Linkage and Sequencing Analysis of Consanguineous Families of Alopecia

By

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- Biotechnology
- Pharmaceutical Chemistry
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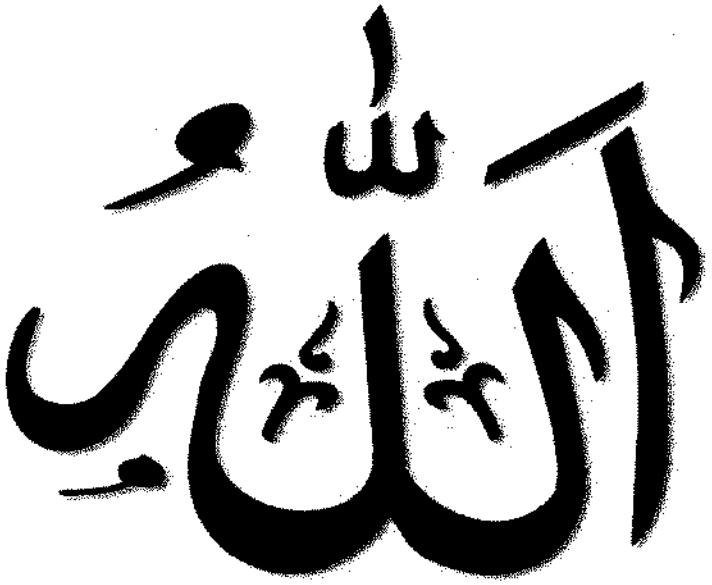

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Department of Bioinformatics and Biotechnology

Faculty of Basic and applied Sciences

International Islamic University Islamabad

FINAL APPROVAL

Department of Bioinformatics and Biotechnology

International Islamic University Islamabad

Dated: 14-7-2015

It is certificate that we have read the thesis submitted by Mr. Iftikhar Ahmad 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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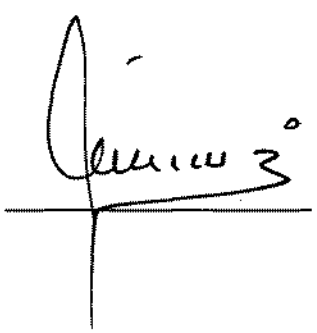

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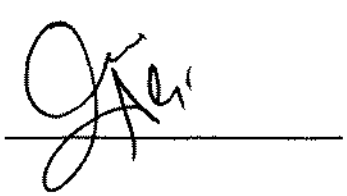

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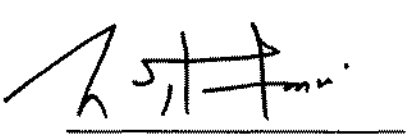

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A thesis submitted to Department of Bioinformatics and Biotechnology Faculty of Basic and applied Sciences, International Islamic University, Islamabad as a partial fulfillment of requirement for the award of the degree of MS Biotechnology.

DEDICATION

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

Rehan Ullah Bhittani and Areeba Iftikhar

&

To my honorable supervisor Dr. Asif Mir.

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 14/07/15

Iftikhar Ahmad

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In the name of Allah, the Most Gracious and the Most Merciful, Alhamdulillah, all praises to Allah for the strengths and His blessing in completing this thesis. I'd like to give my sincere thanks to my supervisor, **Dr. Asif Mir** who accepted me as his MS student without any hesitation. Thereafter, he offered me so much advice, patiently supervising me, and always guiding me in the right direction. I've learned a lot from him, without his help I could not have finished my dissertation successfully. I also wish to my deepest thanks to **Adnan Yousaf, Abdul Kareem, Saad Abbasi** and **Adnan Tahir** for their great support.

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Iftikhar Ahmad

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	Autosmal recessive
AR	Androgentic Receptor
AU	Alopecia Universalis
BOS	Board of Study
bp	Base Pair
^0C	Degree 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
HR	Hair less

IRB	Institutional review board
KB	Kilo base pair
LHA	Localized Autosmal Recessive Hypotrichosis
LIPH	Lipase H
mg	Milligram
ml	Milliliter
mM	Milli molar
MR	Mental Retardation
MUHH	Marie Unna Hereditary Hypotrichosis
NCBI	National center for biotechnology information
ng	Nano gram
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
μ M	Micro meter
μ L	Micro litter

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Abstract

Homozygosity mapping is an effective gene mapping technique in consanguineous families with inherited diseases. The study presented here includes homozygosity mapping of two large consanguineous families (AP5 and AP10) with Alopecia Universalis.

To elucidate the genetic defects in family AP10, linkage analysis via homozygosity mapping was performed. The genotyping results obtained in this family excluded the possibility of presence of reported *HR* locus and strongly suggestive of the involvement of other locus as a cause of Alopecia Universalis.

Linkage was established in family AP5 at *HR* locus. The results for marker D8S1106 located at 27.23 centiMorgan showed five affected individuals were homozygous while normal individuals were heterozygous. The same result was supported by marker D8S1827 located at 27.67 centiMorgan, *HR* gene resides at this locus. Sequencing of affected individual (AP5-6) and normal (AP5-8) shows the deletion of adenosine at c.2123 in *HR* gene. As a consequence mutation resulted in a premature stop codon at position 194 instead of 483 amino acid position. The present study identified this novel mutation in *HR* as a causative gene of Alopecia Universalis in family AP5 (**Novel Mutation report submitted to BMC Medical Genetics**).

During the bioinformatics phase of study, the normal and mutated structure of HR protein for novel mutation was predicated. Our result indicated that normal HR structure comprised of 19 helixes, 7 strands and 28 coils. While in mutated HR protein model, number of helixes, strands and coils are reduced to 4, 1, and 6, respectively. Due to frameshift mutation and creation of stop codon, size of protein and number of structure features gets affected.

Stitch4 was used to identify functional partner of *HR*. The closest one with highest interaction score of 0.824 with *HR* was selected for further analysis and considerd as protein ligands i.e. HDAC1, which plays an important role in the regulation of transcription, progression of cell cycle, and in events of development. We assume both normal and mutated structures of HR were docked with this ligand protein to study the effect of mutation. Mutation not only affects the structure and conformation but also the function of protein, leading to the diseased state.

Chapter# 1

INTRODUCTION

Introduction

During the past decade an extraordinary explosion in molecular biology and genetic research has helped to enhance understanding of pathogenesis of many human diseases. In recent years, genes for several genetic disorder has been identified. Among normal individuals difference in the body trait such as height and intelligence can be accounted by remarkable genetic variability (polymorphism). These genetic differences include environmental factors such as infectious agents and ionizing radiation that produces different diseases in human. Therefore, all human diseases is a result of interaction between environmental and genetic makeup (Scrimgeour, 2009).

Transmission of single mutant gene follow three pattern of inheritance in all human genetic disorder, X-linked, autosomal recessive, autosomal dominant disorders. In autosomal dominant disorders the disease will be expressed if she or he is hetrozygote for gene. Example include waardenburg syndrome and Achondroplasia. The trait is expressed in recessive disorders only if he or she is in homozygote state. From each parents the homozygote receive one abnormal (recessive) gene. In population where consanguineous marriages occur commonly, i.e. arrange marriages between cousins, certain recessive conditions are more common. Examples include cystic fibrosis, sickle cell anemia, seckel syndrome. In X-linked disorders, the mutant allele is located on the X chromosomes. It manifests in female only when it is in double dose (homozygote state). Therefore females are hardly affected. The trait is expressed in single dose, when the male who is hemizygous for X-linked genes. This means males are affected when they have single mutant gene on their X chromosomes, the full syndrome will always be manifested if he inherits the mutant gene e.g. Haemophilia (Scrimgeour, 2009).

Most recently research have overlay with studying using homozygosity mapping and human genome diversity projects to indicate diseased gene in particular sub population. Pakistan demographic and health survey (DHS) (1990-1991) shows that two third of marriages in Pakistan are consanguineous (Hussain and Bittles., 1998). There are three common reasons for such marriages are (religious, economic, and cultural). Among these 60% of marriages are consanguineous in which 80% were between first cousins. Religion put a great effect on consanguinity. In common, consanguineous unions are allowed with in Buddhism, Judaism, and in Zoroastrian/Parsi tradition (Bittles *et al.*, 2001). In Pakistan and many of the Middle

East Muslim countries, the occurrence of close-kin marriages exceed 50%. In Quran there is no specific guidance that could be taken as encouraging consanguinity (Hussain, 1999). Economical factors is the second reason for consanguineous marriages. Ruling and land lords families from Western and Asian countries prefer close kin marriages to maintain their dignity (Bittles, 1994).

The actual reason for which the consanguineous marriages were given preference are primarily social. Most of the families believed that if they merry with in the family the family bonds will be strengthened. In traditional Arabs society, the head of the family consider it as an insult if they merry outside their family (Chaleby, 1988). In Arabs Muslims community first cousin union between a man and his father's brother's daughter (FBD) are favoured (Khlat, 1997). Han Chinese (Wu, 1987) and the tuareg people of North Africa (Degos *et al.*, 1974). The risk of expression of autosomal recessive disorder in the offspring of close kin marriage is inversely proportional to the rate of the mutated allele in the gene pool. Frequency of close kin marriages among French people is less than 0.2%, the ratio of cystic fibrosis among first cousin marriages were 1.4% in comparison with cystinosis (7.1%) and person with Achromatopsia (12.5%) (Tchen *et al.*, 1997).

1.1 Linkage Analysis

Possible linkage to all known Alopecia loci/genes were performed using highly polymorphic microsatellite markers. Microsatellite markers used for genotyping were in (Table 2.1). Oligoneucleotide sequences to amplify microsatellite markers can be obtained from The Genome Database (http://www.ncbi.nlm.nih.gov). Haplotypes were constructed and analyzed for linkage or exclusion to known loci. The basic concept of the technique to find genes involved in infrequent recessive traits is thus to examine for regions of homozygosity that are shared by different affected individuals.

1.2 Homozygosity Mapping

Homozygosity mapping is an effective gene mapping technique relevant to rare recessive conditions in inherited populations. Certainly, the method taking advantage of the fact that inherited affected people are probable to have two recessive duplicates of the disease allele from a common progenitor. Since small chromosomal regions tend to be transferred whole, affected individuals will also have identical-by-descent alleles at markers situated close to the

disease locus and thus will be homozygous at these markers. The homozygosity maps are used to detect genes that are involved in rare recessive characters is thus to find the regions of homozygosity that are shared by different affected people.

1.3 Hereditary Human Hair Loss

Hereditary human hair loss has many forms collectively called Alopecia. Alopecia is one of the genetic disorder. Alopecia is generally derived from Greek word meaning baldness or hair loss. Alopecia is a multifactorial disorder, association between genetical and environmental factors that causes Alopecia (Green and Sinclair, 2000). Ophiasis is example of this disorder, in which hair loss affects the occiput scalp cadaver hairs, nail pitting, frequently reduce the diagnosis straightforward (Gilhar *et al.*, 2012). Circumstantial evidence suggest that Alopecia is autoimmune disease where hair follicle from producing hair fibre is prevented by cell of own immune system (Mehran *et al.*, 2012).

Hair follicle are formed from different types of keratinocytes and trichocytes due to the mesenchymal epithelial interaction. As a result of this continued interaction hair follicle is retained in a cyclical manner between growth, regression, and quiescence in adulthood (Schmidt-ullrich and Paus, 2005). The major defects reported to be related with partial or total absence of hair, either singly or in different combination, include mental retardation, dwarfism, nail dystrophy, epilepsy, total or partial anodontia, impaired sweating, hyperkeratosis, retinas pigmentation (Pinheiru and Freire, 1985; Wali *at al.*, 2007). Inheritance pattern is usually autosomal recessive in familial case, but in certain families' autosomal dominant and X-linked recessive inheritance have also been reported (Aslam *et al.*, 2004). The mode of inheritance was thought to be autosomal dominant with variable penetrance. Alopecia occurring in monozygotic twins have been also reported (Amer *et al.*, 2009).

On different human chromosomes eight isolated autosomal recessive loci of Alopecia have been mapped and in most of such forms the resultant genes have been identified till now. Mutation in hairless (HR) gene which is mapped on chromosomes 8q21 responsible for development Congential atrichia (MIM 203655), hair on scalp and body are completely absent, are the features of congential atrichia and there has been also evidence of presence of papules frequently on scalp (Ahmad *et at.*, 1998; Nothen *et al.*, 1998). There are three types of localized inherited hypotrichosis include LAH1 (MIM 607903) on chromosomes 18q12.1 (Rafique *et*

al., 2003). LAH2 (MIM609167) on chromosome 3q27.2 (Aslam *et al.*, 2004) and LAH3 (MIM 611452) on 13q14.11-q21.32 (Wali *et al.*, 2007).

Mutation in desmoglein4 (DSG4, MIM 607892) as a result, three mentioned types have been developed (Kljuic *et al.*, 2003; Rafiq *et al.*, 2004). Lipase-H (LIPH, MIM 607365) (Kazantseua *et al.*, 2006;Ali *et al.*, 2007) and G-protein coupled receptor (P2RY5, MIM 609239) gene (Pasternack *et al.*, 2008;Tariq *et al.*, 2009; Azeem *et al.*, 2008; Shimomura *et al.*, 2008) when desmoglein-3 (DSC3, MIM 600271) gene mutated which is present on chromosome 18q21.1 that is responsible for inherited hypotrichosis along repeated skin vesicles (MIM 613102) (Ayub *et al.*, 2009). Spares scalp hair is another form autosomal recessive of hypotrichosis that has been plotted on chromosomes 10q11.23-22.3 but no gene has been identified (Naz *et al.*, 2010). Digenic inheritance reported first time in autosomal recessive hypotrichosis phenotype and another novel locus mapped on chromosome 12q21.2-q22 but no gene has been identified yet for it (Basit *et al.*, 2011).

Table: 1.1: Various Types of Alopecia and their causative genes.

S/N	Types	Clinical symptoms	Reported gene /locus	References
1	APL	Patient have no hair on axilla, scalp and body. Eye brows and eyelashes are completely absent.	HR	(Ahmad *et al.*, 1993)
2	Monilethrix	In mild form only nape the neck and occiput are affected, but in severe case eye brows, eyelashes, secondary hair, and entire scalp are involved	hBb6 DSG4	(Salamon and Schnyder, 1962), (Steven et at., 1996), (Birch-Machin e al., 1997), (Zlotogorski *et al.*, 2006)
4	Localized autosomal recessive hypotrichosis (LAH)	LAH is a recessive type of Alopecia. Hypotrichosis is restricted to arms, chest, legs, and scalp. Pubic and Axillary hairs are spares. The patient have normal sweating, hearing, nails, and teeths.	LAH1 LAH2 LAH3 LIPH P2RY5	Kljuic *et al.*, 2003; Whittock and Bower, 2003), (Jelani *et al.*, 2008), (Tariq *et al.*, 2009). (Ali *et al.*, 2007), (Azeem *et al.*, 2008)
5	(MUHH) Marie Unna hereditary hypotrichosis	Patient have abnormal hair at birth and lost progressively in adult stage. Patchy hair lost or complete baldness is the feature of (MUHH).	HR	(Unna, 1925), (vansteenel *et al.*, 1999; Cichon *et al.*, 2000; Lefevre *et al.*, 2000; Sreekumar *et al.*, 2000)
6	Alopecia with mental retardation syndrome (APMR)	Patient of this syndrome shows absence of axillary, scalp, pubic, eye lashes, and eye brows hairs. Affected individual's shows mental retardation. They shows normal hearing, teeths, nails, and sweating.	APMR1 APMR2 APMR3	(Vogt *et al.*, 1998; John *et al.*, 2006; Wali *et al.*, 2007)

1.4 Complex hair disorders

The term complex hair disorder, is applied to common genetic disorder that are demonstrable under polygenic phenomenon. There are marked variations in the disease phenotype among affected individuals.

1.4.1 Androgenetic Alopecia (AGA)

Androgenetic Alopecia depends upon male androgens (AGA; MIM 109200). Androgenetic Alopecia follow a defined pattern W shape in male, in which thinning of hair take place progressively. In male early baldness of this ordinary type has been thought to be autosomal dominant and in female are autosomal recessive. If the female who transmit the trait is in heterozygous state, she is not affected, they show baldness if they are in homozygous state (Osborn, 1916; Synder and Yingling, 1935). Androgenetic Alopecia in male are referred as male pattern baldness (MPB), while in female it is referred as female pattern baldness. 50% of males are affected by the age of 50 years, and about 70% of males are affected by in the upper age (Norwood, 1975).

Nuclear receptor superfamily contain androgen receptor (AR). The action of androgen depends upon androgen receptor, which acts as ligand dependent transcription factor (Gelmann *et al.*, 2002). Mutation in the androgen receptor which results in androgen insensitivity (Gottlieb *et al.*, 2004). The pattern of inherited Androgenetic hair loss is not effected by deletion mutation in one of the allele of HR gene (Sprecher *et al.*, 2001). The presence of AR on X-chromosomes stressed the importance of maternal inheritance of Androgenetic Alopecia (Hillmer *et al.*, 2005).

There was significant association between Androgenetic Alopecia, AP and ectodysplasin anhidrotic2 receptor (EDA2R), but genetically and functionally still need to understand the role of these genes (Prodi *et al.*, 2008). Level of expression of AR gene in baldness can determined by mutation in or near AR gene. If any mutation take place in AR gene, result in the balding scalp.

1.4.2 Alopecia Areata

Alopecia areata (AA) is an autoimmune human disorder, inflammatory scalp and body hair loss disorder (AA; MIM 104000). The scalp has patchy hair which can ultimately involve the whole scalp, this condition is known as Alopecia totalis. If hair on the entire body is absent such condition is known as Alopecia universils. Alopecia areata affect the male and females in equal ratio of all ages (Price, 1991; Sawaya and Hordinsky, 1992; Schwartz and Janniger, 1997; Madani and Shapiro, 2000). It was recently assumed that the antigen associated with melanocytes can induced hair damage in Alopecia areata (Gilhar *et al.*, 2012) but identification of an autoantigen for AA is still unknown.

The onset of this disease can be sudden, the amount of hair follicle appear to remains unchanged in the initial phases of this disease; however, this figure diminished and reduction of the anagen hair follicle is perceived in the more advanced stages (McDonagh and Messenger, 1996). The difference in the severity of AA depends on account of disease, early stage, individual and family history (Xiao *et al.*, 2005).

Two new positive association have been described. MHC class 1 chain-related gene A (M1CA) have also been identified both as a potential candidate gene and as a fragment of an extended HLA haplotype. It has an important role in susceptibility and severity of AA (Barahmani *et al.*, 2006). Another gene (PTPN22) that encode the lymphoid protein tyrosine phosphate shows to be associated with severe forms of AA (Kemp *et al.*, 2006).

The study for Alopecia was done in Turkey, which is founded on poor predictive reasons. It was conclude that primarily, AA and ophiasis were severely linked together, which indicates that these parameters were poor prognostic factor for AA. Family history related only through early start of disease and is more common in females as compared to males (Kavak *et al.*, 2008).

Alopecia areata is multifactorial disorder, environmental and genetic disorders association, which result in the development of disease (Green and Sinclair, 2000; McElwee and Hoffmann, 2002; McElwee *et al.*, 2001; McDonagh and Tazi Ahnini, 2002). Although no distinct resources of confirmation for polygenic inheritance exist for Alopecia areata.

Various cytokines like TNF-α, interleukins, and TNF-$_{\gamma}$ were studied extensively for AA. It was hypothesized that cytokines and their pathways plays an important role in the pathogeny of AA. It was suggested that T-cell mediated autoimmune response was activated by internal or external stimulus. Inability to mark medicinally a single cytokine

suggest that the pathogenicity of AA is either so multifactorial or it was little bit known about it (Gregoriou *et al.*, 2010).

1.4.3 Alopecia Universalis

Alopecia Universalis is characterized by general scalp and body atrichia with papular lesions (Ahmad *et al.*, 1998). Atrichia with papular lesion (APL) is an autosomal recessive form of Alopecia Universalis (MIM 209500). The individuals have normal hair at birth, but the hair are completely shed during first week or 2-3 months of the life and never regrows. The patient shows the presence of papules cyst filled with keratin like substance on their face and scalp (Zlotogorski *et al.*, 2002; Kruse *et al.*, 1999). The patient have normal hearing, sweating, and nails and mentally fit.

Mutation in HR gene plays an important role in the development of (APL). There are more than twenty different mutations identified in hair less gene that were present on entire HR gene. Mutation in HR gene results in the disintegration of hair follicle and new hair never regrows (Panteleyev *et al.*, 1998). Thyroid hormone receptor regulate HR gene at transcription level (Thompson and bottcher, 1997). The presence of HR gene products in the nucleus and their interaction with TR, HDAC, and VDR is of great importance as regards to hair growth cycle (Thompson and Bottcher, 1997; Potter *et al.*, 2001; Potter *et al.*, 2002; Djabali and Christiano, 2004; Hsieh *et al.*, 2003). HR, TR and VDR are consider to have important role in controlling hair cycle pathway. Different types of mutation in HR gene have been identified in several families with atrichia with papular lesions, these mutation are missense, deletion mutation and insertion mutations. These mutation have been identified in close-kin unions in homozygote (Paradisi *et al.*, 2005).

1.5 Bioinformatics Analysis

Computational analysis has been carried out in the current study to analyze its wild and mutant type of HR gene. Using bioinformatics approach, structure prediction, conservation analysis and docking studies were executed to analyze the mutation. Computational analysis has opened many insights into disease understanding in low cost and time and it gives a way towards accurate diagnosis and better therapeutic strategies. Apart from mutational studies, analysis about HR normal structure, conformation, interaction site and

residues actively involved in interaction during protein-protein docking is very helpful and can be used to develop drug therapy for this disease. Through STITCH4 database protein interaction framework was analyzed and closest relative functional partner was identified (Kuhn *et al.*, 2012). Through protein modelling we can also predict normal and mutated protein structure and also find out the effect of mutation on protein structure and function.

Table.1.2: Functions of reported genes.

Candidate gene	Functions
HR	This gene encode for protein which is involved in hair growth cycle. This protein function as a transcriptional co-repressor of several nuclear receptor, containing thyroid hormone receptor and vitamin-D receptor and it interact with histone deacetylase
DSG4	The DSG4 gene provides instruction for making a protein called desmoglein4 (DSG4). This protein is found in specialized structure called desmosome that are located in membrane surrounding certain cells. Desmosomes helps attach cells with one another and play a key role in communication between cells. The DSG4 protein in the cell in different region of hair follicle, including the inner compartment of the hair (shaft) known as cortex. Desmosome provide strength to the hair and are involved in signalling between neighbouring cells within the hair shaft.
LIPH	The LIPH gene provide instruction for making enzyme lipase-H. This enzyme is found in many cells and tissues, where it breaks down the molecule phosphophatidic acid (LPA) and free fatty acids. LPA has multiple receptor and play role in different functions, such as cell growth, division, and (apoptosis).
P2RY5	The protein encoded by this gene belongs to family of G-coupled protein receptor, uridine and adenosine nucleotides are important for activation of these genes. This gene aligns with an interior intron of the retinoblastoma susceptibility gene in contrary alignment, alternate splice result in several alternatives.

Chapter#2

MATERIALS AND METHODS

Materials and Methods

2.1 Families Selection

Two families of alopecia were ascertaining in the present study. The study was approved by the Board of Study (BOS), International Islamic University. Informed written consents were obtained from family members. All the research work was performed in Human Molecular Genetics Lab, Department of Bioinformatics and Biotechnology, International Islamic University Islamabad.

2.1.1 Families Clinical Evaluation

Both families were visited at their place of residence. Members of families were interviewed to get all maximum information about the disease. The clinical evolution was performed at the local hospital by Dermatologist.

2.1.2 Pedigrees Drawing

Pedigrees are used to depict the inheritance patterns. 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 indicates consanguineous marriages.

2.1.3 Blood Collection and EDTA Tube Labelling

The blood samples from both the normal and affected individuals were collected by using 10 ml syringes and transferred in standard potassium EDTA 10 ml BD vacationer (ACD). Before the collection of blood a written consents from obtained from the entire individual. Family members were given details about the research study. EDTA tubes were labelled with permanent markers.

2.1.4 DNA Extraction Method

Human blood samples collected in field were processed by organic method.

2.1.4.1 Standard Organic Method in Lab

Genomic DNA extraction from human blood samples was performed using standard phenol-chloroform procedure.

1. 0.5 ml of blood was taken in a 1.5 ml eppendorf tube and mixed with equal volume of solution A. Solution was kept at room temperature for 15-20 minutes and shaken the Eppendorf tubes as well as moved them upside down after regular intervals in order to mix the blood in solution A. The tubes were centrifuged at 13,000 rpm for 1.12 minute in a micro centrifuge (Model 1-14, Sigma, Germany 2010-11).
2. Discarded the supernatant. Pellet was re-suspended in 400µl of solution A and vortexed (vortex Model: MX-S GR-Biotech UK 2010-11) the tubes for 15 minutes in order to mix solution A with pellet. The solution was then centrifuged again at 13,000 rpm for 1.12 minute.
3. Discarded supernatant with care. Added 400µl of solution B, 12µl of 20% SDS solution and 25µl of proteanase –K (25mg/ml) Fermentas. Vortexed the eppendorf tubes for about 15 seconds to mix the solutions and the pellet. After that incubated them for 6 hours at 37°C (Memmert incubator Model INB-400, Germany 2010-11).
4. After the incubation period added 500µl of solution D and mixed them by shaking vigorously. Then centrifuged them at 13,000rpm for 1.12 minutes.
5. Collected 600µl of supernatant in new eppendorf tube. Added equal volume of solution C and left the tubes for 3-4 minutes after slightly shaking them. Then centrifuged again at 13,000rpm for 1.12 minutes.
6. Collected 800µl of supernatant in new eppendorf tube and added 700 µl of chilled iso-propanol. The tubes were left for 3-5 minutes in order to let DNA precipitate at bottom. Then again centrifuged at 13,000rpm for 1.12 minutes.
7. Discarded the supernatant and added 50µl of 70% ethanol. After the evaporation added 20µl of 100% ethanol in order to remove any impurities from the DNA.
8. Added 100µl of TE buffer and stored it at 4°C.

The following solutions were used for DNA extraction.

1. **Solution A:** 0.32 M sucrose, 10 mMTris pH 7.5, 5 mM MgCl2, 1% Triton X-100.

2. **Solution B:** 10 mMTris pH 7.5, 400 mMNaCl, 2 mM EDTA pH 8.0.

3. **Solution C:** Buffered phenol.

4. **Solution D:** Chloroform (24 ml), Isoamyl Alcohol (1ml).

2.1.4.2 Gel Electrophoresis

Products of DNA extraction were analyzed on 1% agarose gel prepared by melting 0.5 g of agarose in 50 ml 1 X TBE (89 mM Tris-Borate, 2.5 mM EDTA, pH 8.3) in a microwave oven for one minute. 1 µl of ethidium bromide solution (0.1 µg/ml final concentrations) was added to the gel to facilitate the visualisation of DNA after electrophoresis stain DNA. After gentle shaking, the mixture was poured into the gel caster. Gel was solidified after 20-25 minutes.

Amplified PCR products were analysed on 2% agarose gel which was prepared by using 1 g of agarose while other parameters were kept the same as above.

DNA/PCR samples (2 µl) were mixed with 3 µl Bromophenol Blue dye (0.25% bromophenol blue with 40% sucrose solution) and loaded into the wells. Electrophoresis was performed at 120 volts (400 mA) for 30 minutes in 1 X TBE buffer. Amplified products were visualized by placing the gel on Dolphin-Doc, WEALTEC, USA 2010-2011

2.2 Genotyping

To identify the gene defects in the alopecia family's initial search for linkage was carried out by using polymorphic microsatellite markers corresponding to candidate genes involved in hair loss. Genotyping was done by using standard method by using micro-satellite markers. Micro satellite markers were from the LDB genetic maps Psychiatric University Hospital Zurich, Switzerland.

2.2.1 Polymerase Chain Reaction

Polymerase chain reaction (Labnet Multigene International TC9600-G) was performed in 0.2 ml tubes containing 20µl total reaction mixture. The reaction was prepared by adding

2 µl sample DNA (40 ng),

2 µl 10 X buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl),

2 µl MgCl2 (25 mM),

0.2 µl dNTPs (10 mM),

1.5 µl of each forward and reverse primer (0.1 µM)

0.2 µl Taq DNA polymerase (5 unit /µl, Fermentas, USA)

10.6 µl PCR water

The reaction mixture was vortexed for few seconds for thorough mixing. PCR was carried out for 35 cycles with the help of these following thermal cycling programming 95°C for 4 mints, 55°C for 40 sec, 72°C for 40 sec, followed by final extension at 72°C for 7 mins.

2.2.2 Polyacrylamide Gel Electrophoresis

Non-denaturing polyacrylamide Gel (8%) were prepared to resolve the amplified PCR products. Gel solutions was made in a 250 ml conical flask and was poured in the space between the two glasses plates separated at a distance of 1.5 mm. After placing the comb, it was allowed to polymerize for 45-60 minutes at room temperature. Samples were mixed with 5 µl of loading dye (0.25% bromophenol blue with 40% sucrose) and loaded into the wells. Electrophoresis was performed in a vertical gel tank model V16-2 (Life Technologies, USA) at 130 volts (60 mA) for 120-150 minutes depending upon the size of amplified length. The gel was stained with ethidium bromide solution (10 mg/ml) and visualized on Gel Doc system (Dolphin Doc Wealtec).

Composition of 8% Polyacrylamide Gel is as under;

13.5 ml 30% Acrylamide solution (29 g polyacrylamide, 1 g NN'-Methylene-bisacrylamide)

5 ml 10 X TBE

350 µl 10% Ammonium persulphate (APS)

20 µl TEMED (N, N, N', N'-Tetra Methyl Ethylene Diamine),

31.50 ml distilled water

2.3 Linkage Studies of Reported 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. Genotyping was performed in families with microsatellite markers associated with alopecia HR gene on chromosome eight. After linkage of markers with disease gene, gene specific primers were used for sequencing of the mutated gene.

2.4 Bioinformatics Analysis

HR protein structure can be predicated by using psipred (McGuffin *et al.*, 2000) an online tool. Sequence was retrieved through OMIM database (MIM: 602302) in fasta file formate. T-Coffee (**T**ree-based **C**onsistency **O**bjective **F**unction **F**or alignment **E**valuation) was used for MSA (Multiple Sequence Alignment) to show the consistency of sequence among various ortholog species of Human HR (Notredame *et al.* 2000). Through STITCH4 database protein interaction framework was analyzed and closest relative functional partner was identified.

Table 2.1: Set of Microsatellite markers of *HR* used for linkage analysis.

Microsatellite markers	Primers(forward+reverse)	Annealing Temp.
D8S1754	CAGGGAAGTCTCGGTTTG	55° C
	TCAGGGACACGATTCAGC	55°C
D8S552	CCTGTACCATACCCCTGTATC	55°C
	AAGGTTTGAATCTCTCAGTGG	55°C
D8S1106	TTGTTTACCCCTGCATCACT	55°C
	TTCTCAGAATTGCTCATAGTGC	55°C
D8S1145	TGCTAACTGGCACGGTCAC	55°C
	CAATCCCAGTAATCTATAACTTCA	55°C
D8S1827	GACAGAATCATGTGGCCTTT	55°C
	TTTTGTAAAATGTAAAATTGGCTTT	55°C
D8S511	TTGTCCCTGTTGGCAGA	55°C
	TGATTTTTGTGTCCTGAAACTTA	55°C
D8S549	TCTGATTAGCCAACCTCTAACA	55°C
	CTTACAAACACCACTATGCATG	55°C
D8S261	TGCCACTGTCTTGAAAATCC	55°C
	TATGGCCCAGCAATGTGTAT	55°C
D8S1790	TGACTTTGGAACCGTGG	55°C
	CTGCAAATAAGAGATGTTAGGC	55°C
D8S1704	TCTGGGTGATAGAGCAAGAC	55°C
	AGCTAAAAATTGACACTTGTTTACA	55°C
D8S1731	CCAAGCAAATCATGGAAATC	55°C
	AGCAAACTTATCCCACAAGG	55°C

Table 2.2: Primers sequences of *HR* gene, used for *HR* gene sequencing

EXON	PRIMERS(Forward + Reverse) 5′ __3′	PRODUCT SIZE (bp)	ANNELLING 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

Chapter#3

RESULTS

3. RESULTS

3.1 Families under Investigation

Two families (AP5 and AP10) were identified and recruited for finding the genetic causes of disease. Large consanguineous families are suitable for homozygosity mapping of recessive disorders and disease gene identification.

3.1.1 Family AP5

Family AP5 is diagnosed as Alopecia Universalis. Family includes six affected members i.e three male affected members (IV:3, IV:5 and IV:12) and three female affected members (V:3, V:4 and V:8). Four available members are normal (III: 5, IV: 1 and V: 5, V: 7). Ten members in the pedigree ((Figure 3.1) were willing to participate in our study. The affected individuals shared common phenotype (Figure 3.2) and have age between 15-20 years at the time of initial examination. Blood samples were drawn after pedigree construction.

3.1.2 Family AP10

Family AP10 belongs to Sawabi, district Mardan, Khyber pakthoonkhawa. Family AP10 is also classified as Alopecia Universalis. Three affected male members in family (III: 2, III: 7 and IV: 1) and two male members are normal (IV: 5 and IV: 8). Six affected individuals (I:1, II:3, III:3, III:8, III:10 and IV:2) were not available at the time of sampling. Affected individuals have no hair on their all body and do not have any other abnormalities associated with Alopecia Universalis (Figure 3.3). Blood samples were collected from participating family members after informed written consents.

3.3 Linkage Analysis through Homozygosity Mapping

Microsatellite markers were used for linkage studies and genotype for diseased members and normal members of the families were constructed and given below under each family.

3.3.1 Family AP5

In family AP5, ten DNA samples (III:5, IV:1, IV:3, IV:5, V:3, V:4, V:5, V:7, V:8, and V:12), including six affected and four normal individuals were genotyped. In this, family linkage was establish with two Microsatellite markers i.e D8S1106 and D8S1827 at *HR* locus (Figure 3.6 and 3.7). While affected individual's shows heterozygous Pattern with Microsatellite markers D8S552, D8S511 and D8S549 (Figure 3.5, 3.9 and 3.10). After genotyping the family AP5, the microsatellite markers that are tightly-linked on targeted genes for family AP5, the gene is HR gene. The allelic pattern were determined by Haplotyping of the family (Figure 3.14).

3.3.2 Family AP10

In family AP10, five DNA samples (III:2, III:7, IV:1, IV:5 and IV:8),including three affected and two normal individuals were selected for genotyping the markers linked to the candidate genes. In family linkage was not establish at *HR* locus Microsatellite markers. Exon sequencing is required for the family to search the locus in this family.

3.4 Mutational Screening

In family AP5 linkage was established with Locus harbouring the HR gene. Mutational screening of one affected member (AP5-6) and one normal member (AP5-8) was designed. In mutational screening of *HR* gene, sequence of **exon 8** shows deletion of adenosine at **c.2123** (Figure 3.15). Consequence of the changed frame, a premature stop codon can be found at 194 instead of 483 aa. Identified mutation is not reported in literature till yet.

3.5 Bioinformatics Analysis

3.5.1 Model Predication for Normal and Mutated HR Protein

We predicted normal and mutated structure of HR protein for our novel mutation (Figure 3.16). According to Psipred results for protein secondary structure prediction, it has been predicted that in normal HR structure there are 19 helixes, 7 strands and 28 coils. In mutated HR number of helixes, strands and coils gets affected and becomes 4, 1, and 6, respectively. Due to frameshift mutation, size of protein and number of structure features gets affected. Mutation

not only affects structure and conformation but also function of protein, leading to the diseased state.

Stitch4 was used to identify functional partner of *HR* (Figure 3.17). The closest one with highest interaction score of 0.824 with *HR* was selected for further analysis and considered as protein ligands i.e. HDACI. Histone deacetylase1 is responsible for the deacetylation of lysine residues on the N-terminal parts of the core histones (H2A, H2B, H3 and H4) and plays an important role in the regulation of transcription, progression of the cell cycle and in events of development. We assume both normal and mutated structures of HR can be docked with this highly interacting ligand protein (HDACI).

3.5.2 Sequence Conservation Studies

Multiple sequence alignment was carried out using T-Coffee to find the conservation of the sequence region that got affected due to mutation. Results showed a good conservation of overall sequence and deleted region which also justifies its importance (Figure 3.18).

Pedigree of Family AP5

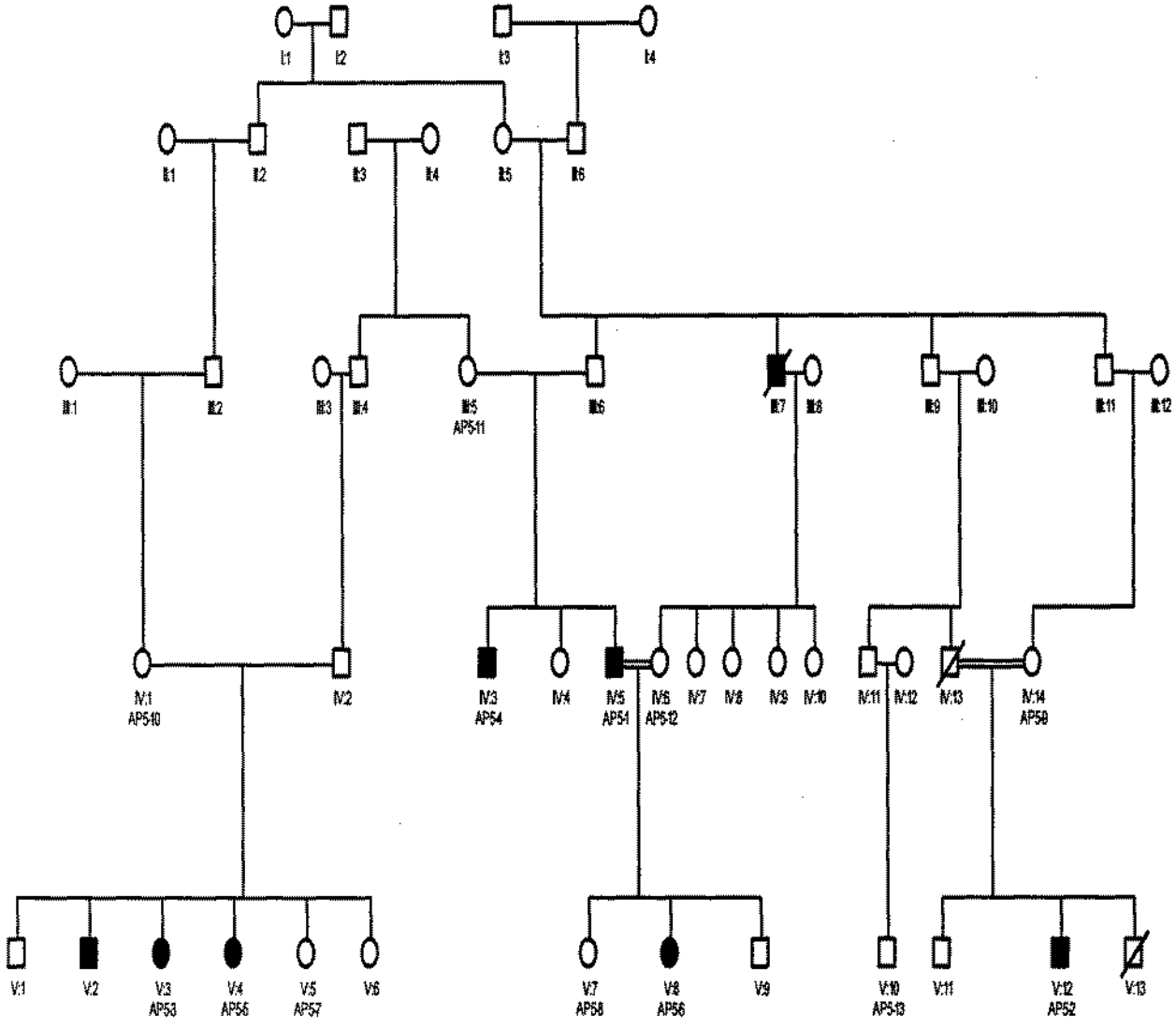


Figure 3.1: Pedigree of family **AP5**. Circles represent females and squares shows males. Filled squares represents affected males and filled circles represents affected females, where double line indicates consanguineous marriages.

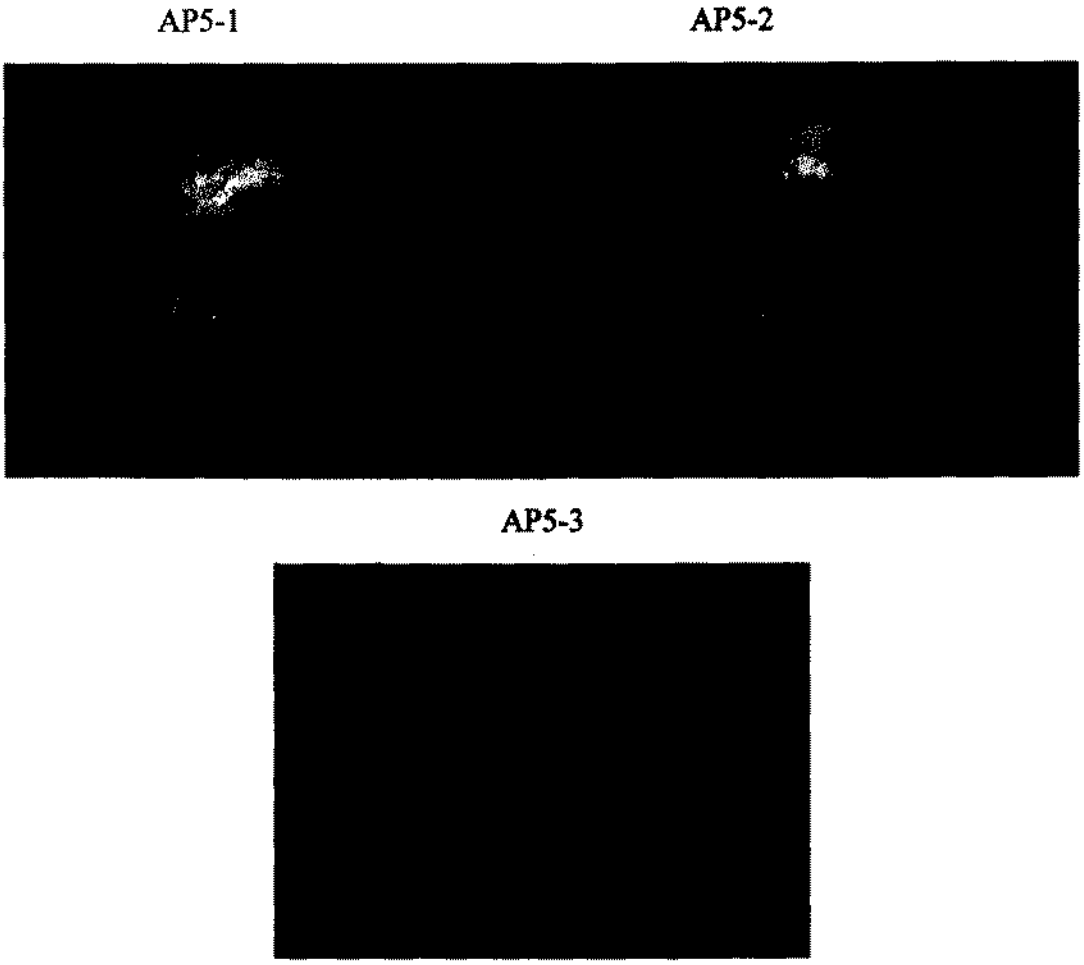


Figure 3.2: Phenotypic appearance of Patient (AP5-1, AP5-2 and AP5-3). Physical appearance of the affected individuals from family showing complete loss of hair from eye brows, eyelashes and absence of hair from all the body.

Pedigree of Family AP10

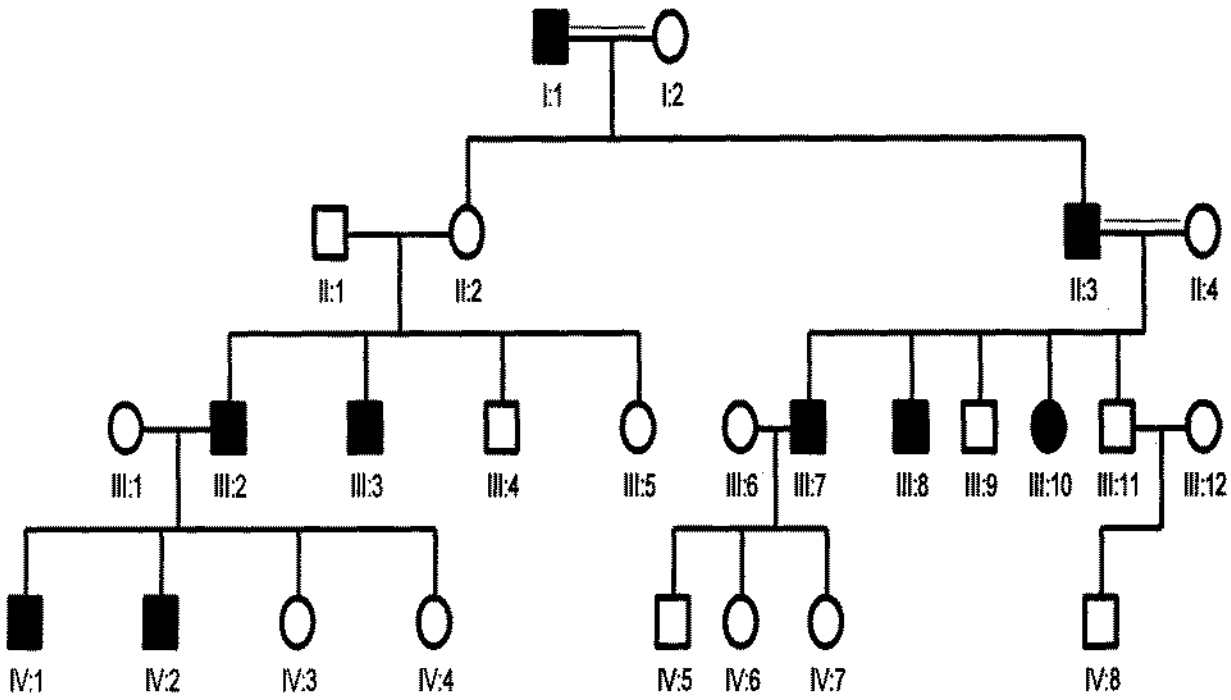
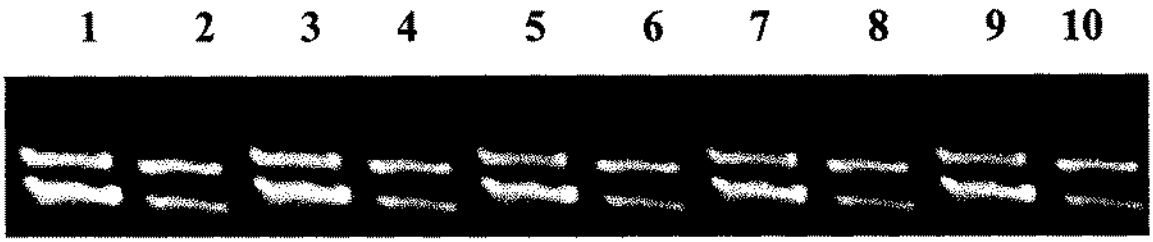


Figure.3.3: Pedigree of family **AP10**. Circles represent females and squares shows males. Filled squares represents affected males and filled circles represents affected females, where double line indicates consanguineous marriages.

Figure 3.4: Phenotypic appearance of Patient AP10-3 (IV: 2) showing Alopecia Universalis.

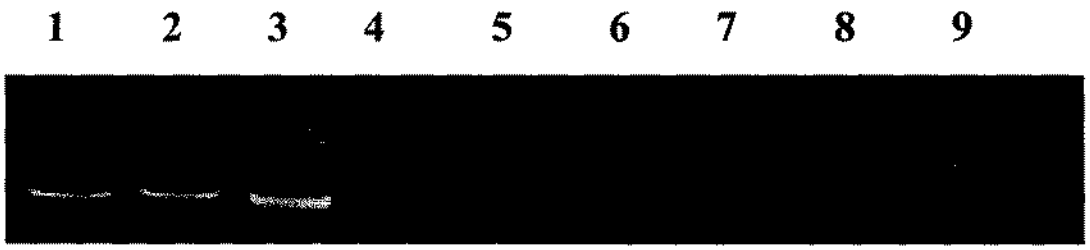
RESULTS OF FAMILY AP5



Family AP5

1. IV: 5 Affected
2. V: 12 Affected
3. V: 3 Affected
4. IV: 3 Affected
5. V: 4 Affected
6. V: 8 Affected
7. V:5 Normal
8. V: 7 Normal
9. IV:1 Normal
10. III: 5 Normal

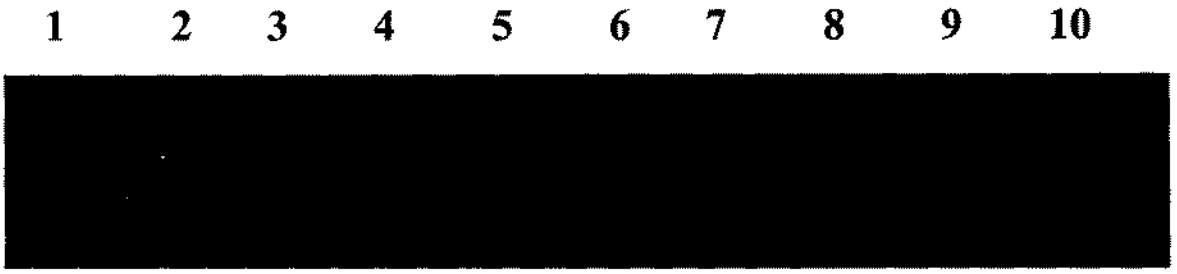
Figure 3.5: Electropherogram of ethidium bromide stained 8% non-denaturing polyacrylamide gel for marker D8S552 at 25.78 cM. The roman numbers and Arabic numbers refers to the individuals in pedigree.



Family AP5

1. IV: 5 Affected
2. V: 12 Affected
3. V: 3 Affected
4. IV: 3 Affected
5. V: 4 Affected
6. V: 8 Affected
7. V:5 Normal
8. V: 7 Normal
9. IV:1 Normal
10. III: 5 Normal

Figure 3.6: Electropherogram of ethidium bromide stained 8% non-denaturing polyacrylamide gel for marker D8S1106 at 27.23 cM. The roman numbers and Arabic numbers refers to the individuals in pedigree. The sample AP5-10 is not shown in figure.



Family AP5

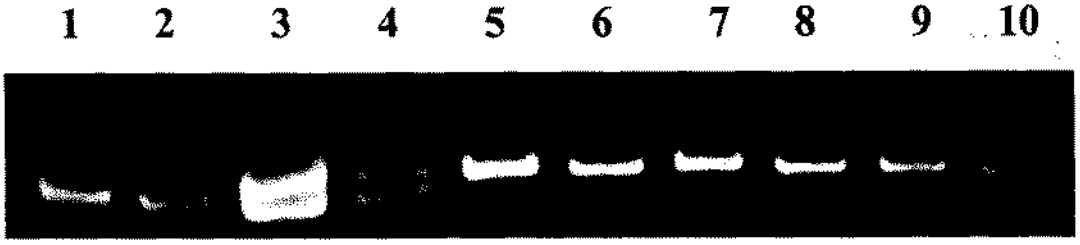
1. IV: 5 Affected
2. V: 12 Affected
3. V: 3 Affected
4. IV: 3 Affected
5. V: 4 Affected
6. V: 8 Affected
7. V:5 Normal
8. V: 7 Normal
9. IV:1 Normal
10. III: 5 Normal

Figure 3.7: Electropherogram of ethidium bromide stained 8% non-denaturing polyacrylamide gel for marker D8S1827 at 27.67 cM. The roman numbers and Arabic numbers refers to the individuals in pedigree.

Family AP5

1. IV: 5 Affected
2. V: 12 Affected
3. V: 3 Affected
4. IV: 3 Affected
5. V: 4 Affected
6. V: 8 Affected
7. V:5 Normal
8. V: 7 Normal
9. IV:1 Normal
10. III: 5 Normal

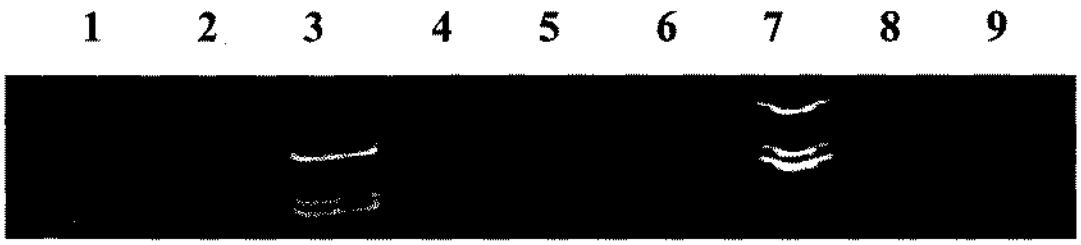
Figure 3.8: Electropherogram of ethidium bromide stained 8% non-denaturing polyacrylamide gel for marker D8S1754 at 28.28 cM. The roman numbers and Arabic numbers refers to the individuals in pedigree.



Family AP5

1.	IV: 5 Affected	4.	IV: 3 Affected	7.	V:5 Normal
2.	V: 12 Affected	5.	V: 4 Affected	8.	V: 7 Normal
3.	V: 3 Affected	6.	V: 8 Affected	9.	IV:1 Normal
				10.	III: 5 Normal

Figure 3.9: Electropherogram of ethidium bromide stained 8% non-denaturing polyacrylamide gel for marker D8S511 at 30.15 cM. The roman numbers and Arabic numbers refers to the individuals in pedigree.

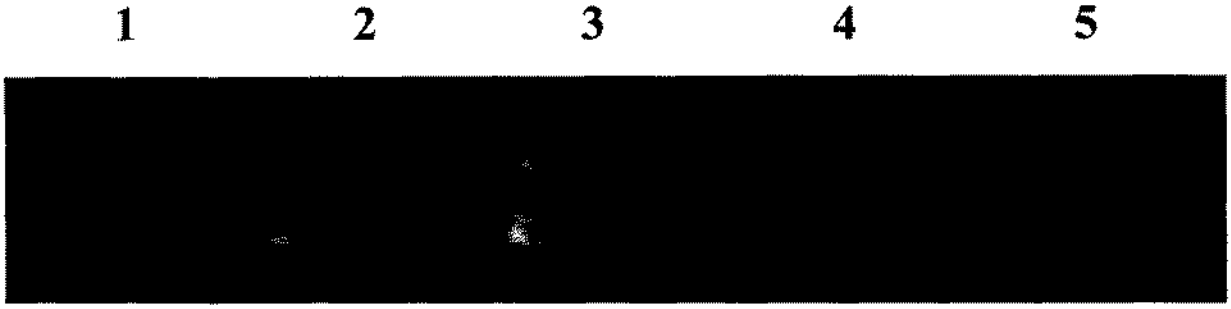


Family AP5

1.	IV: 5 Affected	4.	IV: 3 Affected	7.	V:5 Normal
2.	V: 12 Affected	5.	V: 4 Affected	8.	V: 7 Normal
3.	V: 3 Affected	6.	V: 8 Affected	9.	IV:1 Normal
				10.	III: 5 Normal

Figure 3.10: Electropherogram of ethidium bromide stained 8% non-denaturing polyacrylamide gel for marker D8S549 at 30.41 cM. The roman numbers and Arabic numbers refers to the individuals in pedigree. The sample AP5-10 is not shown in Figure.

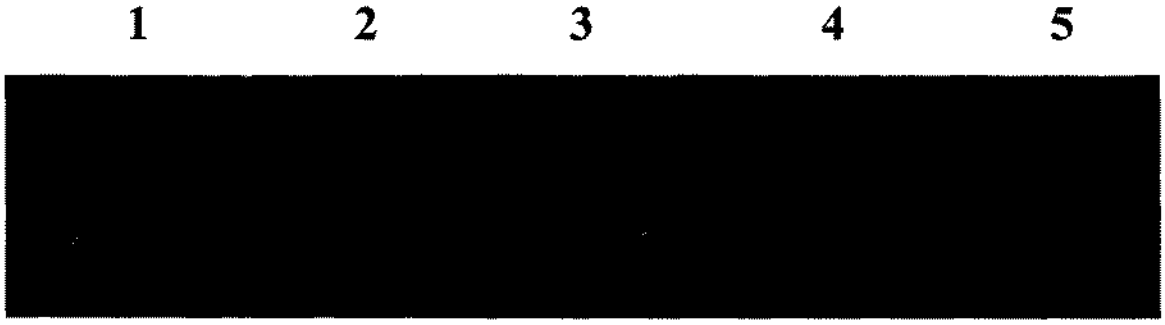
Results of FamilyAP10



Family AP10

1. III: 2 Affected
2. III: 7 Affected
3. IV: 1 Affected
4. IV: 5 Normal
5. IV: 8 Normal

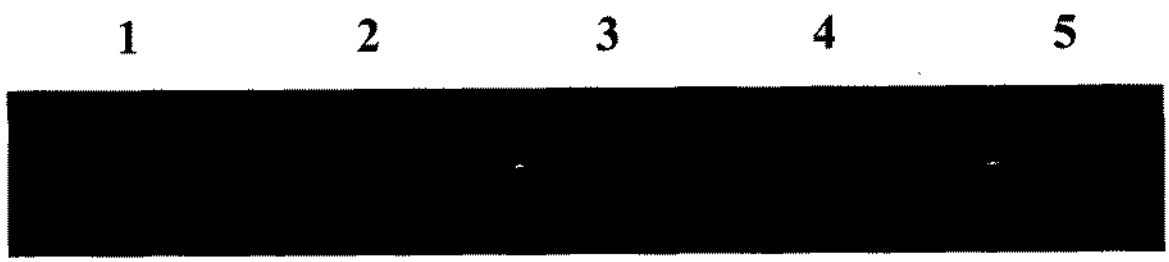
Figure.3.11: Electropherogram of ethidium bromide stained 8% non-denaturing polyacrylamide gel for marker D8S1731 at 27.95 cM. The roman numbers and Arabic numbers refers to the individuals in pedigree.



Family AP10

1. III: 2 Affected
2. III: 7 Affected
3. IV: 1 Affected
4. IV: 5 Normal
5. IV: 8 Normal

Figure 3.12: Electropherogram of ethidium bromide stained 8% non-denaturing polyacrylamide gel for marker D8S1790 at 28.93 cM. The roman numbers and Arabic numbers refers to the individuals in pedigree.



Family AP10

1. III: 2 Affected
2. III: 7 Affected
3. IV: 1 Affected
4. IV: 5 Normal
5. IV: 8 Normal

Figure3.13: Electropherogram of ethidium bromide stained 8% non-denaturing polyacrylamide gel for marker D8S1827 at 30.15 cM. The roman numbers and Arabic numbers refers to the individuals in pedigree.

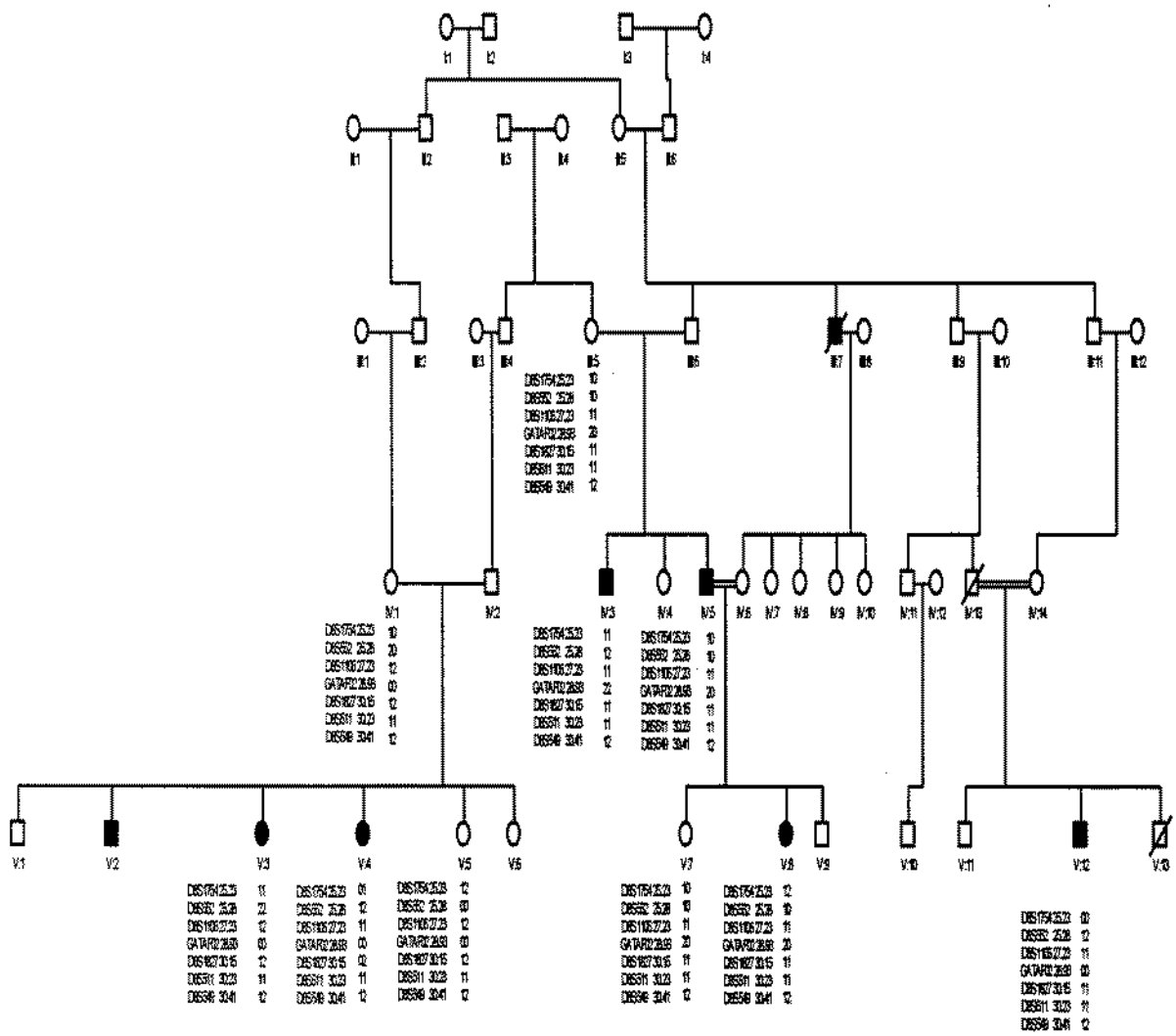


Figure 3.14: Haplotype of family AP5 showing allele pattern:

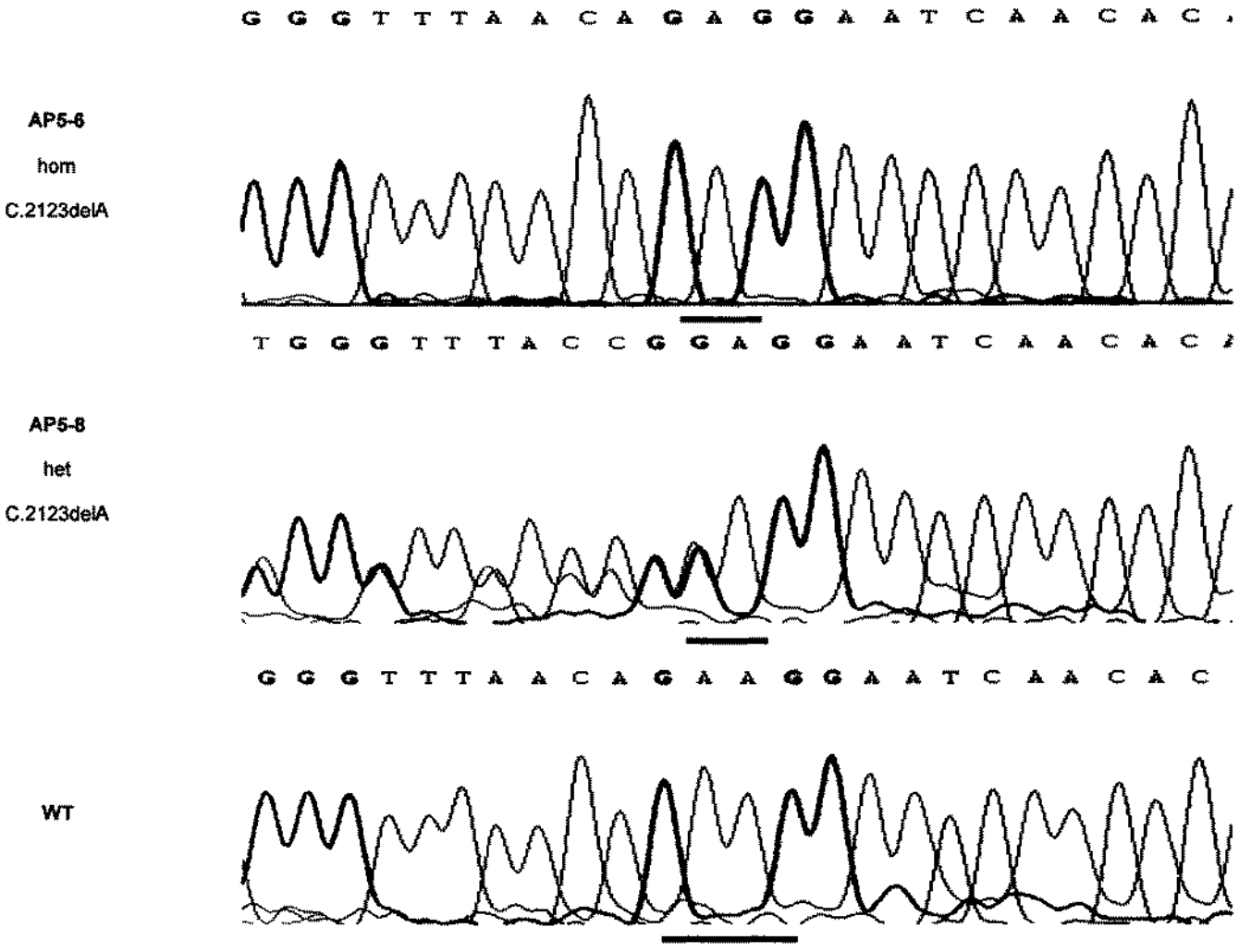


Figure 3.15: DNA sequences of **exon 8** displaying the deletion of the adenosine at **c.2123** of the *HR* gene. The deleted base is underlined in black.

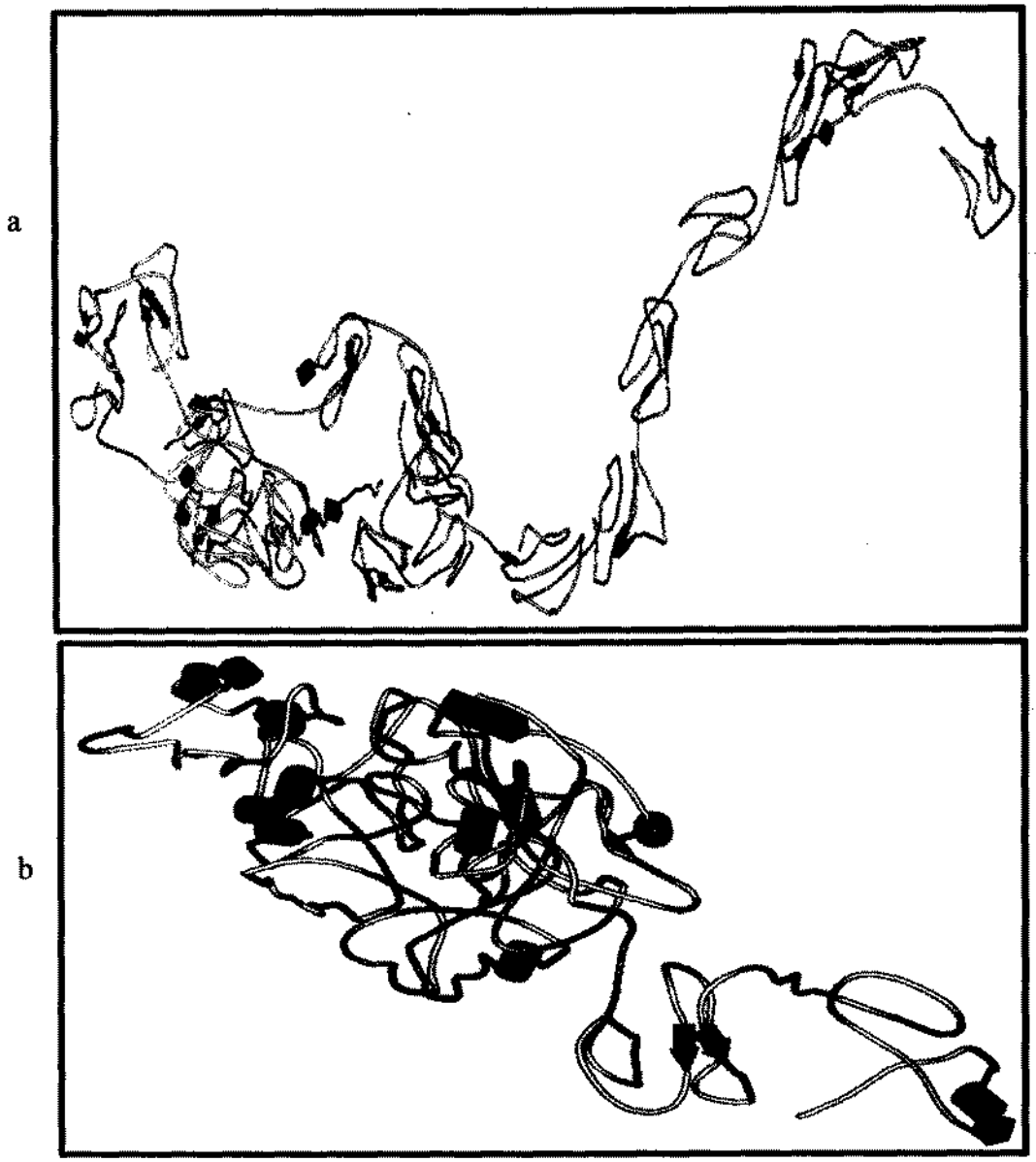


Figure.3.16: a) Predicted 3D-Structure of Human HR, b) Predicted 3D-Structure of Mutated HR. Structures shown via Viewerlite v5.0 (software), color by secondary type and schematic display style.

Stitch4 Results

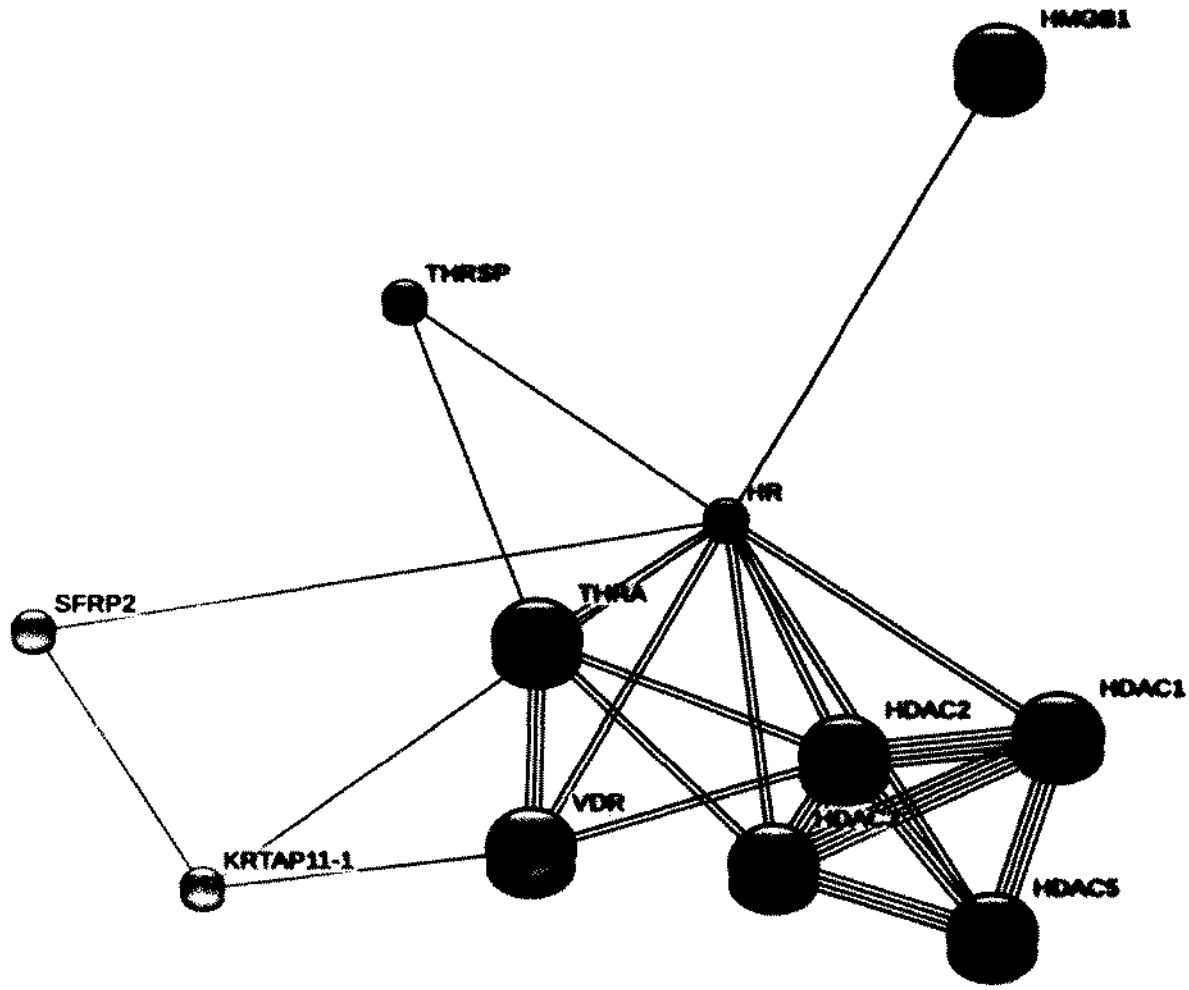


Figure.3.17: Stitch4 Results: Stronger links are represented by thicker lines. Protein-protein interactions are shown in blue, chemical-protein interactions in green and interactions between chemicals in red.

Sequence Conservation Studies

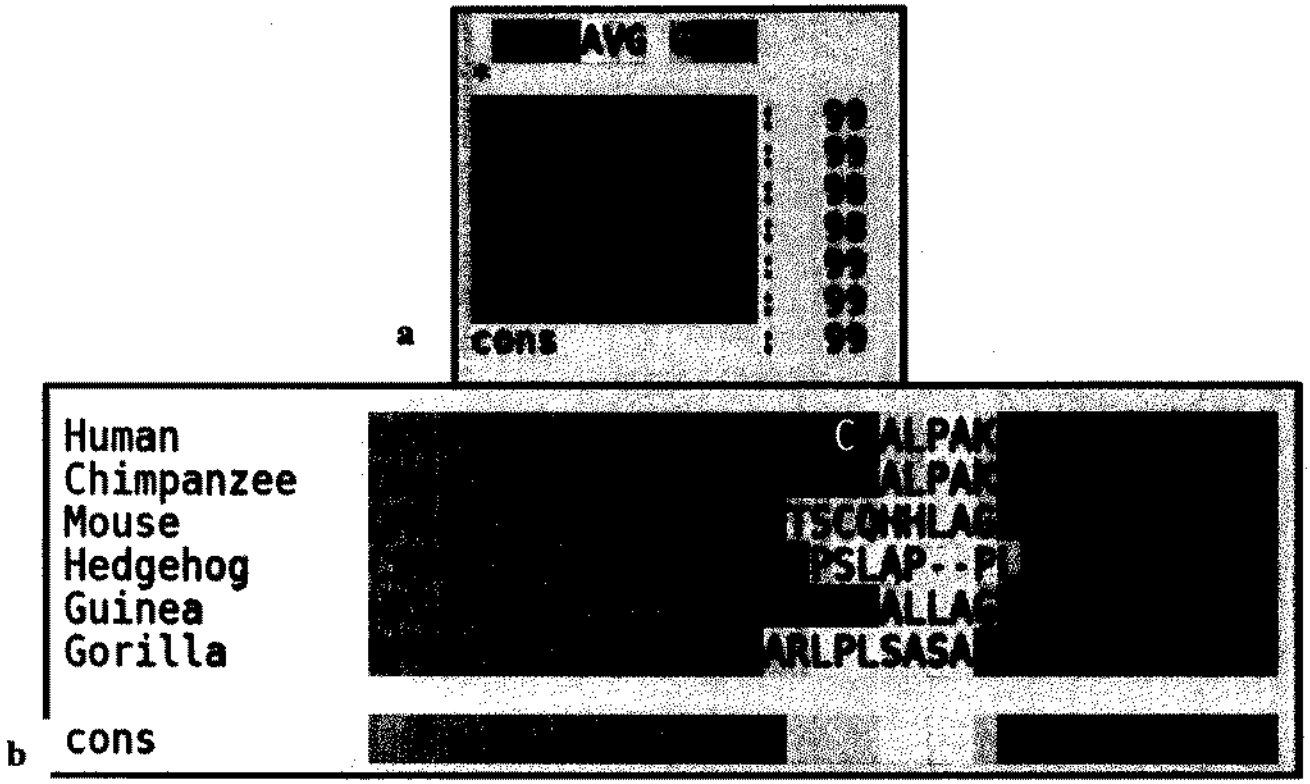


Figure.3.18: T-Coffee Results: a) Multiple Sequence Alignment showing good conversation for overall sequence including deleted region, **b)** 50% conservation of substituted amino acid C highlighted at position 487 in different species.

Chapter#4

DISCUSSION

Discussion

Most recently research have overlay with studying using homozygosity mapping and human genome diversity projects to indicate diseased gene in particular sub population. Pakistan's demographic and health survey (DHS) shows that two third of marriages in Pakistan are consanguineous. There are three common reasons for such marriages are religious, economic, and cultural. Muslim countries of the Middle East and in Pakistan the ratio of consanguineous marriages exceeds 50%. Economical factors is the second reason for consanguineous marriages. The actual reason for which the consanguineous marriages were given preference are primarily social. Most of the families believed that if they merry with in the family the family bonds will be strengthened.

Alopecia is one of the genetic disorders. Alopecia is a multifactorial disorder, association between genetical and environmental factors that causes Alopecia. Circumstantial evidence suggest that Alopecia is autoimmune disease where hair follicle from producing hair fibre is prevented by cell of own immune system. Genetics condition affecting hair growth cycle or hair structure may be isolated or they may be occur as a part of complex syndrome with associated abnormalities of other ectodermal appendages. The major defects reported to be related with partial or total absence of hair, either singly or in different combination, include mental retardation, dwarfism, nail dystrophy, epilepsy, total or partial anodontia, impaired sweating, hyperkeratosis, retinas pigmentation. Alopecia occurring in monozygotic twins have also been reported.

Alopecia Universalis is characterized by general scalp and body atrichia with papular lesions. Atrichia with Papular Lesion (APL) is an autosomal recessive form of Alopecia Universalis (MIM 209500). The individuals have normal hair at birth, but the hair are completely shed during first week or 2-3 months of the life and never regrow. The patient shows the presence of papules cyst filled with keratin like substance on their face and scalp. The patient have normal hearing, sweating, and nails and mentally fit. Mutation in *HR* gene is responsible for Alopecia Universalis.

HR gene functions as a nuclear receptor co-repressor. The nuclear receptors are transcriptional factors. Their transcriptional activity depends on the association of additional proteins. HR

protein interacts with and also influences the transcriptional activity of several nuclear factors. These nuclear factors includes vitamin D receptor and thyroid hormone receptor.

In current study, two families (AP5 and AP10) shows symptoms of Alopecia Universalis. In family AP10 linkage was not establish at *HR* locus Microsatellite markers. Exon sequencing is required for the family to search the locus in this family.

In family AP5 linkage was establish through Microsatellite markers i.e D8S1106 and D8S1827. While affected individuals shows heterozygous Pattern with Microsatellite markers D8S552, D8S511 and D8S549. Mutational screening of one affected member (AP5-6) and one normal member (AP5-8) was designed. In mutational screening of *HR* gene, sequence of **exon 8** shows deletion of adenosine at **c.2123** (Figure 3.15). Consequence of the change resulted premature stop codon at position 194 instead of 483 aa. Identified novel mutation is not reported in literature till yet. We have submitted "**Novel Mutation" in BMC Medical Genetic** and it is under review process. Novel mutation helps to understand the disease pattern of inheritance through *HR* gene varients.

Bioinformatics is new area of research and Insilco data analysis is used to save time and money in experimentation. It also helps in better diagnosis and improved therapeutic strategies. During the study, normal HR protein model was generated along with mutant model to analyse the impact of novel mutation in AP5. Normal HR comprised of 19 helixes, 7 strands and 28 coils in normal HR. In mutated model we have inserted premature stop codon and number of helixes, strands and coils are reduced to 4, 1, and 6, respectively. Due to frameshift mutation, size of protein and number of structure features gets affected. Mutation not only affects structure and conformation but also function of protein, leading to the diseased state.

Stitch4 was used to identify functional partner of *HR*. The HDACI is the closest one with highest interaction *HR*. Histone deacetylase1 is responsible for the deacetylation of lysine residues on the N-terminal parts of the core histones (H2A, H2B, H3 and H4) and plays an important role in the regulation of transcription, progression of the cell cycle and in events of development. We assume both normal and mutated structures of HR can be docked with this highly interacting ligand protein i.e. (HDACI). Further findings will help in drug designing.

In future, our research help to enhance the public awareness to reduce the consanguineous marriages in the population which will result in the reduction of inherited diseases.

By using this research study, we can predict the interaction of mutated protein with other proteins and how it is affecting the pathway and resulting into a disorder.

Our mutated sequence can be checked by the expression of protein in some expression system.

This study can help in future prenatal diagnosis and gene therapy to related disease.

Chapter#5

REFERENCES

References

1) Ahmad M, Abbas H, Haque S., (1993) Alopecia Universalis as a single abnormality in an inbred Pakistani kindred. Am J Med Genet, 46, p. 369-71.
2) Ahmad W, Ul Haque MF, Brancolin V, Tsou HC, Ul Haque S, Lam H, Aita VM, Owen J, de Blaquiere M, Frank J, Cserhalmi-Friedman PB, Leask A, McGrath JA, Peacocke M, Ahmad M, Ott J, Christiano AM., (1998) Alopecia universalis associated with a mutation in the human hairless gene. Science, 279, p. 720-24.
3) Ali G, Chishti MS, Raza SI, John P, Ahmad W., (2007) A mutation in the lipase H (LIPH) gene underlie autosomal recessive hypotrichosis. Hum Genet, 121, p. 319-25.
4) Amer E, Khalid J, Majid S., (2009) Pattern and profile of alopecia areata in Pakistan, JPAD, 19, p. 136-140.
5) Aslam M, Chahrour MH, Razzaq A, Haque S, Yan K, Leal SM, Ahmad W., (2004) A novel locus for autosomal recessive form of hypotricosis maps to chromosome 3q26.33-q27.3. J Med Genet, 41, p. 849-52.
6) Ayub M, Basit S, Jelani M, Rehman F U, Iqbal M, Yasinzai M, Ahmad W., (2009) A homozygous nonsense mutation in the human desmocollin-3 (DSC3) gene underlies hereditary hypotrichosis and recurrent skin vesicles. Am J Hum Genet, 85, p. 515-20.
7) Azeem Z, Jelani M, Naz G, Tariq M, Wasif N, Kamran-Ul-Hassan Naqvi S, Ayub M, Yasinzai M, Amin-Ud-Din M, Wali A, Ali G, Chishti MS, Ahmad W., (2008) Novel mutations in G protein-coupled receptor gene (P2RY5) in families with autosomal recessive hypotrichosis (LAH3). Hum Genet, 123, p. 515-19.
8) Barahmani N, de Andrade M, Slusser JP, Zhang Q, Duvic M., (2006) Major histocompatibility complex class I chain-related gene A polymorphisms and extended haplotypes are associated with familial alopecia areata. J Invest Dermatol, 126, p. 74-78.
9) Barsh G., (1999) Of ancient tales and hair less tails, Nat. Genet, 22, p. 315-316.
10) Basit S, Wali A, Aziz A, Muhammad N, Jelani M, Ahmad W., (2011) Digenic inheritance of an autosomal recessive hypotrichosis in two consanguineous pedigrees. Clin Genet, 79, p. 273-81.

11) Birch-Machin MA, Healy E, Turner R, Haldane F, Belgaid CE, Darlington S, Stephenson AM, Munro C, Messenger AG, Rees JL., (1997) Mapping of monilethrix to the type II keratin gene cluster at chromosome 12q13 in three new families, including one with variable expressivity. Brit J Derm, 137, p. 339-43.

12) Bittles AH, Savithri HS, Venkateshal Murthy HS et al., (2001) Consanguineous marriage: a familiar story full of surprises. In: Macbeth H, Shetty P, eds. Ethnicity and Health. London: Taylor and Francis, p. 68–78.

13) Bittles AH., (1994) The role and significance of consanguinity as a demographic variable. Pop Dev Rev, 20, p. 561–584.

14) Chaleby K., (1988) Traditional Arabian marriages and mental health in a group of outpatient Saudis. Acta Psychiat Scand, 77, p. 139–142.

15) Cichon S, Kruse R, Hilltner AM, Kukuk G, Anker M, Aitland K, Knapp M, Propping P, Nothen MM., (2000) A distinct gene close to the hairless locus on chromosome 8p underlies hereditary Marie Unna type hypotrichosis in a German family. Br J Dermatol, 143, p. 811-14.

16) Degos L, Colombani A, Chaventre A, Bengtson B, Jacquard A., (1974) Selective pressure on HL-A polymorphism. Nature, 249, p. 62–63.

17) Djabali K, Christiano AM., (2004) Hairless contains a novel nuclear matrix targeting signal and associates with histone deacetylases 3 in nuclear speckles. Differentiation, 8, p. 408-410.

18) Gelmann EP., (2002) Molecular biology of the androgen receptor. J Clin Oncol, 20, p. 300115.

19) Gilhar A, Etzioni A, Paus R., (2012) Alopecia Areata: review article. N Engl J Med, 366, p.15-25.

20) Gottlieb B, Beitel LK, Wu JH, Trifiro M., (2004) The androgen receptor gene database (ARDB): 2004 update. Hum Mutat, 23, p. 527-33.

21) Green J and Sinclair RD., (2000) Genetics of alopecia areata. Australas J Dermatol, 41, p.213-218

22) Gregoriou S, Papafragkaki D, Kontochristopoulos G, Rallis E, Kalogeromitros D, Rigopoulos D., (2010) Cytokines and other mediators in alopecia areata. Mediators Inflamm, 2010, p. 1-5.

23) Hillmer AM, Hanneken S, Ritzmann S, Becker T, Freudenberg J, Brockschmidt FF, Flaquer A, Freudenberg-Hua Y, Jamra RA, Metzen C, Heyn U, Schweiger N, Betz RC, Blaumeiser B, Hampe J, Schreiber S, Schulze TG, Hennes HC, Schumacher J, Propping P, Ruzicka T, Cichon S, Wienker TF, Kruse R, Nothen MM., (2005) Genetic variation in the human androgen receptor gene is the major determinant of common early-onset androgenetic alopecia. Am J Hum Genet, 77, p. 140-48.

24) Hsieh JC, Sisk JM, Jurutka PW, Haussler CA, Slater SA, Haussler MR, Thompson CC., (2003) Physical and functional interaction between the vitamin D receptor and hairless corepressor, two proteins required for hair cycling. J Biol Chem, 278, p. 38665-74.

25) Hussain R, Bittles AH., (1998) The prevalence and demographic characteristics of consanguineous marriages in Pakistan. J Biosoc Sci, 30, p. 261-275.

26) Hussain R., (1999) Community perceptions of reasons for preference for consanguineous marriages in Pakistan. J Biosoc Sci, 31, p. 449–461.

27) Jelani M, Wasif N, Ali G, Chishti M, Ahmad W., (2008) A novel deletion mutation in LIPH gene causes autosomal recessive hypotrichosis (LAH2). Clin Genet, 74, p. 184-88.

28) John P, Ali G, Chishti MS, Naqvi SMS, Leal SM, Ahmad W., (2006) Localization of a novel locus for alopecia with mental retardation syndrome to chromosome 3q26.33q27.3. Hum Genet, 118, p. 665-67.

29) Kavak A, Yeşildal N, Parlak AH, Gökdemir G, Aydoğan I, Anul H, Baykal C., (2008) Alopecia areata in Turkey: demographic and clinical features. JEADV, 22, p. 977-81.

30) Kazantseua A, Goltsov A, Zinchenko R, Grigorenko AP, Abrukova AV, Moliaka YK, Kirillov AG, Guo Z, Lyle S, Ginter EK, Rogaev EI., (2006) Human hair growth deficiency is linked to a genetic defect in the phospholipase gene LIPH. Science, 314, p. 982-85.

31) Kemp EH, McDonagh AJ, Wengraf DA, Messenger AG, Gawkrodger DJ, Cork MJ, Tazi-Ahniai R., (2006) The non-synonymous C1858T substitution in the PTPN22 gene is associated with susceptibility to the severe forms of alopecia areata. Hum Immunol, 67, p. 535-39.

32) Khlat M. Endogamy in the Arab world. In: Teebi AS, Farag TI., (1997) Genetic Disorders among Arab Populations. New York: Oxford University Press, p. 63–80.

33) Kljuic A, Bazzi H, Sundberg JP, Martinez-Mir A, O'Shaughnessy R, Mahoney MG, Levy M, Montagutelli X, Ahmad W, Aita VM, Gordon D, Uitto J, Whiting D, Ott J, Fischer S, Gilliam TC, Jahoda CA, Morris RJ, Panteleyev AA, Nguyen VT, Christiano AM., (2003) Desmoglein 4 in hair follicle differentiation and epidermal adhesion: evidence from inherited hypotrichosis and acquired pemphigus vulgaris. Cell, 113, p. 249-60.

34) Kruse R, Cichon S, Anker M *et al* (1999) Novel hairless mutations in two kindred with autosomal recessive papular atrichia. J Invest Dermatol, 113, p. 954-959.

35) Kuhn M, Szklarczyk D, Franceschini A, Mering C, Jensen JL and Bork P., (2012) STITCH 3: zooming in on protein–chemical interactions. *Nucleic Acids Research*, 40, p. D876-D880.

36) Lefevre P, Rochat A, Bodemer C, Vabres P, Barrandon Y, de Prost Y, Garner C, Hovnanian A., (2000) Linkage of Marie-Unna hypotrichosis locus to chromosome 8p21 and exclusion of 10 genes including the hairless gene by mutation analysis. Eur J Hum Genet, 8, p. 273-79.

37) Madani S, and Shapiro J., (2000) Alopecia areata update. J Am Acad Dermatol, 42, p. 549-566.

38) McDonagh AJ, and Messenger AG., (1996) The pathogenesis of alopecia areata. Dermatol Clin, 14, p. 661-70.

39) McDonagh AJ, and Tazi-Ahnini R., (2002) Epidemiology and genetics of alopecia areata. Clin Exp Dermatol, 27, p. 405-409.

40) McElwee, Darvari B, Miller J *et al.*, (2001) Genetic susceptibility and severity of alopecia areata in human and animal model. Eur J Dermatol, 11, p. 11-16.

41) McElwee KJ, and Hoffmann R., (2002) Alopecia areata-animal models. Clin Exp Dermatol, 27, p. 414-421.

42) McGuffin LJ, Bryson K, Jones DT., (2000) The PSIPRED protein structure prediction server. *Bioinformatics*, 4, p. 404-405.

43) Mehran G, Rohaninasab M, Hanifnia A.R, Darvari B and Mehrnahad Z., (2012) The Impact of Recent Life Events on Patients with Alopecia Areata: A Study from Iran, J Clin Exp Dermatol Res, 3, p. 1.

44) Naz G, Ali G, Kamran-Ul-Hassan Naqvi S, Azeem Z, Ahmad W., (2010) Mapping of a novel autosomal recessive hypotrichosis locus on chromosome 10q11.23–22.3. Hum Genet, 127, p. 395-401.

45) Norwood OT., (1975) Male-pattern baldness. Classification and incidence. South Med J, 68, p. 1359-70.

46) Nothen MM, Cichon S, Vogt IR, Hemmer S, Kruse R, Knapp M, Holler T, Faiyaz ul Haque M, Haque S, Propping P, Ahmad M, Rietschel M., (1998) A gene for universal congenital alopecia maps to chromosome 8p21–22. Am J Hum Genet, 62, p. 386-90.

47) Notredame C, Higgins GD, Heringa J., (2000) T-coffee: a novel method for fast and accurate multiple sequence alignment, J Mol Biol, 302, p. 205–217.

48) Osborn D., (1916) Inheritance of baldness. Various patterns due to heredity and sometimes present at birth-a sex-limited character-dominant in man-women not bald unless they inherit tendency from both parents. J Hered, 7, p. 347-55.

49) Panteleyev AA, Natalia V, Botchkareva, Sundberg JP, Christiano AM, Ralf P., (1999) The role of hairless (hr) gene in the regulation of hair follicle catagen transformation. Am J Pathol, 155, p. 159-71.

50) Paradisi M, Masse M, Martinez-Mir A, Lam H, Pedicelli C *et al.*, (2005) Identification of a novel splice site mutation in human hairless gene underlying atrichia with papular lesions. Eur J Dermatol, 15, p. 332-338.

51) Pasternack SM, von Kügelgen I, Aboud KA, Lee YA, Ruschendorf F, Voss K, Hillmer AM, Molderings GJ, Franz T, Ramirez A, Nurnberg P, Nothen MM, Betz RC., (2008) G protein-coupled receptor P2Y5 and its ligand LPA are involved in maintenance of human hair growth. Nat Genet, 40, p. 329-34.

52) Pinheiro M, Freire MN., (1985) Atrichias and hypotrichiosis: a brief review with description of a recessive atrichia in two brothers. Hum Hered, 35, p. 53-55.

53) Potter GB, Beaudoin GMJ, *et al.*, (2002) The thyroid hormone-regulated corepressor hairless associates wit histone deacetylases in neonatal rat brain. Mol Endocrinol, 11, p. 2547-2560.

54) Potter GB, Beaudoin GMJ, DeRenzo CL, Zarach JM, Chen SH, Thompson CC., (2001) The hairless gene mutated in congenital hair loss disorders encodes a novel nuclear receptor corepressor. Genes Dev, 15, p. 2687-2701.

55) Price VH., (1991) Alopecia areata: Clinical aspects. J Invest Dermatol, 96, p. 68.

56) Prodi DA, Pirastu N, Maninchedda G, Sassu1 A, Picciau A, Palmas MA, Mossa A, Persico I, Adamo M, Angius A, Pirastu M., (2008) EDA2R is associated with androgenetic alopecia. J Invest Dermatol, 128, p. 2268-70.

57) Rafiq MA, Ansar M, Mahmood S, Haque S, Faiyaz-ul-Haque M, Leal SM, Ahmad W., (2004) A recurrent intragenic deletion mutation in DSG4 gene in three Pakistani families with autosomal recessive hypotrichosis. J Invest Dermatol, 123, p. 247-48.

58) Rafique MA, Ansar M, Jamal SM, Malik S, Sohail M, Faiyaz-Ul-Haque M, Haque S, Leal SM, Ahmad W., (2003) A locus for hereditary hypotrichosis localized to human chromosome 18q21.1. Eur J Hum Genet, 11, p. 623-28.

59) Salamon T, and Schnyder UW., (1962) Ueber die Monilethrix. Arch Klin Exp Derm, 215, p. 10536.

60) Sawaya ME, and Hordinsky MK., (1992) Advances in alopecia areata and androgenetic alopecia. Adv Dermatol, 7, p. 211-226.

61) Schmidt-Ullrich R, and Paus R., (2005) Molecular principles of hair follicle induction and morphogenesis. Bioessays, 27, p. 247-61.

62) Schwartz ME, and Janniger CK., (1997) Alopecia areata. Cutis, 59, p. 238-241.

63) Scrimgeour ME., (2009) Huntington Disease (Chorea) in the Middle Eastin the Middle East, J. Sultan Qaboos Univ Med, 9(1), p. 16–23.

64) Shimomura Y, Wajid M, Ishii Y, Shapiro L, Petukhova L, Gordon D, Christiano AM., (2008) Disruption of P2RY5, an orphan G protein-coupled receptor, underlies autosomal recessive woolly hair. Nat Genet, 40, p. 335-39.

65) Snyder LH, and Yingling HC., (1935) The application of the gene-frequency method of analysis to sex-influenced factors, with special reference to baldness. Hum Biol, 7, p. 608-15.

66) Sprecher E, Bergman R, Richard G, Lurie R, Shalev S, Petronius D, Shalata A, Anbinder Y, Leibu R, Perlman I, Cohen N, Szargel R., (2001) Hypotrichosis with juvenile macular dystrophy is caused by a mutation in CDH3, encoding Pcadherin. Nat Genet, 29, p. 134-36.

67) Sreekumar GP, Roberts JL, Wong CQ, Stenn KS, Pariznoo S., (2000) Marie Unna hereditary hypotrichosis gene maps to human chromosome 8p21 near hairless. J Invest Dermatol, 114, p. 595-97.

68) Stevens HP, Kelsell DP, Bryant SP, Bishop DT, Dawber RPR, Spurr NK, Leigh IM., (1996) Linkage of monilethrix to the trichocyte and epithelial keratin gene cluster on 12q11-q13. J Invest Derm, 106, p. 795-97.

69) Tariq M, Ayub M, Jelani M, Basit S, Naz G, Wasif N, Raza SI, Naveed AK, Ullah-Khan A, Azeem Z, Yasinzai M, Wali A, Ali G, Chishti MS, Ahmad W., (2009)

Mutations in the P2RY5 gene underlie autosomal recessive hypotrichosis in 13 Pakistani families. Br J Dermatol, 160, p. 1006-10.

70) Tchen P, Bois E, Feingold J, Feingold N, Kaplan J., (1997) Inbreeding in recessive diseases. Hum Genet, 38, p. 163–167.

71) Thompson C, and Bottcher MC., (1997) The product of a thyroid hormone-responsive gene interacts with thyroid hormone receptor proc.Natl Acad Science USA, 94, p. 8527-8532.

72) Unna M., (1925) Ueber Hypotrichosis congenital hereditaria. Dermatol Wochenschr, 32, p. 1167-68.

73) van Steensel M, Smith FJ, Steijlen PM, Kluijt I, Stevens HP, Messenger A, Kremer H, Dunnill MG, Kennedy C, Munro CS, Doherty VR, McGrath JA, Covello SP, Coleman CM, Uitto J, McLean WH., (1999) The gene for hypotrichosis of Marie Unna maps between D8S258 and D8S298: exclusion of the hr gene by cDNA and genomic sequencing. Am J Hum Genet, 65, p. 413-19.

74) Vogt BR, Traupe H, Hamm H., (1988) Congenital artichia with nail dystrophy, abnormal facies, and psychomotor development in two siblings. A new autosomal recessive syndrome. Pediatr Dermatol, 5, p. 236-42.

75) Wali A, Chishti MS, Ayub M, Yasinzai M, Kafaitullah Ali G, John P, Ahmad W., (2007) Localization of a novel autosomal recessive hypotrichosis locus (LAH3) to chromosome 13q14.11–q21.32. Clin Genet, 72, p. 23-29.

76) Whittock NV, and Bower C., (2003) Genetic evidence for a novel human desmosomal cadherin, desmoglein 4. J Invest Dermatol, 120, p. 523-30.

77) Wu L., (1987) Investigation of consanguineous marriages among 30 Chinese ethnic groups. Hered Dis, 4, p. 163–166.

78) Xiao FL, Zhou FS, Liu JB, Yan KL, Cui Y, Gao M, Liang YH, Sun LD, Zhou SM, Zhu YG, Zhang XJ, Yang S., (2005) Association of HLA-DQA1 and DQB1 alleles with alolpecia areata in Chinese Hans. Arch Dermatol Res, 297, p. 201-09.

79) Zlotogorski A, Marek D, Horev L, Abu A, Ben-Amitai D, Gerad L, Ingber A, Frydman M, Reznik-Wolf H, Vardy DA, Pras E., (2006) An autosomal recessive form of monilethrix is caused by mutations in DSG4: clinical overlap with localized autosomal recessive hypotrichosis. J Invest Dermatol, 126, p. 1292-96.

80) Zlotogorski A, Panteleyev AA, Aita VM, Christiano AM *et al.*, (2002) Clinical and molecular diagnostic criteria of congenital atrichia with papular lesions. J Invest Dermatol, 118, p. 887-890.

Genome Database: (http://www.ncbi.nlm.nih.gov).