Molecular Genetic Analysis of Alopecia Families from Southern Punjab

By

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Department of Bioinformatics & Biotechnology

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Genetic screening
Genetics of Alopecia
Hair disorder
DNA

Molecular Genetic Analysis of Alopecia Families from Southern Punjab

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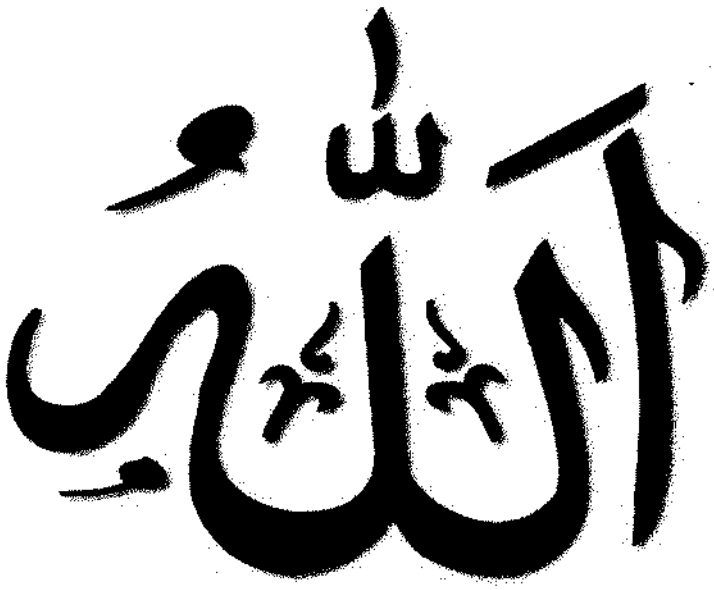

THE MOST MERCIFUL,

THE MOST BENIFICENT

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Dated: 21-05-2015

FINAL APPROVAL

It is certificate that we have read the thesis submitted by Mr. Muhammad Ilyas and it is our Judgment that this project is of sufficient standard to warrant its acceptance by the International Islamic University, Islamabad for the M.S Degree in Biotechnology.

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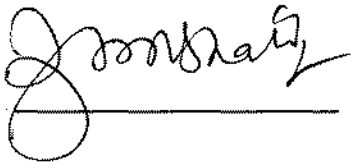

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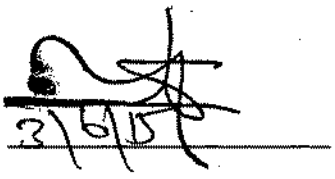

A thesis submitted to Department of Bioinformatics & Biotechnology,
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as a partial fulfilment of requirement for the award of

the degree of MS Biotechnology.

DEDICATION

This thesis is dedicated to my beloved parents whose hands always rise for prayers for my success. Their prayers always helped me in coming out from the complex situations.

DECLARATION

I hereby declare that the work present 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.

Date 21-05-2015

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LIST OF ABBREVIATION

AA	Alopecia Areata
AGA	Androgentic Alopecia
AH	Autosomal Recessive Hypotrichosis
APL	Atrichia with Papular Lesions
APMR	Alopecia with Mental Retardation
APS	Ammonium per sulphate
AR	Androgentic Receptor
AU	Alopecia Universalis
APL	Atrichia with papular lesions
BOS	Board of Study
bp	Base Pair
^0C	Centigrade
CA	Congential Atrichia
CDH	Cadherin
cDNA	Complementary deoxyribonucleic acid
cm	Centimeter
cM	Centimorgan
DNA	Deoxyribo nucleic acid
DSG	Desmoglein
dNTP	di-nucleotide tri phosphate
EDTA	Ethylenediamine Tetra Acetic Acid
HF	Hair Follicle
HGMD	Hypotrichosis with Juvenile Macular Dystrophy
HNP	Human nude phenotype
HR	Hair less
IRB	Institutional review board
KB	Kilo base pair
LHA	Localized Autosmal Recessive Hypotrichosis
LIPH	Lipase H

mg	Mili gram
ml	Mili litter
mM	Mili molar
MR	Mental Retardation
MUHH	Marie Unna Hereditary Hypotrichosis
NCBI	National Center for Biotechnology Informatio
ng	Nano gram
NS	Netherton syndrome
OMIM	Online Mendalian Inhertance in Men
PCR	Polymerase Chain Reaction
PK	Proteinase Kinase
RPM	Round per mints
PAGE	Polyacraylamide gel electrophoresis
SNP	Single nucleotide polymorphism
STR	Microsatellite markers
TE	Tris-EDTA
TEMED	Tetra methylethylene diamine
µM	Micro meter
µL	Micro litter

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ABSTRACT

Alopecia is condition in which the hair can be lost in the form of oval patches, mostly on the scalp and in a certain section of the male beards. Genetic disorder that effect human hair growth. Alopecia disorders have been elaborated clinically but at molecular level Alopecia not fully understood yet. Different forms of inherited human hair loss collectively recognize as Alopecia. The disease can be rapidly spread, and the progress of the disease can be reached in a stage where all the hair is lost from the entire scalps this condition is known as Alopecia Areata or some time all the hair can be lost from whole body normally is called Alopecia Universalis. Multiple genes responsible for Alopecia disorder that effect human hair growth cycle.

In present study, three families (1Ap, 4AP, 3AP) have been selected from southern Punjab. The disease inheritance pattern for families 1Ap and 4AP has been autosomal recessive while X-linked in family 3AP. Candidate gene approach strategy was used for this study. Linkage analysis was performed by PCR using the STR (short tandem repeats) microsatellite markers for the known loci involved in disease. In the present study, a novel homozygous missene mutation in human hairless gene has been found in 1AP family. Sequence analysis revealed a substitution of G to A at nucleotide position 664 (c.664G>A) in exon 3 of the hairless gene (HR). In family 4AP, sequencing of the HR gene showed a reported nonsense mutation (c.2818C>T) in exon 13. Mutation created a stop codon by substitution of T at C position of the arginine (CGA) codon and resulted in premature termination as a result of p.Arg940X. Identification of novel mutation in family 1Ap will help to further understand the role of HR gene causing the disease.

INTRODUCTION

In recent decades our understanding of the genetics of hereditary disease has made tremendous advancement particularly in term of our understanding at molecular level of gene expression. Concisely gene is part of deoxyribonucleic acid (DNA) encoded on a chromosome. Different genes contain specific set of instruction to code a particular protein. Human have 23 pair of chromosomes out of 23 pair of chromosome, 22 paired is called somatic cell chromosome. These paired chromosomes encode thousand of genes in human genome. Each pair of chromosome contains multiple genes. These genes encode genetic variation in the human body traits such as eye color, height and intelligence among normal people. These genetic changes also help to face the environmental challenges including those that produce diseases in human body. Eventually all genetic diseases can be contemplated to result from interaction between genetic makeup and the environment (Scrimgeour, 2009).

Mutation in single gene is called single gene disorder or monogenic disorder. Single gene disorder shows three different pattern of inheritance. In autosomal dominant manner a single mutated allele inherits from parents to offspring will induce a disease be expressed in heterozygous pattern. Example includes familial hypercholesterolemia and Marfan syndrome. In autosomal recessive condition each parents inherits one mutated allele to the patient and disease will be expressed in homozygous manner. Autosomal recessive condition is more common in those populations when consanguineous marriages occur frequently for example sickle cell anemia. When mutation occurs in X-chromosome gene then genetic disorder that link with X-chromosome is called X-linked disorders. Inheritance pattern of X-linked dominant disorder is very similar with autosomal dominant disorder inheritance pattern but in X-linked dominant disorder female are more affected than male. X-linked dominant disorder is very rare disorder. X-linked recessive disorder is very common in male for example Hemophilia is x-link recessive disorder (Scrimgeour, 2009).

1.1 Skin

Skin is the largest organ of our body and its surroundings. Human skin consists of three layers. These layers are epidermis, dermis and sub cutis (Figure: 1.1). The outer layer, epidermis is composed of epithelial cells and keratinocyte cells along with pigment and melanin producing melanocytes (Khavari, 2006). Between dermis and epidermis there is a

membrane consisting of thin layer of proteins which mediates anchorage of basal keratinocyte. After migration from lower basal layer, keratinocyte differentiate and move into upper layer where they are shed off and continuously replace by new cells from lower basal layer which consists of stem cells and basal keratinocyte. These stem cells and basal keratinocyte proliferate continuously to contribute in epidermal repair and hair regeneration (Braun and Prowse, 2006; Fuchs, 2007).

Following to epidermis there is a dermis which is thicker than epidermis and is divided into outer thin papillary and inner thick reticular dermis. Dermis consists of matrix of collagen fiber which provides support, elastin for flexibility, vascular network for nutrition and nerve processes for cutaneous sensation. Macrophages, mast cells and fibroblasts are also present in dermis. Specialized skin appendages like hair follicle, sweat glands, and sebaceous glands originate from dermis. Subcutis is the deepest layer and consists of fat cells (Junqueira and Carneiro, 2003; Ross *et al.*, 2000).

1.1.1 Hair Formation and its Structure

The hairs are composed of strong structural proteins called keratin and each strand of hair consists of three layers (Figure. 1.2). Cuticle the outermost layer work as a protective scale and overlapping keratin cell, cortex contain natural pigment melanin and moisture, medulla is the inner layer that only present in thick hair. Hairs are presents on all body parts except on thick skin of the palms and soles the side of the fingers and toes. Hair composition is a complex two step process involves many genes and mesenchymal-epithelial interaction. Firstly structure is made up with mesoderm cell sited below an epidermal imagination (hair plug) are insistent. Secondly the dermal papilla stimulates the epithelial derive matrix cell into the hair plug. These cells start dividing swiftly and differentiated into inner root sheath cell and hair shafts cell but it depends on their site to hair follicle (Haerdy, 1992). Those cells that are not near from the cells that are going to under cell division and start to differentiate forming the structure making up the mature hair. Keratin protein that makes the hair is also formed the outer layer of skin and nails. Hair grows 3-5 mm under the surface of the skin, the hair follicles (Figure. 1.1). Matrix cells which are germination center presented near the papilla. Dermal papilla control matrix cell and is fed by blood to produce new hair. This is important for hair growth because it have the receptors for androgen which responsible for hair growth regulation (Powell and Rogers, 1997). The bulge made up of bunch of different

cells in outer root sheath that are founded near the insertion of arrector of the pilli muscle (Figure. 1.2a). Each hair reaches the skin surface in an oily substance sebum, secreted by sebaceous glands, present in the upper parts of the hair follicle. The sweat glands present in the dermis, wet the hair shaft (Pragst *et al.*, 1998; Pecoraro and Astore, 1990). Melanocytes is the responsible for hair color presented at the ground layer in association with the basal layer (Figure. 1.2b) Keratin accounts 30% of the cellular protein of the living cells and 85% of the cellular proteins of the dead cells. Keratin further divided into two group's hard keratin protein and soft keratin protein (Marshall *et al.*, 1991).

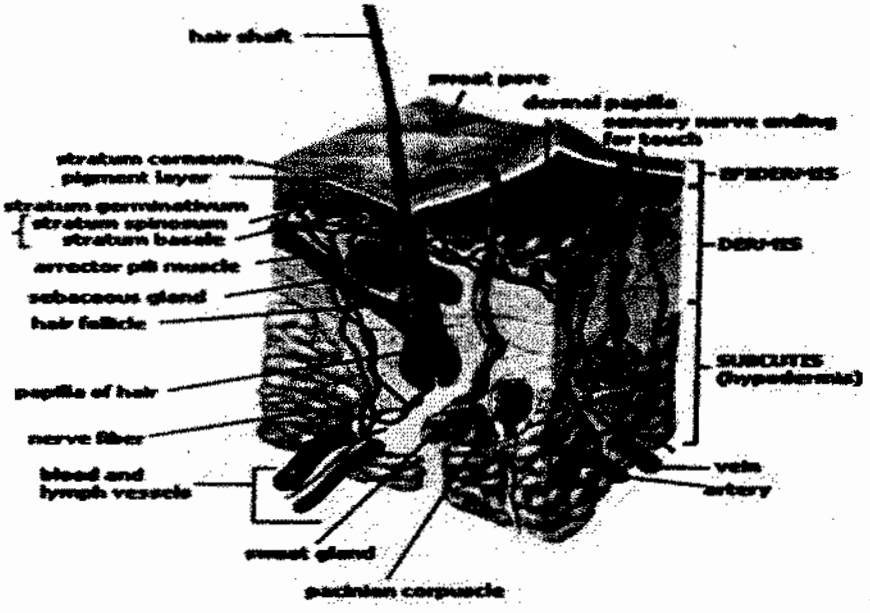


Figure1.1: The Skin showing main structures in epidermis (Anne and Allison, 2004).

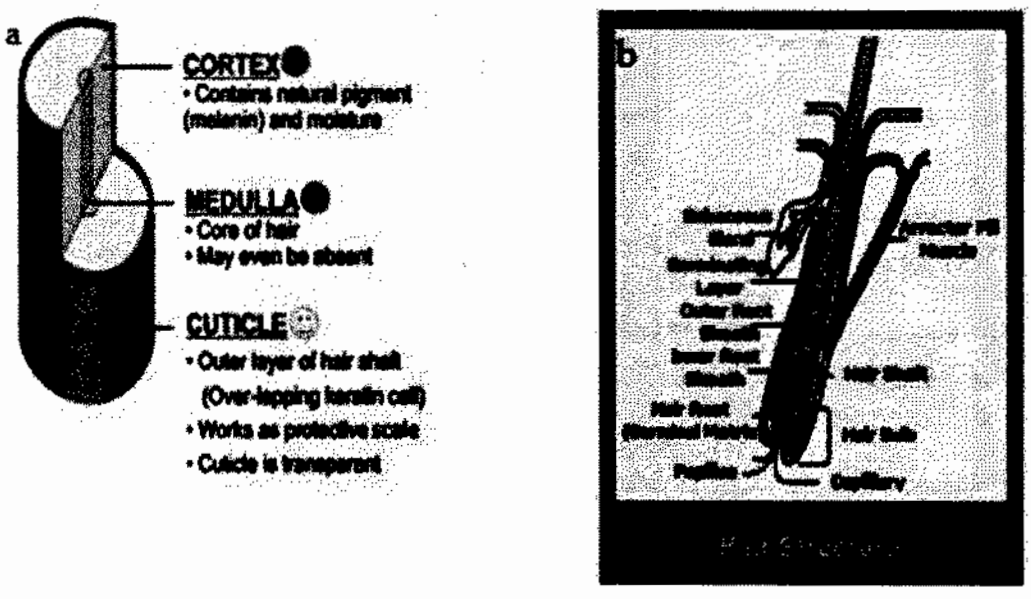


Figure 1.2(a): Three strands of hairs cortex, medulla and cuticle. (b) Formation of hair follicle from matrix cell on the basement membrane of the hair shaft (Pragst and Balikova, 2006).

1.1.2 Hair Follicle and Growth Cycle

The hair follicles are a complex structure, formed of several concentric cylinder root sheath which warped the hair shaft (Sperling, 1991). It is the organs in the mammalian organism that under goes rapid growth. Hair follicle consists of epithelial and mesenchymal parts and varies significantly in shape and size, depending on their location but they have basic structure. Hair follicle formation occur during embryo formation and depends upon signal sent between epidermal cell and epithelial cell that change in cell population, finally hair shaft, dermal papilla and root sheaths differentiated (Haerdy, 1992). The first signal derives from dermas instruct the overlying epidermal to thicken from a placode and then a down growth into derms known as the hair plug; dermal papilla is formed when second signal started from epidermis to dermis. Division of overlying epithelial derived matrix cell into hair plug stimulated by the dermal papilla. These cell divided swiftly and differentiated into inner root sheaths cell are hair shaft cell. Hair follicle has three stages. First stage is called anagen that is growth phase, the second stage is catagen that is involuting phase and the third stage is resting which is called telogen. Most of the information and knowledge derived from the clinical study about the human hair growth, studies and observations in mice which have shows some of the molecular base association with cycling of hair follicle. Anagen is the longest phase which lasts 3 to 10 years with 90% of follicles on a normal scalp of human in this active growth stage. The beginnings of anagen phase recapitulate the development of hair follicle for the development of new lower hair follicle starts with the propagation of secondary germ cell in the bulge. The hair enters into a catagen phase after the anagen stage which lasts about one week. This is the transitional stage that hair follicle shrinks and detaches from the dermal papilla. Hair follicle goes through controlled progression of infolding during the catagen phase. In this stage a programmed cell death occur that is called apoptosis in the majority of follicular keratinocytes. The telogen phase pursues the catagen stage. The hair does not grow but stays attached to the follicle during this time and the dermal papilla remains in the resting phase. The hair shaft mature into a club hair that is finally shed from the follicle during exogen as the new hair grows in. The telogen phase typically lasts for two to three month before the scalp follicle re-enter the anagen stage and the growth cycle is repeated (Figure 1.3) (Cotsarelis, 2006).

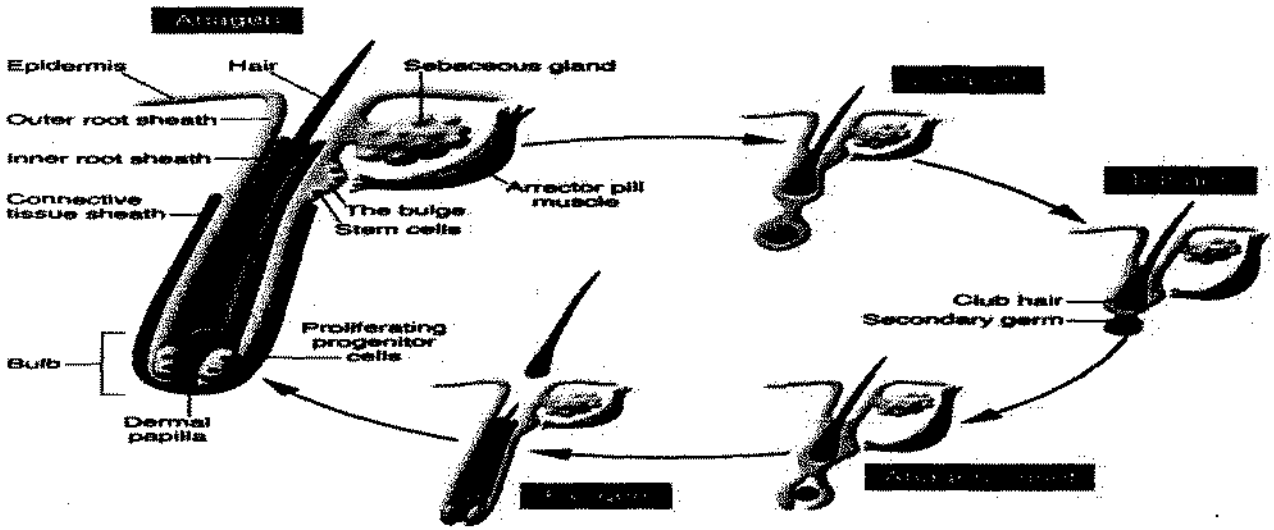


Figure 1.3: Figure shows different stages of hair growth cycle, Anagen is the growth phase Catagen is the involuting phase, Telogen is the resting phase and Exogen is the hair shading phase (Cotserlies, 2006).

1.2 Genetics of Alopecia

There are many forms of hereditary human hair loss collectively called Alopecia, which vary in age of onset, severity and associated ectodermal abnormalities. Hair follicle is responsible for hair growth and hair formation infect all hair disease is actually hair follicle diseases. The hair follicles are composed of concentric layer of different type of keratinocyte and trichocyte and arise as a result of mesenchymal epithelial interaction. It is maintain in a cyclic fashion between regression, growth and quiescence in adulthood mostly because of this continues interaction (Schmidt-Ullrich and Paus, 2005).

Hair loss or Alopecia can result either from a failure to re-grow hair fibers from existing hair follicles, from extra-follicular environmental factors that affect follicular stem cell activity, or alternatively from the loss of HFs themselves (Chueh *et al.*, 2013). Partial or total hair loss occurs either alone or in combination with other abnormality as parts of syndrome of a very different nature. The major dysfunction reported to be associated with partial or total absence of hair, either singly or in diverse combination, including MR (mental retardation), nail dystrophy, impaired sweating, hyperkeratosis, retinas pigmentation,

cataracts, hearing etc (Pinheiru and Freire-Maia, 1985; Wali *et al.,* 2006). Inheritance Pattern of Alopecia is normally autosomal recessive, but autosomal dominanant inheritance pattern also present in families and X-linked recessive are also observed (Ahmad *et al.*, 1993; Anzari *et al.*, 1996; Ahmad *et al.*, 1998; Wali *et al.*, 2007a).

1.2.1 Isolated Alopecia

Molecular genetic analysis of different types of Alopecia in inherited families lead to the detection of novel loci and genes which control hair growth. Genetic conditions that affect human hair growth cycle or hair structure can be isolated as part of complex syndrome. Different genetic defects for isolated types of Alopecia have been identified. Both autosomal dominant and recessive patterns of inheritance have been described in these isolated Alopecia.

1.2.2 Monilethrix

Monilethrix (MIM 158000) is a genetic disorder of the hair shaft inherits as a penetrate condition with changeable expression. Individual those are affected, at birth their hair grows normally, but within short period of time they develop weak and fragile hair and form different type of dystrophic Alopecia. Monilethrix is usually an autosomal dominant defect. However, autosomal recessive transmission has been suggested in some cases (Zlotogorski *et al.*, 2006).

1.2.3 Atrichia with Papular Lesions (APL)

Atrichia with papular lesions (MIM 209500) is a rare form of Alopecia with an autosomal recessive pattern of inheritance. Affected individuals have no hairs on the scalp, axilla and body. Affected scalp skin show the nonappearance of mature hair follicle when histological examination was performed. Several families develop this condition of popular lesions as an extra phenotypic feature. Affected individuals do not show any delay in growth or developmental. They have usual hearing, nails, teeth's, and sweating. Histological examination of scalp skin of the patient shows irregular hair follicle and hair shaft that are atrophic, thin and often appeared coiled up within the skin due to their inability to penetrate into the epidermal layer (Ahmad *et al* 1998; Panteleyev *et al* 1998). Atrichia with papular lesions (APL) is a very common disease but this disease previously misdiagnosed as Alopecia Universalis (AU). In most cases this disease considered as Alopecia Universalis. This misdiagnosis is occurring due to less diagnostics facility available and also there is lack of

awareness about this disease (Zlotogorski *et al*, 2002). Proper diagnostics between AU and APL is of very important. All mutations in APL patients is homozygous mutations but only single affected patient with compound heterozygous mutation had been described in a small non consanguineous family (Henn *et al*, 2002). Case of APL (Atrichia with papular lesions) is more common as three compound heterozygous mutations were also reported in these patients from families without consanguinity marriages (Amy *et al*., 2003). Mutation in HR gene is responsible for APL. Hairless mutation involved in papular atrichia, novel mutation were reported in a study and affected individuals with C-to-T change at different position in the amino acid sequence with homozygous nonsense mutation and the other 2 mutations Q323X (CAG-TAG) in exon three, in exon six there is mutation mutation Q502X (CAG-TAG) in two families and one family had a mutation at R940X (CGA-TGA) in exon fourteen (Kim., *et al* 2007). Sequencing of the HR gene in Pakistani families show homozygous mutation, different families show novel mutation in different exons of the HR gene. In other study two novel mutations in the hair less gene has been descried. These mutation are homozygous 2bp deletion in exon six leading to a frame shift change and a downstream premature termination codon in exon eight (1782-83delAG) and the other mutation was the deletion of a G nucleotide at the exon fifteen-intron 15 boundary that was novel homozygous mutation (Kraemer *et al*., 2008). APL was also founded in Chinese families that have no consanguineous marriages. A study in Chinese families also shows mutation in exon 10 in HR as well as the second novel compound heterozygous mutations. The mutation is a nonsense mutation T 2265 A in the patient and his father as well as a 2bp deletion (3482 deletion CT) in the patient and his mother was detected (Wang *et al*., 2011).

1.2.4 Hereditary Hypotrichosis

Hereditary hypotrichosis is a medical term occurs either alone or with combination of ectodermal as a part of syndrome of diverse nature. Medical term for Varity of phenomena related to thinning of scalp hair without any abnormalities of hair shaft morphology. Hereditary hypotrichosis shows autosomal dominant or show recessive pattern in few cases variability observed in clinical feature such as age of onset severity and involvement of eyebrows and eyelashes.

1.2.5 Autosomal Recessive Hypotrichosis

LAH1 (Autosomal Recessive Hypotrichosis) is a genetic disorder that form Alopecia characterized by thin hair on scalp. Typical hairs to break easily and fragile leaving shorts light scalp hair with characteristic appearance. Histological analysis shows the abnormal hair follicle and shafts. This disease maps within the desmoglein gene complex on chromosome 18q12.1 called as LAH 1 (MIM 607903). This cluster contains four desmoglein genes (DSG1, DSG2, DSG3, and DSG4). The desmoglein genes are cluster within area of 15 kilo base pair in the following order desmoglein gene complex one, desmoglein gene complex four, desmoglein gene complex three and desmoglein gene complex two. It is significant in anchor the telogen hair to outer root sheath of hair follicle and demonstrates that desmosomes are significant in regular function and structure of hair (Kljuic *et al* 2003). LAH2, MIM 609167 was located on chromosome 3q26.33-q27.3 in Pakistani family with consanguineous marriage. This genetic disease is called autosomal recessive hypotrichosis (Aslam *et al.*, 2004). Sparse hair was present on the scalp in the affected individuals at birth but did not re-grow. All affected individuals were normal eyelashes, eyebrows, axillary hair, and body hair. Woolly hairs on scalp were seen in affected individuals. Nails and teeth were normal in all affected individuals. Male patients of the family had normal beard hair but they have no hair on their leg and arm. Another locus for (LAH3, MIM 611452) was mapped on chromosome 13q14.11-q2l.32 with autosomal recessive form of hypotrichosis. The patient shows typical skin of the genetics of hypotrichosis. LAH3 has been shown into the result from the mutation in G-protein coupled receptor (*P2RY5*, MIM 609239) gene and also designated as lysophosphatidic acid receptor 6 (*LPAR6*) (Tariq *et al.*, 2009).

1.2.6 Marie Unna Hereditary Hypotrichosis (MUHH)

Marie Unna hereditary hypotrichosis (MUHH; MIM 146550) was first reported in 1925 by the German dermatologist Marie Unna. MUHH is autosomal dominant hair loss disorder characterized by abnormal hair at birth, with progressive hair loss in adults. Affected individuals with this disorder are very little or no hair at birth. In early childhood thin and twisted hair developed on the scalp and these hairs shaded first few year of life. Alopecia is formed when the hair is lost from the scalp but the loss of hair varies, sometimes hair lost in the form of patches and some time hair lost and it developed into complete baldness. Axillary, and body hair as well as beard hair in men are sparse or absent. Genome wide scanning in several families mapped the MUHH gene to chromosome 8p2l (Van *et al.*, 1999). Human

hairless (HR) gene is responsible congenital atrichiain in which the whole coding region and intron splice junction of the hair less gene and nine other genes present in this region were sequenced. All of these genes were excluded for mutational analysis. The MUHH gene was mapped to a 1.1 cM region which flank by D8S282 and D8S1839 and these result provided confirmation for the presence of a gene different from HR gene in chromosomal region 8p21. This study played a significant role in the understanding of hair follicles mechanism (He *et al.*, 2004).

1.3 Complex Hair Disorders

These are genetic disorder with marked variation in the disease phenotype among affected individual. The most common complex disorder is Alopecia Areata and Androgenetic Alopecia, Alopecia Universal and Alopecia Totalis.

1.3.1 Alopecia Areata

Alopecia Areata (AA) is a common genetic disease. Alopecia Areata is equally prevalent among all ethnicities. The female to male ratio is 1 to 1 until adolescence when the disease becomes more common in females. Alopecia Areata is caused by a combination of genetic susceptibility and environmental triggers. Alopecia Areata is most common in families with different diseases of autoimmune system such as thyroid disease, vitiligo, and rheumatoid arthritis. One recent study has shown the pattern of inheritance is constant with a polygenetic additive model like to vitiligo. Alopecia Areata with autoimmune basis has been supported by connection with multiple HLA antigens. These antigens are HLA-DR4, HLA-DR5, and HLA-DQ3. The immune process in AA is a type of antibody mediated cellular immune system, this scheme supported by the "swarm of bees" emergence of lymphocyte near the hair follicle seen on biopsy. Morphologic analysis of hair follicles in AA suggests that degeneration of precortical keratinocyte and melanocyte of the hair bulb are the targets of immune attack (Hawit *et al.*, 2008). Genome wide study of Alopecia Areata shows 139 SNPs associated with disease. These associations show several genes controlling the activation of T cells and also found association for region containing gene expressed in the hair follicles. It proved evidence that both the immunity involved in the pathogenesis of Alopecia Areata (Petukhova *et al.*, 2010). A study on Turkish people that affected with Alopecia Areata shows that MTHFR gene C677T mutation may be showing effect on the risk of Alopecia Areata. It

is the first study reported association between Methylene-tetrahydrofolate reductase (MTHFR C677T) genotype and Alopecia Areata (Kalkan *et al.*, 2013).

1.3.2 Androgenic Alopecia (AGA)

AGA is male pattern baldness; it is most common type of thinning of hair in males. It is also called Androgenetic Alopecia; hair loss can be occurring as early as late adolescence. AGA is dermatological condition that affects mostly males and females can also be affected from Androgenetic Alopecia (AGA). Hamilton referred that the combination of age factor, androgen receptor and genetics play role in the origin of AGA and describes a specific system for the clinical assessment of Alopecia. Hamilton theory in the end was changed by Norwood and this is still used. Testosterone male hormones play vital role and Polygenetic inheritance is assumed to be the primary cause of AGA. Testosterone hormones convert into more active metabolite di-hydro testosterone (DHT) catalyzed by the enzyme 5-alpha reeducates in the hair follicle cells. Testosterone hormones attach to androgenic receptors in the hair follicle and cellular processes is activated by specific bond which decrease the anagen growth phase of the hair cycle. For this reason hair passes through the next stage that is telogen phase and hair falls out (Bienova *et al.*, 2005). Androgenic Receptor (AR) gene located on Xq12 chromosome, between baldness and a synonymous coding SNP (rs6152) was identified (Ellis *et al.*, 2001).

1.3.3 Alopecia Totalis

Alopecia Totalis is a sever type of AA in which hair loss occur only on the scalp. In this condition hair are presented in all body, eyebrows and eyelashes. It is intermediate condition between Alopecia Areata in which hair loss can be occurred in the form of patches in scalp and Alopecia Universalis is the loss of hair from total body. Children and young adults suffer from Alopecia Totalis though it affects people of all ages. The onset of Alopecia totalis can be sudden or it may come progressively because of AA. It is the severe form of AA. The prevalence of Alopecia totalis is one per million (1 in 100,000) (Letada *et al.*, 2007).

1.3.4 Alopecia Universalis

It is the most severe type of Alopecia Areata in which hair loss can be occurred and no hair can be grow over the entire scalp and body parts, is an autoimmune disease, in which the immune system attacks on the hair follicle mistakenly. In Alopecia Universalis (AU) no hair present on the full scalp and the eyelashes eyebrows and all over the body. Onset of the Alopecia Universalis can be sudden and in some case patients have no hair at the birth. Affect individuals have few sparse hairs at the crown of head which sheds within few days and never re-grow (Tosti *et al.*, 2006). The Alopecia disease has different stages clinically based on the different stages of hair loss. The most common loss of hair is patchy hair loss also known as Alopecia Areata and the next stages of Alopecia Areata is Alopecia Totalis, in which the loss of hair can be occurred from scalp only, or Alopecia Universalis, in which there is no hair on all body parts. It presents all types of hair loss histopathology show changes according to the disease stage. The occurrences of the disease and treatment of the disease is also unpredictable. Different types of therapy modals are used including topical and systemic agents, although none are healing or preventive (Alkhalifah, 2011).

1.3.5 Syndromic Alopecia

Syndromic types of Alopecia show connection of hair loss with different clinical condition. These clinical phenotypes include mental retardation, immunodeficiency, erythroderma and blindness. Inheritance pattern in these syndromic Alopecias has been described both as autosomal dominant and autosomal recessive.

1.3.6 Alopecia with Mental Retardation Syndrome (APMR)

Alopecia with mental retardation syndrome (APMR) (APMR; MIM 203650) is a rare type of Alopecia with autosomal recessive condition. In APMR the affected individual shows complete loss of scalp hair loss of eyelashes, eyebrows, pubic and axillary hair. Mild to severe mental retardation has also been reported in affected individuals. Nails, teeth, hearing and sweating were normal in these individuals. Seizures and other clinical signs were not detected in any of the affected individual. Genome wide scan mapped the first locus of Alopecia with mental retardation (APMR1; MIM 203650) on chromosome 3q26.33-q27.3 (John *et al.*, 2006). A second Alopecia with mental retardation locus (APMR2; MIM 610422) was mapped on chromosome 3q26.2-q26.31 and the third locus for Alopecia and mental

retardation syndrome (APMR3) was mapped on chromosome 18q11.2-q12.2 (Wali *et al.*, 2007a).

1.3.7 Netherton Syndrome (NS)

Netherton syndrome is a grievous syndrome first time reported by Netherton in 1958. This syndrome (NS; MIM 256500) is skin autosomal recessive disorder which is characterized by congenital ichthyosi-form erythro-derma, hair shaft defect and atopic manifestation (Wilkinson *et al.*, 1964). Scalp hairs are brittle and sparse in this syndrome. Congenital ichthyosiform erythrodermic (NBCIE; OMIM 242100) is a clinically and genetically heterogeneous form of autosomal recessive congenital ichthyosis (ARCI). NBCIE is characterized by generalized white, fine, superficial scaling all over the body with erythrodermic background and without blister formation. Sometimes, it is accompanied with palmoplanter keratoderma and ectropian or eclabium and madrosis (Lefevre *et al.*, 2003).

1.3.8 Hypotrichosis with Juvenile Macular Dystrophy (HJMD)

Hypotrichosis with Juvenile Macular Dystrophy (OMIM no 601553). Hypotrichosis with juvenile macular dystrophy is odd genetic disorder manifest at birth with thin and short hair. It is characterize by progressive deterioration which leads to blindness in the second decade of life. This disease is caused by mutation in cadherin 3 (CDH3; OMIM 114021) gene which encodes classical cadherin molecule P-cadherin. This is the only classical cadherin expressed at certain stage of their development. This *CDH3* gene spans 55 kb on chromosome 16q22.1, and comprised of 16 exons (Xu *et al.*, 2002).

1.3.9 Human Nude Phenotype

New mouse phenotype that characterized by loss of the hair. This mouse showed an abnormal keratinization in hair fibers, with follicular infundibulum unable to enter the epidermis. The human Nude is an autosomal recessive disorder. Affected individuals in human nude phenotype hair disease having incomplete hair shafts and hair follicles (Koepf-Maier *et al.*, 1990). The human homologues of the nude mouse share the characteristics of no eyelashes, no eyebrows, and absence of hairs on full body and scalp and immunodeficiency (Frank *et al.*, 1999). In 1996 two sisters first time describe the Human nude murine phenotype after more than thirty year from the first mouse description and associated to FOXN1 gene alteration. The human Nude phenotype is an autosomal recessive disorder with the T-cell

immunodeficiency because thymus is completely absence. This immunodeficiency is very similar to the SCID phenotype and more severe than Desmoglein. Along with the severe infection, other feature of the syndrome is Alopecia and nail dystrophy (Abitbol *et al.*, 2015).

Table 1.1 Different types of Alopecia and associated Responsible gene

S/ No	TYPES OF ALOPECIA	RESPONSIBLE GENES	GENE LOCIS	REFERENCES
1	Monilethrix	hHb3 gene	-	Horve *et al.*, 2003
2	Atrichia with Papular Lesions	HR gene	8p21	Kim *et al.*, 2007
3	Autosomal Recessive Hypotrichosis	LIPH gene	18q12.1	Kazantseva *et al.*, 2006
4	Marie Unna Hereditary Hypotrichosis	MUHH gene	8p21	He *et al.*, 2004
5	Alopecia Areata (AA)	FCRL3 gene	6q25.1	Xiao *et al.*, 2005
6	Androgenetic Alopecia	AR gene	Xq12	Hillmer *et al.*, 2005
7	Alopecia Totalis	Gene encodes protein tyrosine	6p25.1	Kemp *et al.*, 2006
8	Alopecia Universalis	HR gene	8p21-22	Ahmed *et al.*, 1998
9	Alopecia with Mental Retardation Syndrome	No gene identified	3q26.2-q26.31	Jhon *et al.*, 2006
10	Netherton Syndrome	SPINK5 gene	5q31-q32	Stoll *et al.*, 2001
11	Hypotrichosis with Juvenile Macular Dystrophy	Cadherin gene (CDH3)	16q22.1	Kjaer *et al.*, 2005
12	Human Nude Phenotype	FOXN1	17q11-q12	Adriani *et al.*, 2004

1.4 Objectives of the Study

In this study, families from southern Punjab defining the clinical phenotypes of Alopecia were ascertained. This work includes defining the inheritance pattern, clinical phenotypes and molecular genetic studies of Alopecia.

The Main Objectives of this Research

- To identify the loci/gene involved in the disease
- Known gene will be screened for mutations

MATERIALS AND METHODS

2.1 Selection of Families

Three families of Alopecia were ascertaining in the present study from different areas of south Punjab districts. The study was approved by the Board of Study (BOS), International Islamic University, Islamabad and Institutional ethics committee of Institute of Biomedical and Genetic Engineering Islamabad KRL Hospital (IB&GE) Islamabad. Informed consents were obtained from all the members who participated in this research.

2.1.1 Identification of Families

All the families were visited at their native homes. The elders and relatives of the families were interviewed at their homes to get all the information of the hair loss and age of onset of the disease and other related matters of the affected individuals and associated defects were noted carefully. The clinical examination was performed at the local hospital for all the families. The affected individuals were examined by the dermatologist and skin specialist of the local hospital of the areas. All affected individuals were also photographed for phenotypic representation in all families.

2.1.2 Construction of Pedigrees

Pedigrees are used to show the history of inherited traits through a family. Pedigrees are required to describe the genetics relationships among the normal individuals and patients. After discussing elders of these families pedigrees were constructed according to standard method. In a pedigree, males are represented by squares and females by circles. Filled circles and square represents affected individuals while normal individuals are shown with unfilled symbols. The demised individuals in the families were shown by the crossed line on the square or circle. Double lines in the pedigrees represent consanguineous marriages [Cyrillac software was used for the construction of the pedigrees].

2.1.3 Blood Sampling from the Families

Blood samples from both the normal and affected individuals including their parents were collected by using 10 ml syringes and transferred in BD Vacutainer (ACD). Before the collection of blood a written consents form obtained from the participating individuals. The

collected blood samples were stored 4^0C in the laboratory, before extraction of genomic DNA.

2.1.4 Protocol for DNA Extraction with Organic Method

5 ml blood collected in a 50 ml falcon tube and the tube was raised 3 times cell lyses buffer. Tubes were shaken gently and incubate for 30 min on ice and centrifugation was performed at 1200 rpm for 10 min at 4^0C. The supernatants was discarded and the pellet was re-suspended with 5 or 10 ml cell lyses buffer to performed second wash at 1200 rpm for 10 min at 4^0C. The supernatants were discarded again and pellet was completely dissolved. 5 ml STE buffer was added in to each tubes and 250µl, 10% SDS was added drop wise while vortex, 10µl proteinase K (20mg/ml) was also added in the samples. The samples were placed in water bath at 55^0C for overnight. Next day equal amount of phenol was added into the each tube and tube was shaked for 10 min and also kept on ice for 10 min. The centrifugation was carried out at 3200 rpm for 30 min at 4^{oC} after centrifugation aqueous layer was transferred with cut tips into separate labeled tubes. 5 ml of chilled chloroform isoamyl-alcohol (24:1) was added in the aqueous layer and tubes was shaken for 10 min and also kept on ice for 10 min. The centrifugations were performed at 3200 rpm for 30 min at 4^0C. Aqueous layer was transferred with cut tips into separate labeled tube. 500 µl of 10 molar Ammonium Acetate and 5 ml of chilled Iso-propanol was added and tube was shaken until DNA precipitated as visible white threads and the samples was placed overnight at -20 ^{0}C. The following day the samples were spin at 3200 rpm for 60 min at 4^0C. Supernatants were discarded and the pellet was re-suspended and washed with 5ml of chilled 70% ethanol and centrifugation was performed at 3200 rpm for 40 min at 4^0C, supernatants were discarded and sample was placed at room temperature to dry the pellet. After drying the pellet samples were re-suspended in 10 mM TE buffer (PH 8.00) and DNA was stored at 4^0C. Solution used for DNA extraction was given in Table 2.1.

2.1.5 Quantification of Extracted DNA

Extracted DNA was quantified and analyzed on nano drop by taking DNA samples optical density (OD) at 260 nm to 280 nm. The DNA absorbed the light at 260 nm wavelength. All the DNA samples ratios was between 1.7 to 2. If the ratio is 2 or greater than

2 then we should consider it phenol contamination and if the ratio is less than 1.7 than it should be consider as protein contamination. After taking the (OD) of the DNA samples, DNA dilution was prepared for PCR amplification and 40 ng DNA was used in PCR.

2.2 Genotyping

To identify the gene defects in the selected Alopecia families' initial search for linkage was carried out by using polymorphic microsatellite markers corresponding to candidate genes involved in Alopecia. Used microsatellite markers genetics distances were checked from the LDB genetic maps Psychiatric University Hospital Zurich, Switzerland.

2.2.1 Polymerase Chain Reaction (PCR)

Reaction was carried out by using 40 ng of the genomic DNA, which was prepared from stock solution of genomic DNA. PCR amplification of microsatellite markers was performed according to standard procedure in a total reaction volume of 10 µL, containing 40 ng genomic DNA, 1µL 10X PCR buffer [200 mM Tris (pH 8.4), 0.3 µL $MgCl_2$ (50 mM); [Fermentas, San Diego, CA, USA)], 0.3 µL nucleotides (dNTPs; 25 mM), 0.25 µL of each primer (10 µM), and 0.2 µL Taq DNA Polymerase (5 U/µL; Fermentas, San Diego, CA, USA)]. PCR was carried out for 35 cycles with the help of these following thermal cycling conditions: 95°C for 4 min, 55°C for 40 sec, 72°C for 40 sec, followed by final extension at 72°C for 7 min. PCR reaction mixture and the thermal cycling condition are also mention in the (Table 2.2) and (Table 2.3) respectively. Microsatellite markers were used for linkage analysis and the list of microsatellite markers was given (Table 2.4).

2.2.2 Polyacrylamide Gel Electrophoresis (PAGE)

For allele separation amplified PCR products were loaded in 8% Poly-Acrylamide gel. The gel composition regents were shown in Table 2.5. All reagents were mixed in cylinder. Before poring the gel between two plates 50 ml of gel with 300 µl APS and 300 µl TEMD was poured in base. The polyacrylamide gel solution was poured between two glass plates separated by spacers of 1.5 mm thickness. Wells-comb was inserted and the gel was allowed to solidify for overnight at room temperature. Amplified products were mixed with 6x loading dye (bromophenol blue), loaded into the wells and allowed to run about 2-3 hours depending upon size of the microsatellite markers at 100 watts. The gel was subsequently stained with

ethidium bromide (10 mg/mL) solution and visualized on UV transilluminator for photography by digital camera DC120 (Kodak, USA).

2.3 Linkage study

2.3.1 Linkage to Targeted Gene Loci

Literature and genetic data bases were studied to search which gene and loci are already known for Alopecia. Reported microsatellite markers also selected for genotyping that has been linked with Alopecia. After linkage of markers with disease gene; gene specific primers were used for sequencing of the mutated gene. The list of gene specific primers was shown in (Table 2.6). PCR master Mix regents are shown in Table 2.7.

2.3.2 Purification of PCR Product for Sequencing Reaction

In order to remove primers and dNTPs PCR product was purified by precipitation method. To 15 µl of PCR product 1.5 µl of 10mM ammonium acetate and 30µl of absolute ethanol was added. It was then left on ice for 20 minutes. After centrifugation at 13000rpm for 10 minutes at room temperature, the supernatant was discarded and pallet was washed with 100 µl of 70% ethanol. After centrifugation at high speed the supernatant was again discarded and pallet was vacuum dried for one hour in desiccators at 40 °C and re-suspended in 20 µl of deionized water. The purified product was further amplified by using single primer through sequencing PCR.

2.3.3 Purification of the Sequencing Reaction Product

Sequencing PCR product was again purified to get rid of dye-dNTPs. To each 10 µl sequencing reaction product 2.5 µl of 125 mM EDTA and 30µl absolute ethanol was added, vortex mixed and left on ice for 15-20 minutes. Samples were then centrifuged at 14000 rpm for 20 minutes. Supernatant was removed and 30µl 70% ethanol was added to each tube. Pellet was re-suspended with the help of pulse vortex for 1-2 minutes and again centrifuged at 14000 rpm for 10 minutes. Supernatant was discarded and tubes were vacuum dried. Pellet was re-suspended in 10 µl of HiDi formamide. Then 10 µl of each sample was loaded in ABI plate and denatured at 95°C for 3 minutes. Immediately samples were placed on ice for at least 5 minutes and placed in ABI 3130 Genetic analyzer.

Table2.1: Solution for DNA Extraction

LYSIS BUFFER	STE BUFFER	PHENOL	CHLOROFORM ISOAMYL ALCOHOL
NH_4Cl 8.29g	NaCl 4. 39 g	Buffer phenol with Tris pH # 8	Chloroform (24ml)
$KHCO_3$ 1g	Tris_HCl pH 8		
Na_2EDTA 200 µl 0.5 M	Na_2EDTA 50ml 0.5 M		Isoamyl alcohol(1ml)
pH # 8.00	pH# 7.4		

Table 2.2: PCR Reaction Mixture for 1x

S No	REAGENTS	AMOUNT
1	DNA	2 µl
2	10x PCR buffer	1 µl
3	50mM $MgCl_2$	0.3 µl
4	dNTP	0.8 µl
5	Taq	0.2 µl
6	Primer F	0.3 µl
7	Primer R	0.3 µl
8	dH_2O	5.1 µl

Table 2.3: PCR Reaction Program for Linkage Analysis

STEPS	TEMPERATURE	TIME	CYCLE
Initial Denaturation	96^0C	5 min	1
Denaturation	94^0C	30 sec	35
Annealing	55^0C	45 sec	
Extension	72^0C	30 sec	
Final Extension	72^0C	7 min	1

Table 2.4: List of Microsatellite markers and their sequences used for linkage analysis

Gene	Microsatellite markers	PRIMERS(FORWARD+ REVERSE)	ANNEALING TEMP
HR	D8S1145	TGCTAACTGGCACGGTCAC	55°C
		CAATCCCAGTAATCTATAACTTCA	55°C
	D8S136	GCCCAAAGAGGAGAATAAA	55°C
		CTGTTTCCACACCGAAGC	55°C
	D8S1477	AGCAAACTTCATCCACTTGG	55°C
		TTCCTGTCCTCTAATGTAACTTACC	55°C
	D8S1130	GAAGATTTGGCTCTGTTGGA	55°C
		TGTCTTACTGCTATAGCTTTCATAA	55°C
	D8S1106	TTGTTTACCCCTGCATCACT	55°C
		TTCTCAGAATTGCTCATAGTGC	55°C
	D8S258	CTGCCAGGAATCAACTGAG	55°C
		TTGACAGGGACCCACG	55°C
	D8S264	GGGAGATTCGGAACATCTGCGTC	55°C
		TGAGTCAGCATAAAGTAAGAGAGA	55°C
	D8S277	CCAGGTGAGTTTATCAATTCCTGAG	55°C
		TGAGAGGTCTGAGTGACATCCG	55°C
AR	DXS7132	AGCCCATTTTCATAATAAATCC	55°C
		AATCAGTGCTTTCTGTACTATTGG	55°C
	DXS6800	GTGGGACCTTGTGATTGTGT	55°C
		CTGGCTGACACTTAGGGAAA	55°C
	DXS6797	TTCCCTCTCTCCCTCTGTCT	55°C
		ACACACACCCAAAACCAGAT	55°C

Table 2.5: Recipe for 8% polyacrylamide gel composition

CHEMICAL	AMOUNT
40% Acryl amide Gel	62.5 ml
10x TBE	25 ml
TEMED	300 µl
25% APS	200 µl
dH_2O	162 ml
Total	250 ml

Table 2.6: List of Primer Sequences of HR gene used for the Sequencing

EXON	PRIMERS(FORWARD + REVERSE)	BASE PAIR SIZE	OPTMIZED TEMPERTURE
HR-2A	GCCTTACTGGTTTGAGCTGC TGAGATGGCCACCACTATGC	549	55^{0}C
HR-2B	TCCTGAGCACCCCAGACTCC CTTGGGGTTGACTGTGGGGC	614	60^{0}C
HR-3A	GAGGGCTTCAGTATTCTCCC AGTGGGTGGGTAGGATGAAC	494	61^{0}C
HR-3B	GAATCCTTGCCCGCTCTTCC CTGAGGAACTCCCAGAGAGC	777	55^{0}C
HR-4, 5	CATCCTCAGACTCCCTGCTC CCTTAGGTCTAGGAGCTGGC	724	60^{0}C
HR-6	CTCTCCATGGAAGCTGCTCC GCCAACGAATGACCACAGGC	361	58^{0}C
HR-7	GCTGTGTCTCTATGTGACCC GGTGGTGAGTCTAGACCAAC	393	56^{0}C
HR-8	AGCTTCCCGTCTGATTGTCC CCATTTGCAGGCACGATACC	291	60^{0}C
HR-9	GGTAGAAGTCCATGAGCAAC AAGGTGTTTGGAGGCATGTC	422	53^{0}C
HR-10	TGCAGGAAAAGCAGTAGAGC ATGTTGGTGATGCGGTCATC	462	52^{0}C
HR-11	AGCGAATACACATGGCCTTC TAAGGGCAGTAGAACAGCCTC	529	55^{0}C
HR-12	TCCCCGAGCTGTTCTACTGC ACAGGAGGAGACAGAACGGC	430	60^{0}C
HR-13	AGCGTAAGTGTCCCCAACAC ACATGAGAGTACCAGGGACC	358	57^{0}C
HR-14	CCTGGTAACTCTCATGTTTGC TGGAATCAGAGAAGCGCTTC	358	55^{0}C
HR-15	ACTCCTGACCTCAGGTGATC TCCAGGCCTGAAAGGAAGTC	357	56^{0}C
HR-16	TCAGCATCCTGGTGCATGCC TTGGGTCTGTGCAGCTCACC	400	61^{0}C
HR-17	CTGCCCTTCAAGACTTGACC CTCAGTGACTTCAAGGCCTC	465	56^{0}C
HR-18	GAATCTGCTCTCTGAGAGCC AGGGTGGGATCTGCTATGTC	348	55^{0}C

Table 2.7: PCR Reaction Gradients for Amplification of HR Gene Specific Primers

S No	REAGENTS	COMPOSITION FOR 25µL MASTER MIX
1	DNA	5
2	10x PCR buffer	2.5
3	50 mM $MgCl_2$	0.75
4	2.5 mM dNTP	2
5	Taq	0.5
6	Primer F	0.75
7	Primer R	0.75
8	dH_2O	12.75

RESULTS

3.1 Analysis of Pedigrees

Three families have been studied under the research design. Two families i.e. 1AP and 4AP were affected with Alopecia Universalis and the family 3AP was suffering with androgenetic Alopecia.

3.1.1 Family 1AP

Family 1AP belongs to Behal, District Bakhar, Punjab. Family 1AP was having Alopecia Universalis phenotype. Family consists of seven members (IV: 4, V: 1, V: 2, V: 3, V: 4, V: 5 and VI: 1), including four affected members (V: 1, V: 3, V: 4 and VI: 1). The affected individual shared common phenotype. Affected individual have no hair on all the body parts. Three affected individual have normal parents while one affected individual have affected parent. The disorder is transmitted in autosomal manner (Figure 3.1). Affected individuals was shown in generation (V) and (VI) of the pedigree. Blood sample were collected from seven (7) family members and DNA extraction was carried out.

3.1.2 Family 4AP

Family 4AP belongs to Taunsa, District Dera Ghazi Khan, Punjab. Family 4AP is affected with genetic disorder Alopecia Universalis. Family consists of four members (IV: 3, IV: 4, V: 1 and V: 2), including three affected members (IV: 4, V: 1 and V: 2). Affected individual shared common phenotype. Affected individual have no hair on all body parts. The disorder is transmitted in autosomal manner (Figure 3.2). Affected individuals are shown in generation (IV) and (V) of the pedigree. Blood sample were collected from four family members and DNA extraction was carried out.

3.1.3 Family 3AP

Family 3AP belongs to Tehsil Tribal Area Distt Dera Ghazi Khan, Punjab. Family 3AP phenotype is androgenetic Alopecia or more commonly called male pattern baldness. Family 3AP consists of nine members (II: 1, II: 2, III: 1, III: 2, III: 3, III: 4, III: 5, IV: 2 and IV: 3), including four (III: 1, III: 2, III: 3, IV: 2 and IV: 3) affected with androgenetic Alopecia. In

this family all male members are affected. The family members share common phenotype of baldness. Affected individual have normal parents. The disorder is X-linked (Figure 3.3). Affected individuals are shown in generation (III) and (IV) of the pedigree. Blood sample were collected from nine family members and DNA extraction was carried out with organic method.

3.2.1 Clinical Symptoms

Clinical symptoms of families (1AP and 4AP) shows Alopecia Universalis, the affected individual have no hair on all the body parts. Affected individuals lost their hair after birth within two months. In Alopecia Universalis no hair present on the full scalp and the eyelashes eyebrows and all over the body. Onset of the Alopecia Universalis can be sudden and in some case patients have no hair at the birth. Physical feature of the Alopecia families (1AP and 4AP) was shown in Figure 3.2 and 3.4 respectively. A clinical symptom of family 3AP indicates that family was affected with androgentic Alopecia. The Physical feature of the family 3AP was shown in Figure 3.6.

3.3 Linkage Analysis

Microsatellite markers were used for linkage studies corresponding to candidate genes involved in HR gene for Alopecia universals and AR gene for Androgenetic Alopecia (male pattern baldness). All the microsatellite markers used for linkage analysis were shown in (Table 2.4). PCR reaction was performed for microsatellite markers linkage with diseased gene. PCR products were checked on 8% non-denaturing polyacrrylamide gel and gel was stained with ethedium bromide. The Genotype for affected members and normal members of the families were analyzed in gel doc for visual inspection.

3.3.1 Family 1AP

In family 1AP have total seven DNA sample (IV: 4, V: 1, V: 2, V: 3, V: 4, V: 5, IX: 1) including three normal and four affected individuals were selected for genotyping the markers linked to the candidate genes. In family 1AP linkage was established with three microsatellite markers D8S1130, D8S1106 and D8S1145 and the results was shown in Figure 3.7, 3.8 and 3.9 respectively.

3.3.2 Family 4AP

In family 4AP have total four DNA sample (IV: 3, IV: 4, V: 1, V: 2,) including one normal and three affected individuals were selected for genotyping the markers linked to the candidate genes. In family 4AP linkage was established with two microsatellite markers D8S1106 and D8S1130 and the 8% polyacrylamide gel picture was given in Figure 3.10 and 3.11.

3.3.3 Family 3AP

In family 3AP total nine DNA sample available from these nine sample, five members of the family affected with Androgenetic Alopecia (III: 1, III: 2, III: 3, IV: 2 and IV: 3) and all others members are normal. Genotyping was performed for all the nine members of the family with x-linked microsatellite markers. Analysis of the results showed the linkage for AR gene with microsatellite marker DXS6797 and the 8% polyacrylamide gel picture was given in Figure 3.12.

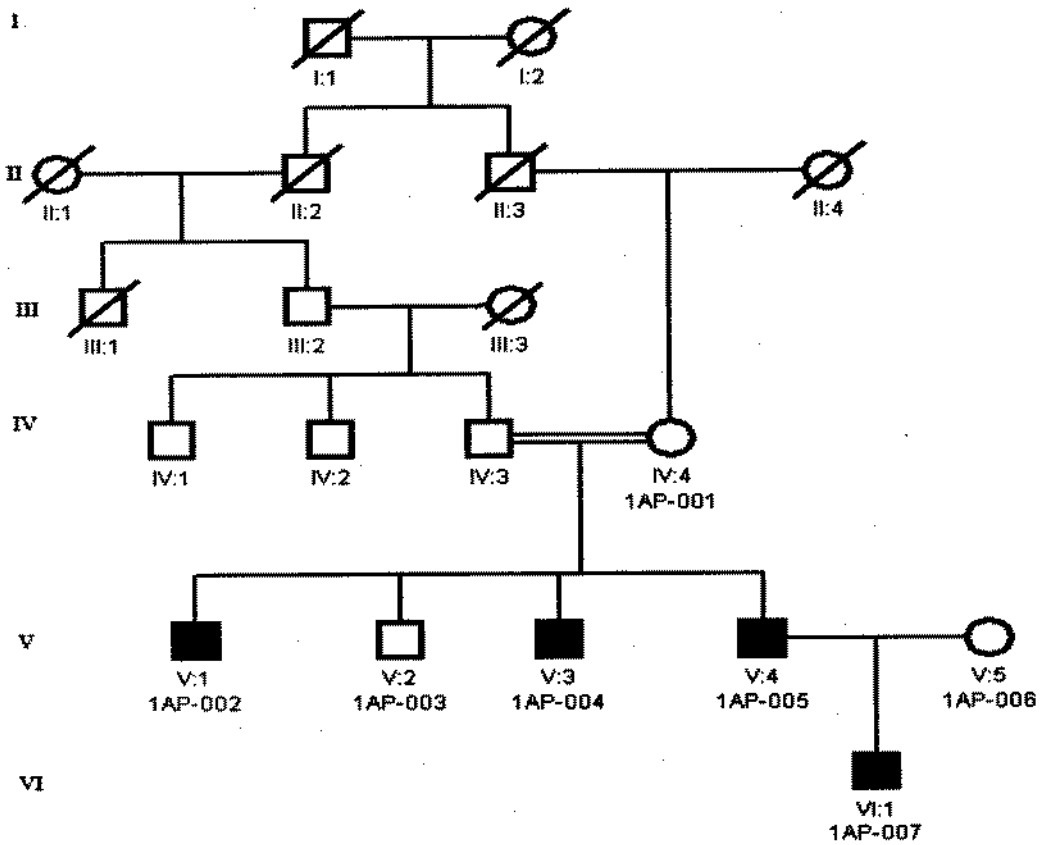


Figure 3.1: Pedigree of family 1AP with Alopecia Universalis. The pattern of pedigree was that, squares represent the male individuals and circle represents the female individuals in the family. All the filled square and circle represents the affected individuals and double line indicates consanguineous marriage. The crossed line on the square and circle represents deceased individuals.

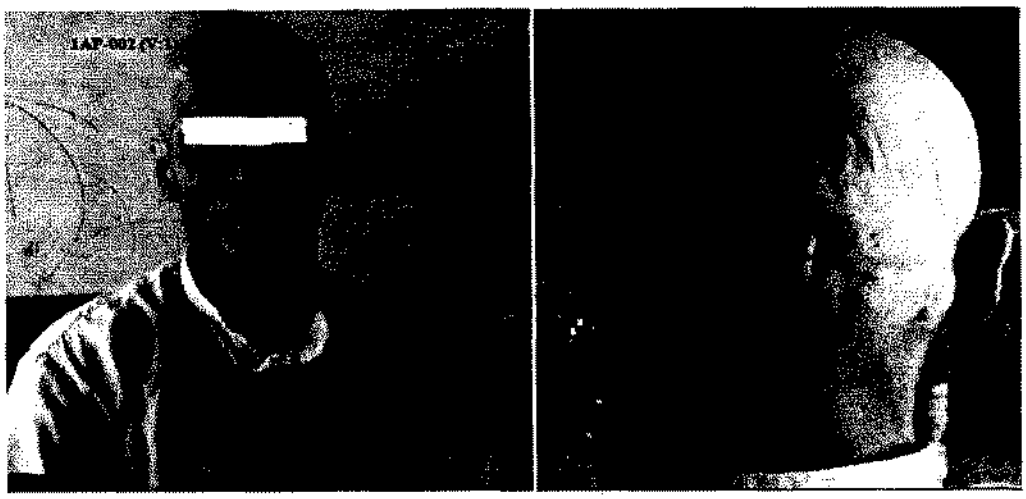


Figure 3.2: Clinical features of Alopecia Universalis in family 1AP represents of affected individuals 1AP-002 (V: 1) and 1AP-003 (V: 3). Physical features of the affected individual from family 1AP showing complete loss of hair from eye brows eyelashes and absence of hair from all the body.

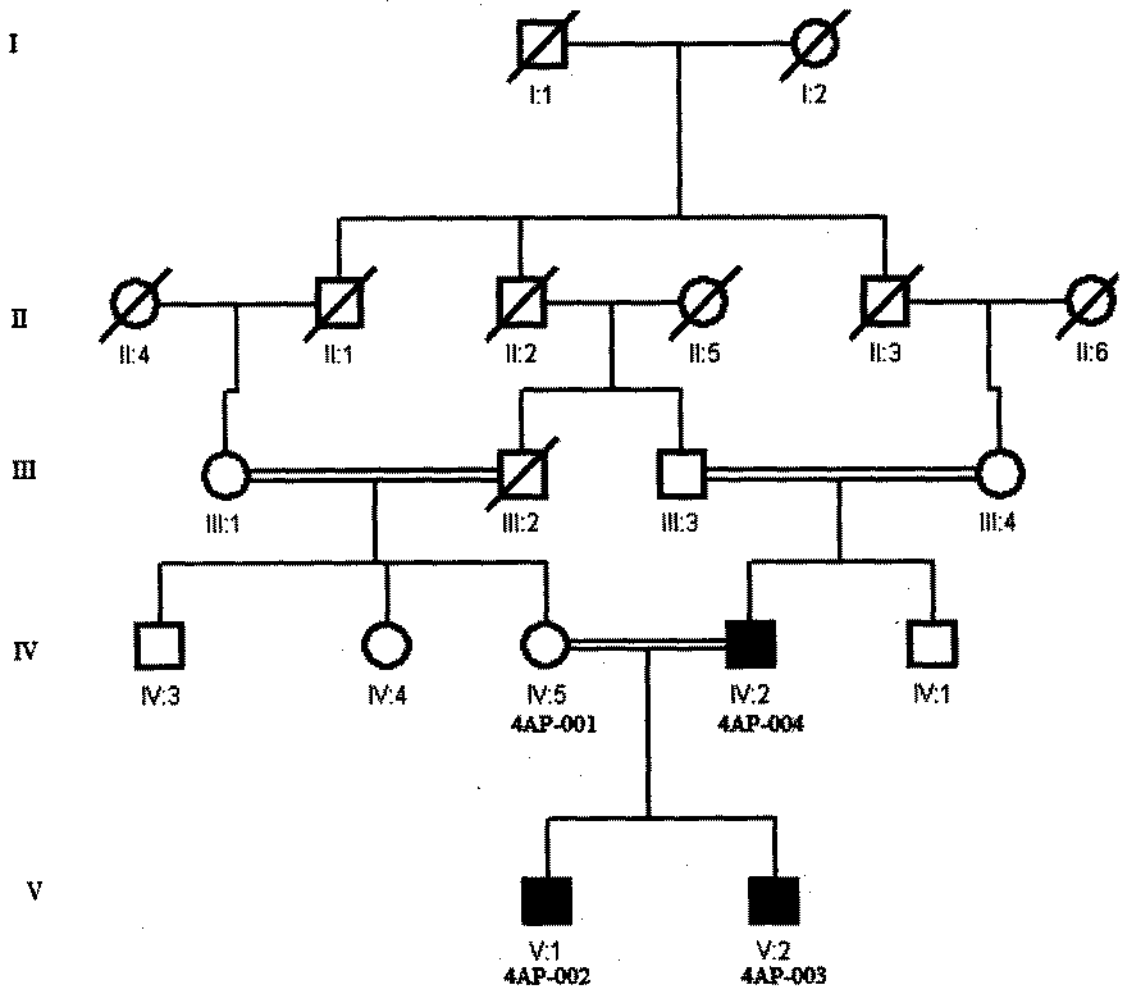


Figure 3.3: Pedigree of family 4AP with Alopecia Universalis. The pattern of pedigree was that, squares represent the male individuals and circle represents the female individuals in the family. All the filled square and circle represents the affected individuals and double line indicates consanguineous marriage. The crossed line on the square and circle represents deceased individuals.

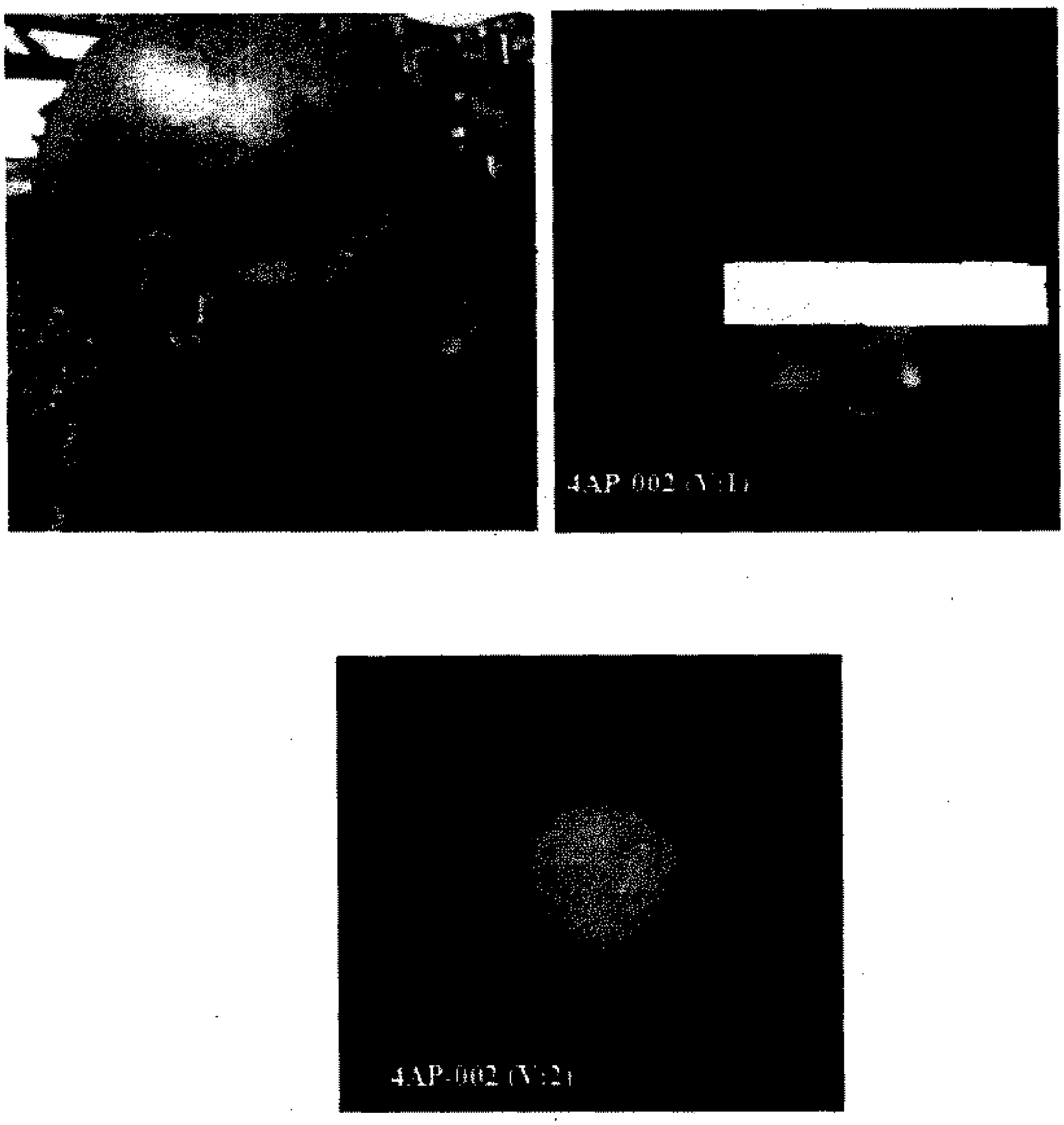


Figure 3.4: Clinical features of Alopecia Universalis in family 4AP-001 represents of affected individuals 4AP-002 (V: 1), 4AP-003 (V: 3) and 4AP-004 (IV: 4). Physical features of the affected individual from family 4AP showing complete loss of hair from eye brows eyelashes and absence of hair from all the body.

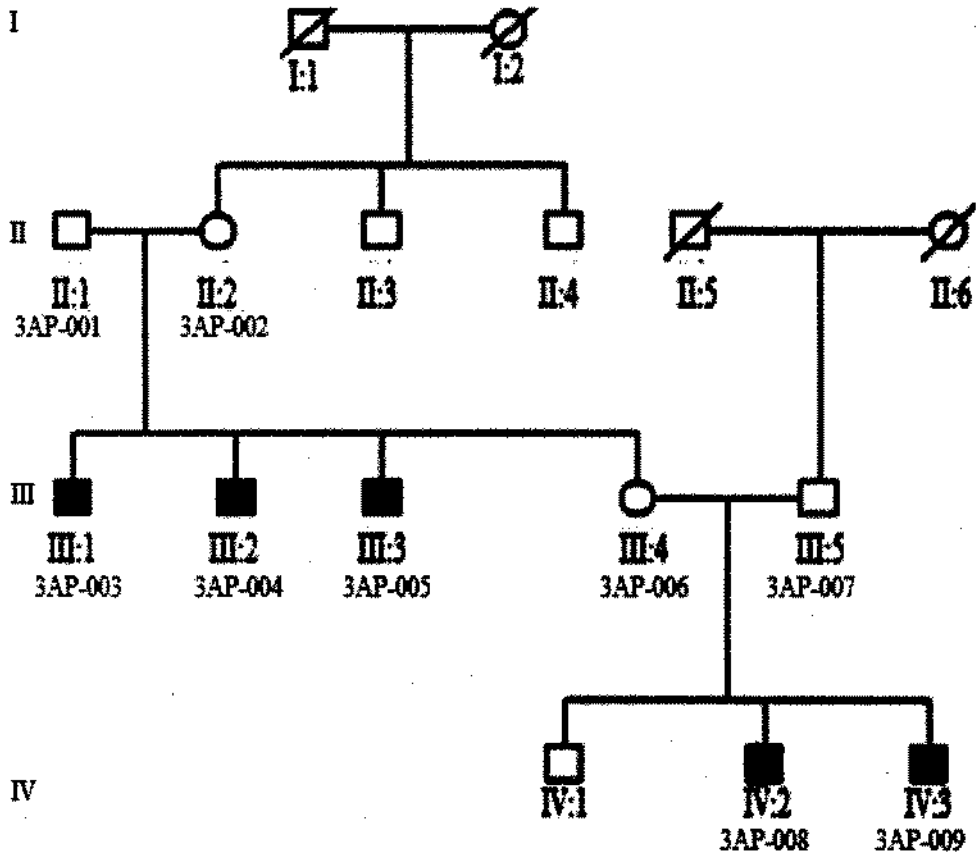


Figure 3.5: Pedigree of family 3AP with Androgenetic Alopecia (male pattern baldness). The pattern of pedigree is that, squares represent the male individuals and circle represents the female individuals in the family. All the filled square and circle represents the affected individuals. The crossed line on the square and circle represents deceased individuals.

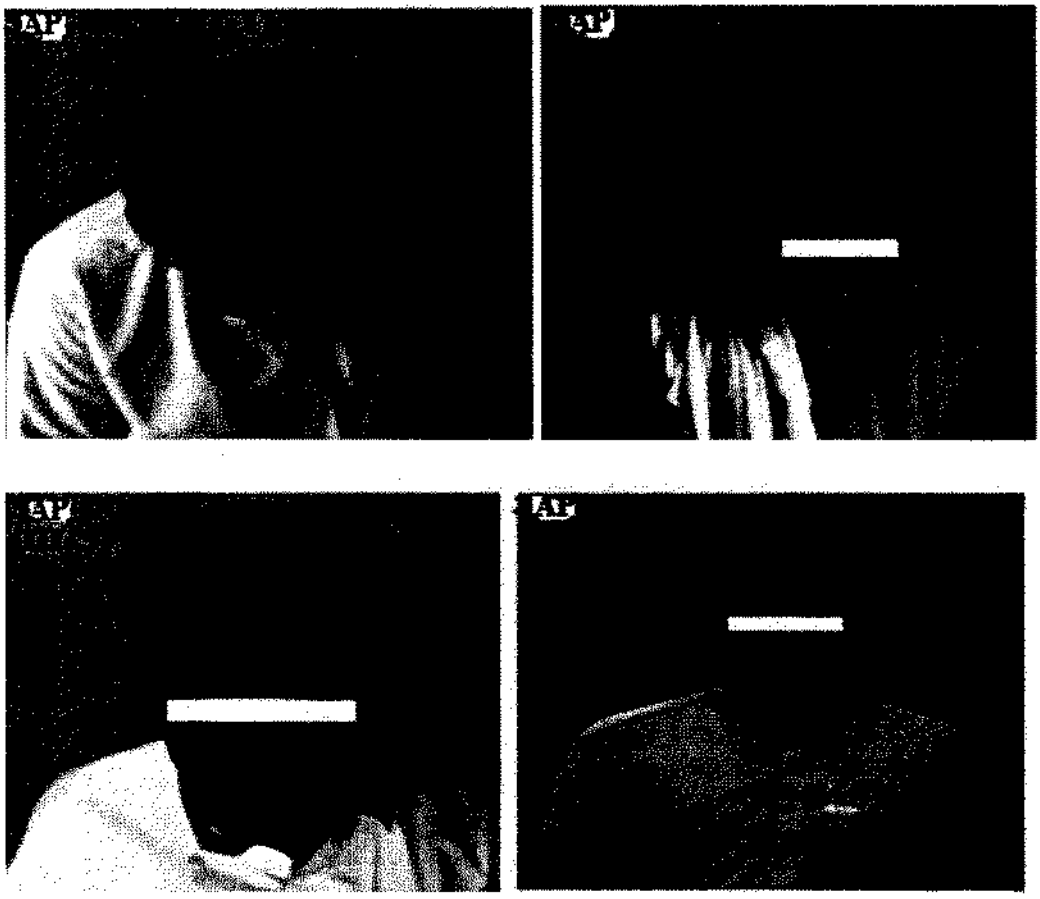


Figure 3.6: Clinical features of Androgenetic Alopecia in family 3AP represents of affected individuals 3AP-003 (III: 1), 3AP-004 (III: 2), 3AP-005 (III: 3), 3AP-008 (IV: 2) and 3AP-009 (IV: 3). Physical features of the affected individual from family showing male pattern baldness and the age of onset is 25 years after birth.

3.3.4 Linkage Analysis of the Families

On the basis of genetic linkage studies in Alopecia Universalis, it is clear that at least some candidate intervals should be tested for linkage or exclusion prior to genotypes the whole genome. In the present study all the families were tested for linkage to known loci by using microsatellite markers within the intervals two families shows linkage to known loci and one family did not show any linkage to the targeted disease.

1. III: 4 Normal
2. V: 1 Affected
3. V: 2 Normal
4. V: 3 Affected
5. V: 4 Affected
6. V: 5 Normal
7. VI: 1 Affected

Figure 3.7: Electropherogram of ethidium bromide stained 8% polyacrylamide gel showing allelic pattern obtained with microsatellite marker D8S1130 at 27.2 cM on chromosome 8q12. The roman numbers and Arabic number refer to the individuals in the pedigree.

1. III: 4 Normal
2. V: 1 Affected
3. V: 2 Normal
4. V: 3 Affected
5. V: 4 Affected
6. V: 5 Normal
7. VI: 1 Affected

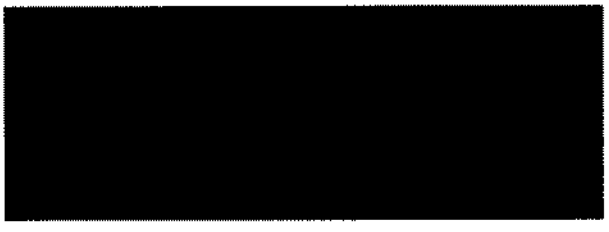
Figure 3.8: Electropherogram of ethidium bromide stained 8% polyacrylamide gel showing allelic pattern obtained with microsatellite marker D8S1106 at 29.1 cM on chromosome 8q12. The roman numbers and Arabic number refer to the individuals in the pedigree.

1. III: 4 Normal
2. V: 1 Affected
3. V: 2 Normal
4. V: 3 Affected
5. V: 4 Affected
6. V: 5 Normal
7. VI: 1 Affected

Figure 3.9: Electropherogram of ethidium bromide stained 8% polyacrylamide gel showing allelic pattern obtained with microsatellite marker D8S1145 at 37.2 cM on chromosome 8q12. The roman numbers and Arabic number refer to the individuals in the pedigree.

1. IV: 3 Normal
2. IV: 4 Affected
3. V: 1 Affected
4. V: 2 Affected

Figure 3.10: Electropherogram of ethidium bromide stained 8% polyacrylamide gel showing allelic pattern obtained with microsatellite marker D8S1130 at 27.2 cM on chromosome 8q12. The roman numbers and Arabic number refer to the individuals in the pedigree.



1. IV: 3 Normal
2. IV: 4 Affected
3. V: 1 Affected
4. V: 2 Affected

Figure 3.11: Electropherogram of ethidium bromide stained 8% polyacrylamide gel showing allelic pattern obtained with microsatellite marker D8S1106 at 29.1 cM on chromosome 8q12. The roman numbers and Arabic number refer to the individuals in the pedigree.

1. II: 1 Normal
2. II: 2 Carrier
3. III: 1 Affected
4. III: 2 Affected
5. III: 3 Affected
6. III: 4 Normal
7. III: 5 Normal
8. IV: 1 Affected
9. IV: 2 Affected

Figure 3.12: Electropherogram of ethidium bromide stained 8% polyacrylamide gel showing allelic pattern obtained with microsatellite marker DXS6797 at 37.2 cM on chromosome X. The roman numbers and Arabic number refer to the individuals in the pedigree.

3.3.5 Haplotyping of Families

After genotyping the family 1AP, 4AP and family 3AP, the microsatellite markers that are tightly-linked on targeted genes for family 1AP, and 4AP the gene is HR gene and for family 3AP the gene is involved AR gene. The allelic pattern of the family 1AP was show in Figure 3.13. Allelic pattern of family 4AP and 3AP was shown in Figure 3.14 and 3.15.

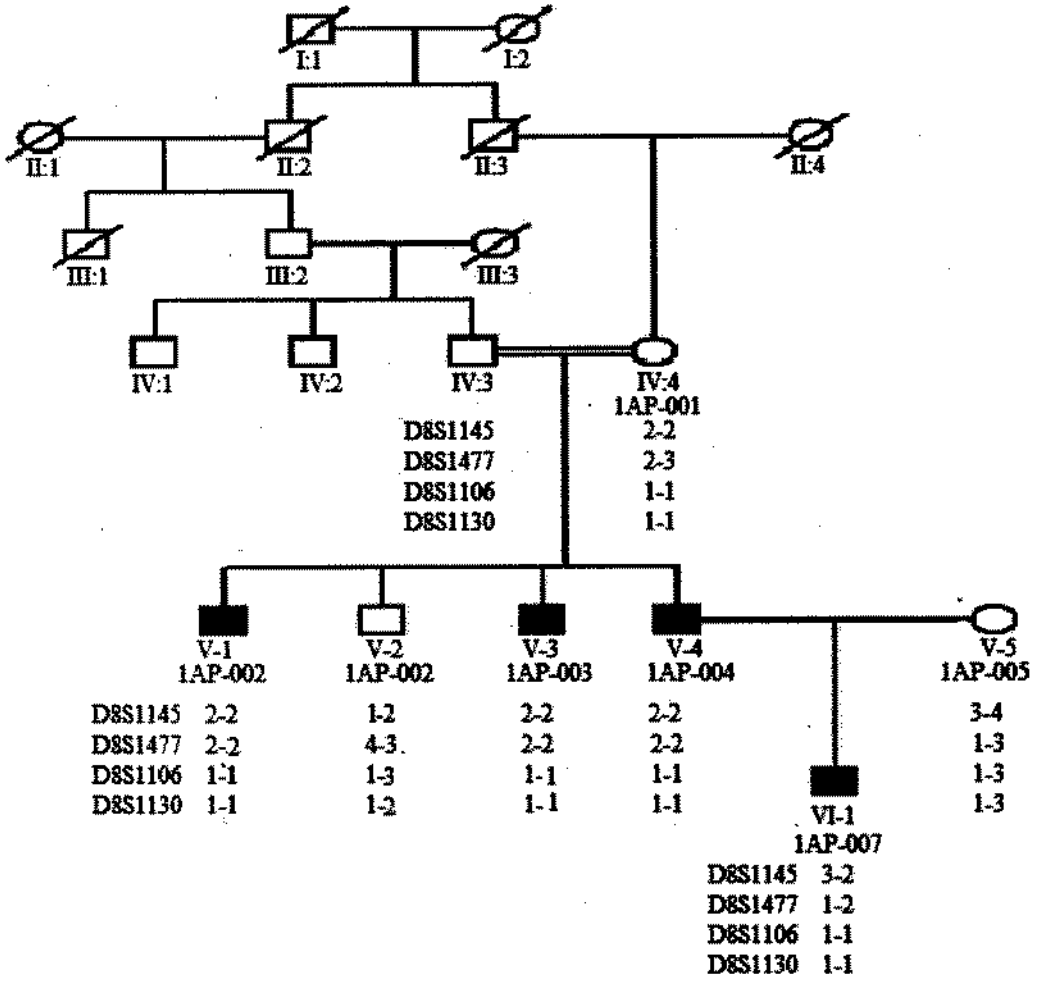


Figure 3.13: In family 1AP, six microsatellite markers used for homozygousity mapping to check the linked region with disease gene or candidate gene. This shows that D8S1145, D8S1106 and D8S1130 linked with candidate gene.

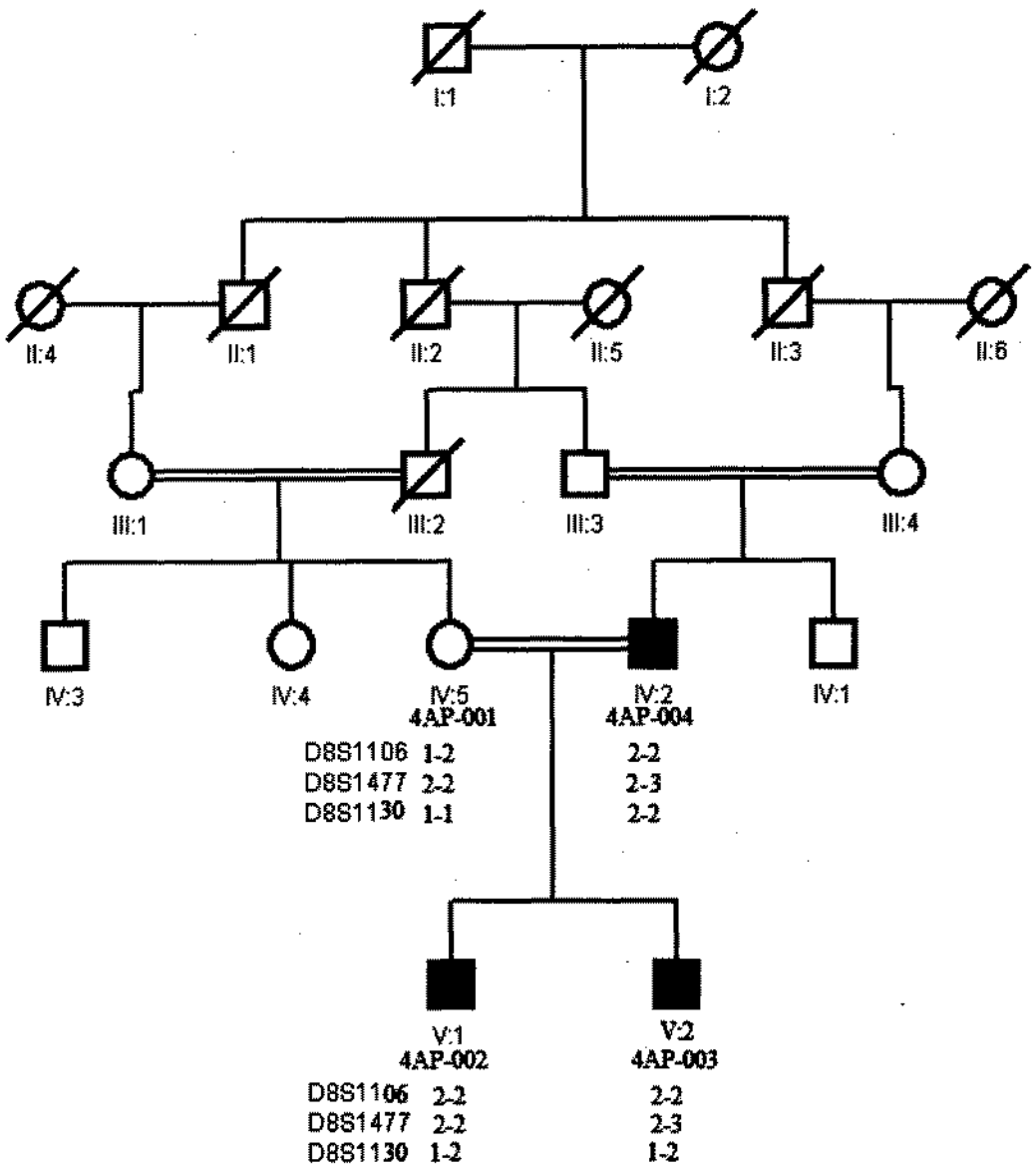


Figure 3.14: In family 4AP three microsatellite markers used for homozygousity mapping to check the linked region with disease gene or candidate gene. This shows that D8S1106 linked with candidate gene.

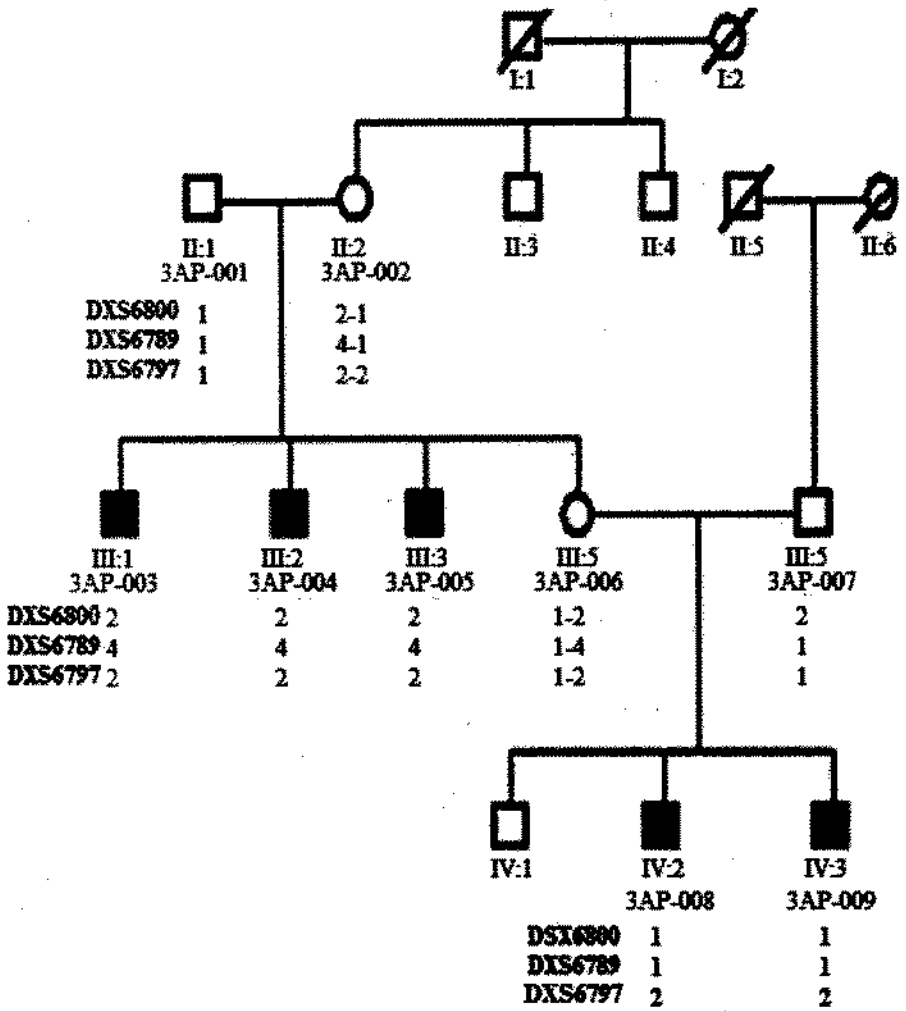


Figure 3.15: In family 3AP three microsatellite markers used for homozygousity mapping to check the linked region with disease gene or candidate gene. This shows that DXS6797 linked with candidate gene other two microsatellite markers shows no linkage

3.3.6 Amplification of HR Gene Family 1AP

Before sequencing of targeted gene, gene specific primers were optimized by using PCR. The HR gene is 19 Exon and all the Exon was optimized at different annealing temperature. The Exon HR-3A was optimized at 61^0C. The total reaction volume for PCR was 25µl. Out of 25 µl, 5 µl used for check gel.

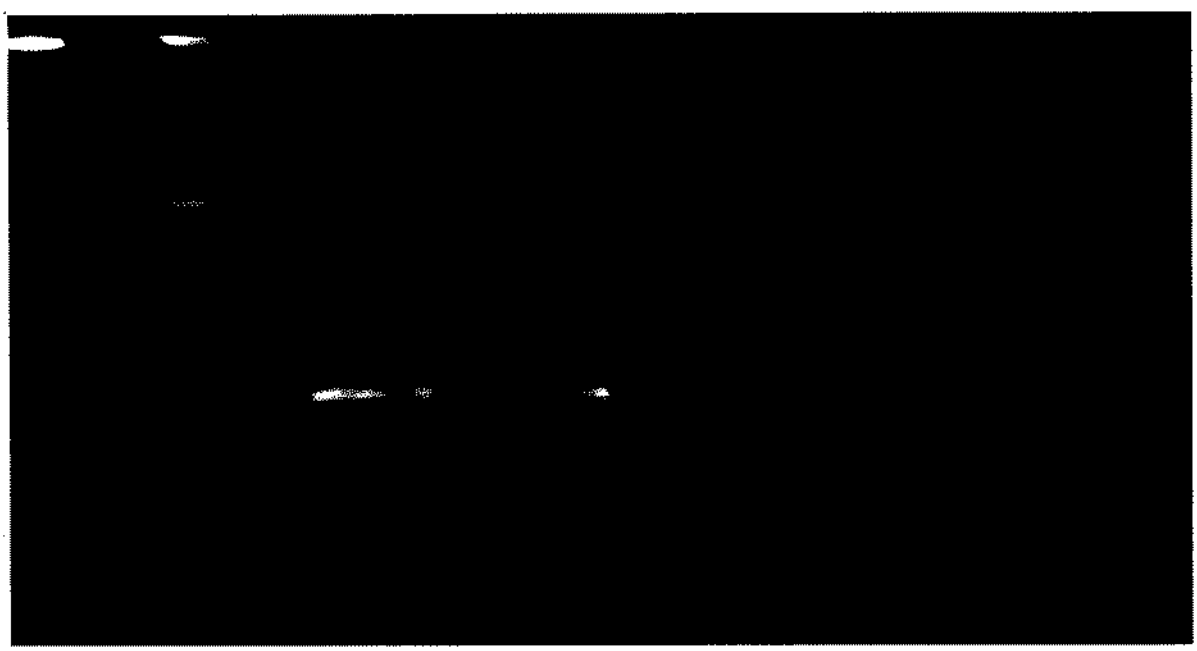


Figure 3.16: The Exon HR-3A with 494bp size was optimized at 61^0C.

3.3.7 Amplification of HR Gene Family 4AP

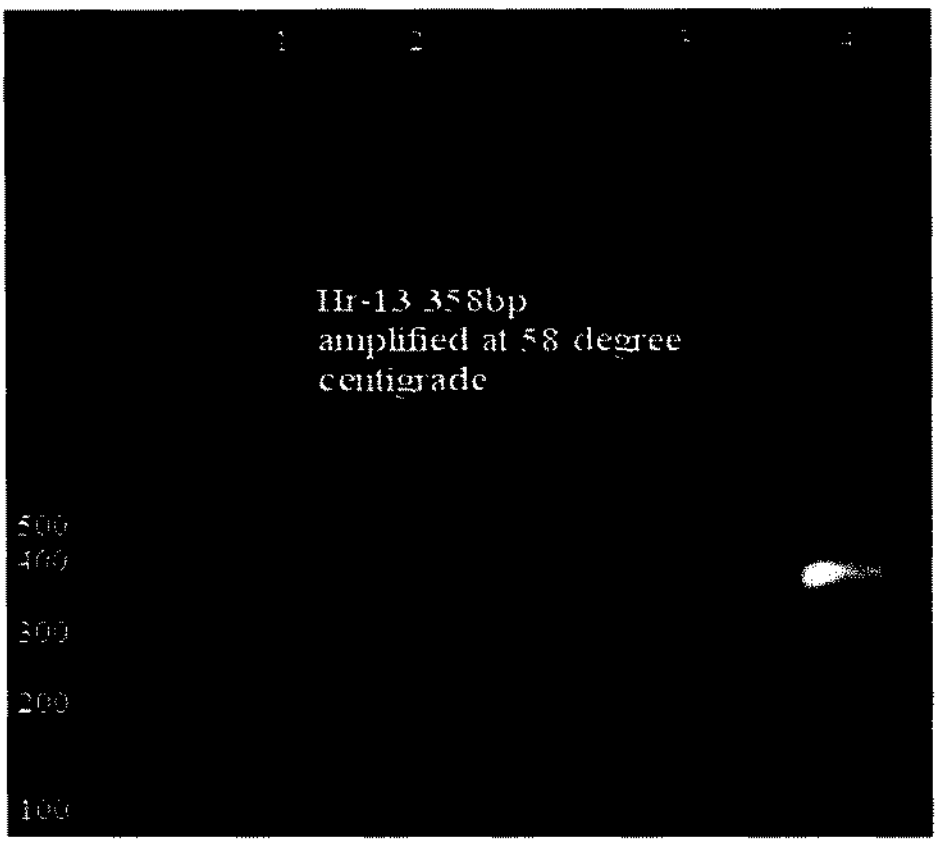


Figure 3.17: The Exon HR-13 with 358 base pare size was optimized at 58°C.

3.3.8 Mutational Screening of Hairless Gene Family 1AP

In the family 1AP, (HR) gene investigated for mutational screening. The sequence analyses of the HR gene shows a novel homozygous missene mutation in exon 3A at the amino acid position 222. This missene mutation changes the amino acid alanin (GCA) to thorine (ACA). Sequence Electropherogram of the affected and carrier individuals for the mutation is shown in figure 3.18.

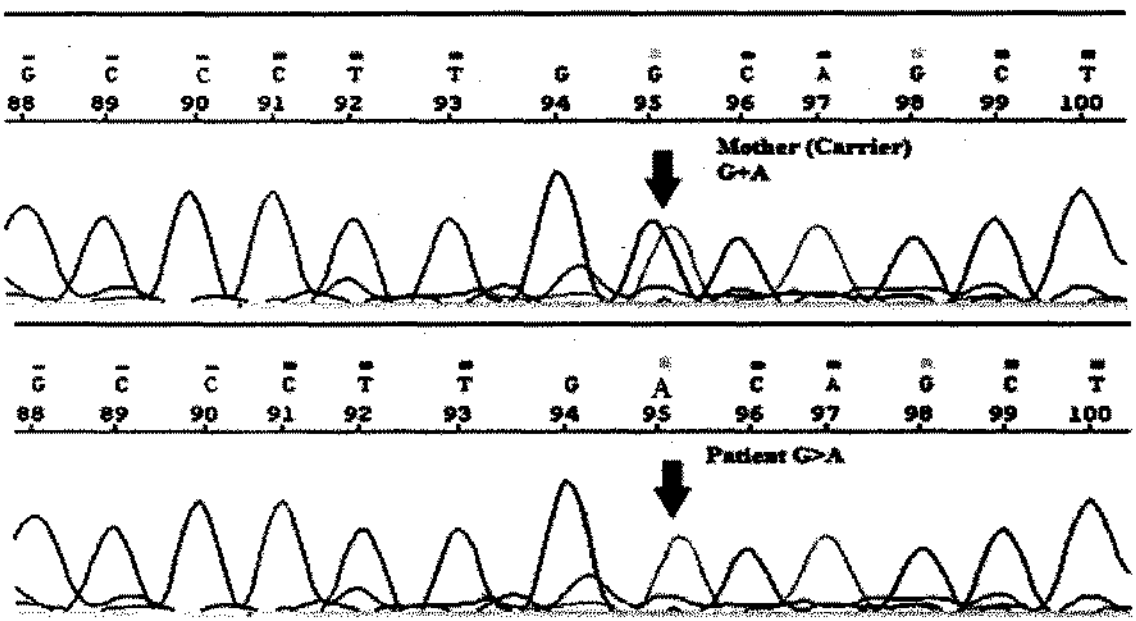


Figure 3.18: DNA sequence chromatograms from exon 3A of HR gene Showing the novel homozygous missene substation mutation of nucleotide G to A at position 664 (c.664 G>A), for family 1AP. homozygous mother (upper panel III: IV), and homozygous affected male (V: 1; lower panel) was shown in this above Figure.

3.3.9 Mutational Screening of Hairless Gene for Family 4AP

HR gene Consisting of 18 exons, the patients of this family were indentified to carry a nonsense mutation, c.2818C>T, in exon 13, which at this position substitutes the arginine (CGA) with the stop codon TGA (p.Arg940X). The results were shown in Figure 3.19. This mutation has been reported in 2007 by (Kim *et al.*, 2007) for an 'atrichia with papular lesions' family.

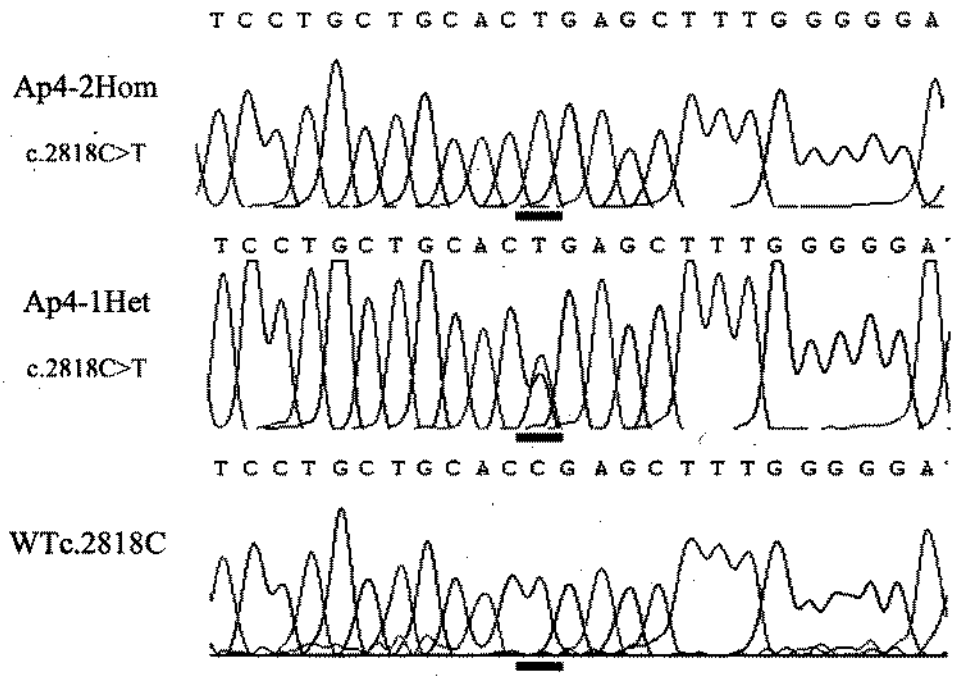


Figure 3.19: Sequencing of the HR gene revealed the stop codon generating nonsense mutation c.2818C>T in exon 13. The mutated base is underlined with a black bar. The affected son, 4AP-002, and the heterozygous father, 4AP-001.

DISCUSSION

Different forms of inherited human hair loss collectively recognize as Alopecia. Alopecia is condition in which the hair can be lost in the form of oval patches most commonly round shapes can be formed on the normal appearing skin, mostly on the scalp and in the certain section of the male beards. The disease can be rapidly spread, and the progress of the disease can be reached in a stage where all the hair is lost from the entire scalps this condition is known as Alopecia Areata, Alopecia Totalis or some time all the hair can be lost from whole body normally is called Alopecia Universalis. The identification of more causative genes for Alopecia disorder is the main source of approaching into the molecular mechanism of epidermal development and differentiations. Multiple genes responsible for Alopecia disorder that effect human hair growth cycle. Genetic conditions that affect hair structure and the hair growth cycle may be isolated or they may occur as a part of other complex syndromes with associated defects of other ectodermal appendages like teeth, nails, sweat glands, brain, etc. Isolated hereditary Alopecia includes congenital atrichia, Marie Unna hereditary hypotrichosis, hypotrichosis simplex and three forms of localized autosomal recessive hypotrichosis. The degree of Alopecia is variable among patient and during various stages of life for the same individual in all the above case.

In this study, three families (1AP, 4AP and 3AP) were ascertained from southern Punjab Pakistan. The mode of inheritance in two families i.e. 1AP and 4AP was autosomal recessive. The inheritance pattern of family 3AP was X-linked. Linkage analysis was performed by Homozygosity Mapping to identify the gene(s) causing different forms of hereditary Alopecia in the selected families. Homozygosity Mapping was performed by using STR matching to targeted gene involved in Alopecia. In family 1AP and family 4AP, linkage was established to hairless gene (HR) on chromosome 8p21. In family 3AP, linkage was established with X-linked Androgen Receptor (AR) gene.

All the exons of HR gene were amplified and sequenced for both normal and affected individuals of the family 1AP and family 4AP. A novel homozygous missence mutation identified in the present study at exon 3 of HR gene at position c.664 G>A. This missence mutation change the amino acid alanine (GCA) (non polar) to threonine (ACA) polar amino acid. In family 4AP, a nonsense mutation, c.2818C>T, in exon 13 was identified. This

mutation has been reported in 2007 by Kim et al (Kim *et al.*, 2007) for an 'atrichia with papular lesions' family. This mutation (p.Arg940X) substitutes the Arginine (CGA) with the stop codon TGA.

Hairless (HR) work as nuclear receptor co-repressor. HR gene also play key role in the maintenance of human hair growth. The function of HR gene is work at the cellular transition from the natal to postnatal hair cycle in human. This gene encodes a putative zinc-finger transcription factor. This transcription factor protein has 1189 amino acids (Potter *et al.*, 2001). HR gene is a major cause of Alopecia in most of the families worldwide. We identified mutation in HR gene in family (1AP, 4AP). It will further strength the idea of the involvement of HR gene in hair loss.

Genotyping the family (3AP) of Androgentic Alopecia, and the family showed linkage with AR gene. In future AR gene sequencing will identify the mutation in this family. AR gene mutation identification will increase our understanding about Androgentic Alopecia.

In spite of the limitations, this study shows that Alopecia can be problematic for society in number of ways. Combined with previous research, the current study has contributed a considerable amount of valuable information regarding the Alopecia (hair loss).

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