proposed gene and no linked was established which means that proposed dwarfism may not be because of mutation in this gene.

In family B there were 6 individuals in whom two were having phenotype of dwarfism and all the other 5 individuals were normal and this was also familial autosomal recessive disorder. All the individuals were genotyped for approximately 9 microsatellite markers of GHRHR gene in this family.

There was no linked observed for the locus of that proposed gene in all members of the family so it was concluded from the recent study that family B also might have no mutation of GHRHR gene which was a proposed reason of dwarfism in the families of Distract Karak, Khyber pakhtunkhwa, Pakistan. In contrast to recent study individual with dwarfism in Sindh are reported by (Baumann and Maheshwari, 1997) with GHRHR mutation gene.

In the study a nonsense mutation in GHRHR gene is mention in exon 3 which produces a truncated protein product GAG –TAG that replace Glu^{50}-stop codon due to missense mutation (Baumann and Maheshwari,1997).

A new mutation in GHRHR gene is also reported in individuals with dwarfism in the two families Israeli-Arab by Haskin. Haskin reported that there was mutation in exon 11 of GHGHR gene in 11 patients and was C-T transversion CGC-TGC and due to this mutation Acil restriction

site is removed as a result of this mutation, which also contradicts recent study (Haskin *et al.*, 2006). A novel mutation called an indel mutation that refers to insertion, deletion mutation in the GHRHR gene in sequence *(AGAGATCCA)* is reported by Kale *et al.*,(2014).

The novel indel has resulted in the loss of 5′ regulatory region and exon 1 of the *GHRH-R* gene impairing the *GHRH-R* expression (Kale *et al.*,2014). Different approaches are used during research nationally and internationally, in which some direct and indirect approaches. In human molecular genetics there is also the same approaches used to identify genetic disorders, analyzing/screening the mutated gene through linkage analysis and the direct one is detection of mutation of the gene using microarray and whole genome sequencing. This study was designed for the purpose to identify diseased phenotype at molecular level and also interaction of genotype with associated phenotype because dwarfism is becoming a lethal genetic disorder in the particular distract which has affected more than 40 families in this distract which has negative impact on society both socially and biologically.

CONCLUSION

This study of research was designed to find out the biological and genetic changes that are affecting body growth or height. The effect of gene on the body growth was studied in two of the families of distract Karak Khyber pakhtunkhwa who were having individuals with dwarfism. A selected gene GHRHR was investigated for its role in these individuals growth which was reported previously by many researchers that it have a prominent role in body size extension although this research results shows a wide range of differences compared to other scientist because no linkage was found when the gene was amplified using respective primers. So this study conclusively declares that the reason to dwarfism in distract Karak individuals is not because of GHRHR gene alteration and suggesting some other genes study may involve in the maintenance of body growth.

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APPENDICES

Appendix List 1

Chemical Name	Function	Amount
MgCl2	Work as co factor for polymerase	2.3 µl/sample
PCR Buffer	Maintains pH	2.5 µl/sample
Primers	Help in initialization of polymerization	0.6 µl/sample
Taq Polymerase	Catalysis the process of polymerization	0.3 µl/sample
PCR Water	Used as standard solvent	17.5 µl/sample
DNA	Used as template	1 µl/sample
dNTPs	Used as a precursor	0.5 µl/sample
Total Volume	----------	25 µl/reaction

Appendix List 2

Name	Function	Amount
dATP	Precursor	10 µl
dTTP	Polymerization unit	10 µl
dCTP	----------	10 µl
dGTP	----------	10 µl
PCR water	----------	60 µl
Total Volume	----------	100 µl