Clinical and Molecular Characterization of Hereditary Skin Disorders in Consanguineous Families

A thesis submitted in the partial fulfillment of the requirements for the degree of Master of Philosophy

In

Biochemistry/Molecular Biology

by

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This Thesis is

Dedicated

To

My Loving

Parents and Family

For their endless love, support,

encouragement and prayers

In the Name of ALLAH, the Most gracious, the Most Merciful.

CERTIFICATE

This thesis, submitted by **Mr. Abdullah** to the Department of Biochemistry, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan, is accepted in its present form as satisfying the thesis requirement for the Degree of Master of Philosophy in Biochemistry/Molecular Biology.

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Declaration

I hereby declared that the work presented in this thesis is my own effort and hard work and it is written and composed by me. No part of this thesis has been previously published or presented for any other degree or certificate.

Abdullah

List of Contents

Continue..

Continue...

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Abdullah

List of Figures

Continue...

Continue...

List of Tables

List of Abbreviations

%	Percentage
(NH4)2SO4	Ammonium sulphates
AEI	Annular epidermolytic ichthyosis
APS	Ammonium persulphate
ARCI	Autosomal recessive congenital ichthyosis
AREI	Autosomal recessive epidermolytic ichthyosis
ASI	Autosomal syndromic ichthyosis
BM	Basement membrane
bp	Base pair
CCE	Cornified envelope
CDPX2	Chondrodysplasia punctata type 2
CIE	Congenital ichthyosiform erythroderma
cM	Centimorgan
CRIE	Congenital reticular ichthyosiform erythroderma
DEB	Dystrophic epidermolysis bullosa
DNA	Deoxy-ribonucleic acid
dNTP's	Deoxy nucleotide triphosphates
DP	Dermal papilla
DPPK	Diffuse palmoplantar keratoderma
EB	Epidermolysis bullosa
EBS	Epidermolysis bullosa simplex
ED	Ectodermal dysplasias
EDA	Ectodysplasin A1

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EDAR	Ectodysplasin A1 isoform receptor
EDARADD	Ectodysplasin-A receptor associated death domain
EDTA	Ethylene diaminetetraacetic acid
EI	Epidermolytic ichthyosis
EN	Epidermolytic nevi
F	Forward
FLG	Filaggrin
Gm	Gram
HF	Hair follicle
HI	Harlequin ichthyosis
HS	Hair shaft
ICM	Ichthyosis Curth-Macklin
IRS	Inner root sheath
IV	Ichthyosis vulgaris
JEB	Junctional epidermolysis bullosa
KDa	Kilodalton
KS	Kindler syndrome
LEKTI	Lympho-Epithelial kazal-type inhibitor
LI	Lamellar ichthyosis
M	Molarity
MEDOC	Mendellian disorders of cornification
mg	Miligram
MgCl2	Magnesium chloride
ml	Milliliter
mM	Milimolar

Continue…

NaCl	Sodium chloride
NEMO	NF-kB essential modulator
ng	Nanogram
NS	Netherton syndrome
°C	Degree centigrade
p	Short arm of chromosome
PhyH	Phytanoyl-CoA hydroxylase
PPK	Palmoplantar keratoderma
PPK-GN	Gamborg-Nielson palmoplantar keratoderma
PPKI	Punctate palmoplantar keratoderma type I
PPKII	Punctate palmoplantar keratoderma type II
PPKIII	Punctate palmoplantar keratoderma type III
PTC	Premature termination codons
q	Long arm of chromosome
R	Reverse
rpm	Revolutions per minute
RXLI	Recessive X-linked ichthyosis
SB	Stratum basale
SC	Stratum corneum
SDS	Sodium dodicyl sulfate
SEI	Superficial epidermolytic ichthyosis
SG	Stratum granulosum
SL	Stratum lucidum
SLS	Sjogren-Larsson syndrome
SS	Stratum spinosum

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STS	Steroid sulfatase
Taq	Thermus aquaticus
TBE	Tris borate EDTA
TE	Tris- EDTA
TEMED	N, N, N', N'-Tetra methyl ethylenediamine
Tm	Annealing temperature
TTD	Trichothiodystrophy
UV	Ultraviolet
v/v	Volume by volume
µg	Microgram
µl	Microliter

Abstract

Abstract

The skin makes an effective barrier between organism and their surrounding environment, preventing the entry of pathogens, ward off chemical and physical assaults and keep the water-solutes concentration in balance through osmoregulation. Inherited ichthyosis and palmoplantar keratoderma are group of genetic disorders, characterized by dry skin, scaling and hyperkeratosis of the skin surface. It can be in isolated conditions limited to the skin or appear secondarily with involvement of other cutaneous or systemic abnormalities. The molecular basis of these manifestations has been clearly understood by identification of mutations in genes involved in skin barrier formation.

The study presented in the dissertation investigated clinical and molecular characterization of three consanguineous Pakistani families (A, B, C), segregating different types of autosomal recessive congenital skin disorders. Family A presented diffuse palmoplantar keratoderma, family B showed lamellar ichthyosis and family C having ichthyosis vulgaris phenotypes. Genotyping using microsatellite markers and haplotype analysis were used to establish linkage in these families. Sanger sequencing of *ABCA12* and *FLG* was performed by dideoxy chain termination method in family B, C.

Affected members of family A were found heterozygous for different combination of parental alleles thus, excluding this family for linkage to known genes/loci. Family B was found linked to *ABCA12* gene on chromosome 2q35 whereas, Family C showed linkage to *FLG* gene on chromosome 1q21.3.

Sanger sequencing was performed for analysis of linked genes both for family B, C. In family B, selected exons (32 and 49) of *ABCA12* gene were sequenced. Analysis of results failed to show any pathogenic variant, suggesting the involvement of another exon or regulatory sequences. In family C, all three exons of the *FLG* gene were sequenced which revealed no sequence variant, suggesting that mutation might be present in regulatory sequences of the gene or another unknown gene in the same region.

Chapter 1
INTRODUCTION

INTRODUCTION

All organisms have an outer layer that delimits the body and separates it from the outer environment. In humans this outer layer is the '"skin". The human skin is the outermost covering of the body, accounts for about 12-15% of the total body weight of an adult and makes a surface area of about $2m^2$. Two main types of human skin are; (1) Glabrous skin, thick and non-hairy skin present on palms and soles, and possess encapsulated sense organs; (2) Hairy skin, which is present all over the body except palms and soles (MacGrath *et al.*, 2004). Skin thickness varies in different areas but mostly it is 2–3 mm (0.10 inch) thick. The average square inch (6.5 cm^2) of skin holds 650 sweat glands, 20 blood vessels, 60,000 melanocytes, and more than 1,000 nerve endings. The average human skin cell is about 30 micrometers in diameter; it may vary from one region to another. Skin is a major hindrance to external stimuli and provides the body with thermoregulation, immunological surveillance and UV protection.

The mammalian skin and its appendages are derived from embryonic ectoderm and mesoderm (Hardy, 1992; Fuchs, 2007). The human skin has three major layers; the epidermis, dermis, and sub-cutis.

Epidermis is the outer covering of body which functions as "outside-in" and "inside-out" barrier (Elias *et al.*, 2008a; Proksch *et al.*, 2008). "Outside-in" denotes the epidermal function of hindering the infiltration of external materials through the epidermis to inside of cell. "Inside-out" means the ability of the epidermis to prevent the leakage of water and electrolytes from inside to the outer environment. The major cells in epidermis are the keratinocytes, comprising 95 % of the total epidermal cells (McGrath *et al.*, 2004). Besides keratinocytes, melanocytes, Langerhans cells and Merkel cells also reside in the epidermis (James *et al.*, 2005). The epidermis is divided into five distinct layers, which from top to bottom layer are; Stratum corneum (SC), Stratum lucidum (SL), Stratum granolusum (SG), Stratum spinosum (SS) and stratum basale (SB).

The stratum corneum, the outer most layer, constitute approximately 40% keratin, 40% water and 15 to 20% lipids (McGrath *et al.*, 2004). Located within the stratum corneum, the cornified envelope (CCE) is an insoluble protein matrix vital for skin-barrier function and integrity. It replaces the plasma membrane of the granular cells

during cornification. The stratum corneum barrier is maintained by complex interaction of the CCE, intra-cytoplasmic moisturizing factors, and complex lipid mixture in the extracellular space. A constant regulated turnover of keratinocytes moves from the stratum basale and ultimately shed from SC, a process called desquamation. The stratum lucidum a transparent layer found between the SC and SG. It may be few layers thick with dead keratinized cells found only in hairless skin of palms and soles. The stratum granulosum (SG), present in the granular layer still contain all organelles and are characterized by keratohyalin granules and lamellar bodies. In stratum spinosum (SS), the keratinocytes cease proliferation and begin to differentiate. The cells become flatten and the cell-surface extensions or spines end in desmosomes, interconnected by numerous desomosomal plaques. The stratum basale is usually described as single-cell-layer thick. The keratinocyte proliferate in the stratum basale, moves progressively from the epidermal basement membrane towards the skin surface and eventually shed from stratum corneum. Several distinct layers are formed during this process of keratinization (Kaplan *et al.*, 2007).

Melanocytes (3% in epidermis) are the melanin producing cells present in SB of epidermis. Needed for pigmentation of skin, melanin absorbs the UV-B of sun light and provides protection (Agar and Young, 2005). Langerhans cells (2% in epidermis) are the antigen-presenting cell of the skin and works as an immune barrier (Williams *et al.*, 1988; Holikova *et al.*, 2001). Merkel cells (0.5% in epidermis) are situated in the stratum basale of epidermis, essential for light touch response of the skin (Tobin, 2006).

The dermis or corium is a resilient tissue which provides nutrition to the epidermis and support against mechanical injury. The dermis contains few cells, the majority being fibroblasts that secrete dermal constituents. Other cells include mast cells, melanocytes and immunological cells (macrophages and lymphocytes). Polysaccharides and proteins also get linked in the dermis to produce macromolecules with high water binding capacity. These have a major role in the supporting matrix of the connective tissue (Williams *et al.*, 1988). The basement membrane (BM), which physically separates dermis from epidermis, provides a stabilizing as well as dynamic interface (Koster and Roop, 2007).

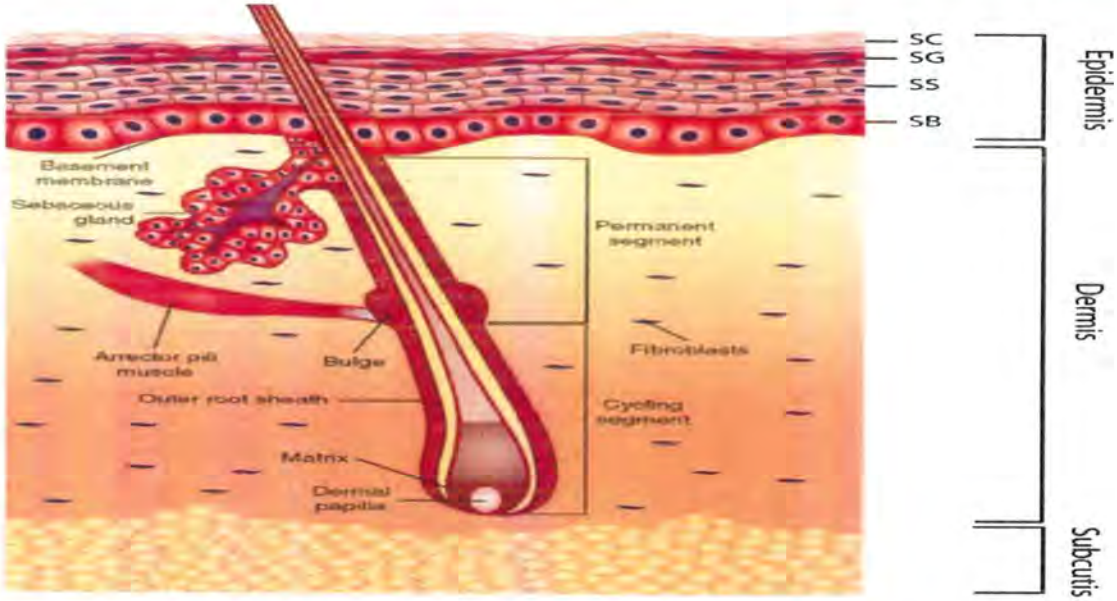


Figure 1.1: Arrangement of the skin layers (Wong and Chang, 2009). SC (Stratum corneum), SG (Stratum granulosum), SS (Stratum spinosum), SB (stratum basale).

Skin Appendages

Some organs are developed from the embryonic epidermis, called epidermal derivatives or skin appendage. These organs include hairs, nails, sweat glands and tooth enamel.

Hairs

Hairs are found over most of the human skin surface. Hairs primarily provide protection by protecting the scalp from mechanical injury and reduce heat loss. Keratinized cells are the major component of hairs. Dermis of skin encourage the formation of hair follicle (HF) both in hair and non-hair producing regions of the skin. However, the non-hair forming sites don't support the development of HF. Hairs development initiate with a localized precursor thickening of the embryonic epidermis, known as placode. The HF is detectable at embryonic day 14-15. HF is capable to regenerate about 7-10 times throughout life. Shortly after placode is formed, dermal condensate is developed from fibroblasts cells under placode. Afterwards, reciprocal signaling cascade starts between the condensate and the placode. This initiates the proliferation of upper epithelial cells and downward movement of follicle into the dermal region (Ohyama *et al.*, 2010; Yang and Cotsarelis, 2010). As soon as the epithelial cells surround the dermal condensate, a fully matured dermal papilla (DP) is created. The DP now signals the nearby

epithelial cells to proliferate. These cells now migrate upward to form different layers of maturing hair shaft (HS) and inner root sheath (IRS). After IRS is developed, epithelial cells around DP start to differentiate into various parts of HS (Welle and Wiener, 2016). HS germinates from the matrix cells of hair bulb (Sequeira and Nicolas, 2012). The HS also gets surrounded by IRS (Millar, 2002; Schneider *et al.*, 2009). As, fibroblast have different embryonic origin hence, DP differ from one position to another (Jinno *et al.*, 2010). The HF has a three phase cyclic growth in the life span of a human. Telogen phase, the resting phase in which the hairs doesn't grow and DP remains in resting condition. During anagen, the cells at the base of HF proliferate; move downward and enclose the DP. As soon as anagen starts, DP activates stem cells in the secondary hair germ leading to new downward growth of follicles. Catagen the last phase, during which the apoptosis of epithelial cells start. The DP is still intact and move upwards, till the telogen starts again (Chi *et al.*, 2010).

Nails

Nails in healthy state could really seem decent and eye catching. But it also has many important functions like, they are used to grip and manipulate objects, help to protect tissues of the fingers and toes from mechanical injuries. In humans the health condition can be checked by physicians directly from their nails. Nail thickness and growth varies among all the digits. The growth also restricted during nail disorders (Rodney Dawber, 1970). Growth rate of fingers nail is around 0.1 mm/day; whereas toenails growth is slower (Gawkrodger, 2002; McGrath *et al.*, 2008; Pirzado, 2012).

Like hairs, the leading constituent in nail is protein keratin. A nail has three main parts, nail bed, nail matrix and nail plate. The skin beneath the nail plate is called nail bed or hyponychium, has both dermis and epidermis tissue. Nail matrix is present beneath nail plate near the root. It is nourished by blood vessels hence; keratinocytes proliferates and push the older cells closer to form the future nail plate. Healthy looking upper most part of the nail is nail plate or nail body. It provides protection to finger tips. At the distal end of nail body lunula is present where it is translucent and the matrix could be seen here. Lunula covers more area in thumb nail while it is smaller in little finger (Lellipop, 2006).

Sweat Glands

Sweat glands are present in skin which secretes their products directly onto skin surface. The number varies from 2-4 millions in human (Landis, 1990). Like most other body systems, human have developed unique thermoregulatory systems which effectively control body temperature during extended physical activities resulting in huge transpiration from the skin. It is estimated that evaporation from skin leads to 80% of total heat loss during exercise (Shibasaki and Crandall, 2001). Sweat glands are present almost everywhere on the skin with exception of nail bed, lips margins, nipples and glans of penis. To keep body temperature in normal range requires the development, distribution and increased number of sweat glands. Apocrine, eccrine and apoeccrine are three types of sweat glands. Glabrous skin holds high density of eccrine units and few hair follicles. That's why non-hairy skin surface such as soles, palms and cheeks also contribute to thermoregulation. Normal sweating rate is nearly 0.5-1mL/min but it can produce up to 10L sweat /day at the time of need like exercise and stress conditions (Groscurth, 2002).

Eccrine glands are distributed almost all over the body, with high density in palms and soles, but less on the trunk and the extremities. Eccrine glands have long tubular structure. It has an extremely coiled secretory portion in dermal or hypodermal region. Two types of cells are present here; clear cells and dark cells, each contribute its product to sweat. A ductular system that open onto skin surface, release the sweat (Sato, 1993). Apocrine sweat glands are restricted to hairy skin areas like axilla, mammary, perineal and genital region and become active at puberty. It secrete an oily odourless substance, containing proteins, lipids and steroids (Wilke *et al.*, 2007). Apoeccrine glands are mixed type of glands developed from eccrine glands at the time of puberty. It secrete eccrine like watery fluid sharing morphology, marker proteins and mode of secretion with eccrine glands. Apoeccrine glands are more sensitive to cholinergic and adrenergic stimuli and secrete more sweat then other types of sweat glands (Sato and Sato, 1987).

Teeth

Human teeth are the most hard calcium phosphate bio-mineral organ of human body (Busch *et al.*, 2001). Human teeth are classified into two types, the deciduous and secondary teeth. The primary function of teeth is food biting and chewing besides

manipulation for sound production. Mammalian teeth are heterodontic, having four major groups: the incisors, canines, premolars and molars, each adopted for their distinctive function. The structure of teeth remains conserved among all types. Crown is superficial part of teeth covered with enamel (2 mm), the most hard and mineralized part in human body. Every tooth is held tight in bone through a single or multiple roots (Al-Rub and Voyiadjis, 2006). The main substance and second hardest tissue which is covered with enamel is called dentin. The central part of connective tissues is called dental pulp, supplied by blood vessels and nerves. Dentine tubules possess a fluid and have cellular structures that open into central nerves inside the pulp and produces pain sensation (Kumar *et al.*, 2005).

Tooth development initiates when a small epithelial thickening arises to form dental lamella. Cell of lamella multiply and invaginate to form placode. As the proliferation proceeds further, bud, cap and then bell shapes of tooth morphology are attained. During the course of development, growth signals between epithelial and mesenchymal cells causes them to differentiate into ameloblasts and odontoblasts cells (Bei, 2009).

Skin Disorders

Human skin has a lot number of developmental disorders. These include hereditary skin disorders like, male pattern baldness, malignant melanoma, ichthyosis, epidermolysis bullosa, alopecia, dyskeratosis congenita, follicular atrophoderma, etc. Ichthyosis and related disorders represent a group of more than 28 distinct disorders with different modes of inheritance and different origins. All these disorders primarily affect the outermost layer of the skin, the epidermis, and can be summarized as disorders of cornification (Uitto and Richard, 2005). Palmoplantar keratoderma (PPK), a heterogeneous group of disorders which may either be hereditary or acquired, characterized by thickening of stratum corneum of the palms and soles (Vinay *et al.*, 2004). Whereas, epidermolysis bullosa and Kindler's Syndrome are mechano-bullous disorders and are termed as fragile skin disorders.

Ectodermal Dysplasia (ED)

Ectodermal dysplasias (ED) are large group of heterogeneous heritable disorders characterized by genetic imperfections of ectodermal structures and their appendages

i.e. hair, nails, teeth, sweat glands (Itin, 2014). Frequently, the clinical manifestations could be seen in more than one ectodermal tissue (skin, hairs, nails, sweat glands). Two main groups of ectodermal dysplasias can be distinguished. Group one is characterized by hypoplasia of ectodermal tissues, which fail to develop and differentiate due to lack of mutual signaling between ectoderm and mesoderm, the other has palmoplantar keratoderma as its most striking feature besides other epithelial structures. Nearly 200 different types of ED have been reported until now (Garcia-Martin *et al.*, 2013). The prevalence ED in the general population estimated to be 7/10,000 cases (Lamartine, 2003). Total 77 genes and 9 chromosomal loci have been identified in 75 different ectodermal dysplasias (Pagnan and Visinoni, 2014).

Classification of Ectodermal Dysplasia

EDs are classified into large number of groups or even sub-groups by different authors according to their own formulas. One classification of EDs proposed by Maia and Pinheiro was, Pure (non-syndromic) and syndromic ED. According to this rule, Pure non-syndromic ED are those having only one distorted ectodermal appendage with no involvement of other epidermal derivative. However, if any other structure of embryonic origin is found malformed, it is categorized as syndromic type of ectodermal dysplasia (Burn J, 1985).

Another classification for ED was suggested by Freire-Maia, EDs were arranged into two groups, A and B. Group A encompasses clinical variation in at least two of the epidermal derivative. Whereas, defect in any single of the epidermal appendages with any other ectodermal defect, were included in group B. Therefore, group A was also called as, "pure ED" and group B as "ED syndrome" (Pinheiro and freire-Maia, 1994).

One recent classification of ED was proposed, based on clinical features, their most relevant genetic and molecular association and protein function in a signaling pathway. By this mode of classification only single major clinical feature of ectodermal origin would be enough to confirm ectodermal dysplasia by diagnosing alteration in gene for ectodermal development. Subclasses were recognized by protein product and their involvement in cell–cell communication, adhesion, transcription, development, aging/DNA-repair and Genes for structure proteins (Itin, 2009).

Based on Priolo clinico-functional features, Martin *et al.* (2013) proposed a new classification for EDs into two groups.

Group 1; comprises those conditions in which abnormal interaction between ectodermal and mesenchymal cells is present. It has two subclasses based on pathophysiological mechanisms. First subclass hold altered signaling pathways that modify activity of NF-kB {ectodysplasin-A receptor associated death domain (EDARADD)} signaling pathway and NEMO (NF-kB essential modulator) regulatory pathway. Second subclass includes regulatory deviations in transcription or expression of genes like *p63* (MIM 603273), *DLX3* (600525), *MSX1* (142983), *EVC2* (607261) and *EVC* (604831). Due to abnormal signals between ectoderm and mesoderm, development and differentiation remain halted and clinically hypoplastic structures develop from ectoderm.

Group 2; encompass abnormalities with defective structural proteins in plasma membrane. Nectin1, connexins and plakophilin are structural proteins that maintain cell-cell communication, growth, development and other responses to external factors. Palmoplantar keratoderma with or without association of deafness and retinal dystrophy are incorporated in this class (Martin *et al.*, 2013).

Palmoplantar Keratoderma (PPK)

Palmoplantar keratodermas (PPK) are heterogeneous group of genodermatosis characterized by abnormal hyperkeratotic thickening of the stratum corneum on the palms and soles. It encompasses approximately 60 genetically distinct diseases (Wilson *et al.*, 2010). Traditionally they have been classified as:

- Hereditary palmoplantar hyperkeratosis
- Acquired palmoplantar hyperkeratosis

Hereditary palmoplantar keratoderma may be autosomal dominant, autosomal recessive, X-linked or mitochondrial. Whereas, Acquired PPK usually occur later in life due to many reasons, such as Keratoderma climactericum, drugs, malnutrition, chemicals, systemic disease, malignancy, dermatoses and infections (Patel *et al.*, 2007). The molecular pathogenesis of hereditary PPK may include various dysfunctions that, among others, affect keratin intermediate filaments, desmosomes, gap junctions, water channels, EGFR signaling. (Has and Technau-Hafsi, 2016).

Types of Hereditary Palmoplantar Hyperkeratosis

Hereditary PPK are divided into 4 types based on morphology and distribution of hyperkeratosis on palms and soles (Sakiyama and Kubo, 2016).

- Diffuse PPK
- Focal PPK
- Striate PPK
- Punctate PPK

Diffuse Palmoplantar Keratoderma (DPPK)

DPPK shows diffuse hyperkeratosis covering the entire surface of the palms and soles. This could be inherited in either autosomal dominant or autosomal recessive manner. DPPK has two major types; DPPK with transgrediens and DPPK without transgrediens.

Diffuse PPK without Transgrediens

Unna– Thost PPK (MIM 600962) is autosomal dominant non-epidermolytic form of diffuse PPK without transgrediens. It is most common type caused by mutation in the gene coding for keratin 9 or keratin 1 (*KRT9*, *KRT1*). Genetic mutations (p.R162W, p.N160I) in *KRT9* (MIM 607606) gene evaluated by Unna-Thost and Vorner were found on the coil-1A segment at the beginning of the central rod domain (Reis *et al.*, 1994; Kuster *et al.*, 2002). Keratin 1 is a constituent of keratin intermediate filaments in the supra-basal epidermal layers, building heterodimers with keratin 9 (Knöbel *et al.*, 2015). So, whenever *KRT9* or *KRT1* (MIM 139350) is mutated it leads to formation of defective epidermal layer.

Diffuse PPK with Transgrediens

Diffuse PPK with transgrediens include several diseases like; mal de Meleda (MIM 248300), acral keratoderma, Nagashima type PPK (MIM 615598), Bothnian (MIM 600231), Gamborg-Nielsen (MIM 244850), Greither (MIM 144200), and Sybert.

Nagashima type of PPK (PPKN) was first described by Masaji Nagashima in 1977, characterized by a whitish spongy change in palmoplantar hyperkeratotic skin upon water exposure (Kubo *et al.*, 2013). It is non-progressive with no constricting bands and spontaneous amputation having skin redness and milder phenotypes than mal de

Meleda (Nagashima, 1977; Kabashima *et al.*, 2008). PPKN is caused by autosomal bi-allelic loss of function mutation in *SERPINB7* (Kubo *et al.*, 2013). Whereas, mal de Meleda is due to homozygous recessive mutations in *SLURP1* gene (Fischer, 2001).

Bothnian PPK (MIM 600231) described in 1994, is an autosomal dominant form of diffuse non-epidermolytic PPK. It exhibits whitish spongy appearance upon water exposure (Lind *et al.*, 1994; Kubo *et al.*, 2013). It is caused by mono-allelic missense mutation in *AQP5* (MIM 600442) gene encoding water channel protein (Blaydon *et al.*, 2013). *AQP5* genes encode channel proteins primarily responsible for osmoregulation across the plasma membrane (Cao *et al.*, 2016).

Gamborg-Nielson PPK (PPK-GN) is morphologically very similar to mal de Meleda. It has been reported with less severe hyperkeratosis except for the knuckle pads and no nail deformities (Kastl *et al.*, 1990). PPK-GN may be either due to homozygous or compound heterozygous mutation in *SLURP1* gene (Zhao *et al.*, 2014).

Greither PPK and Sybert PPK both are autosomal dominant forms of DPPK with transgrediens and progressive features with age. Sybert PPK show more severe and wider range of hyperkeratosis than does Greither PPK (Sybert *et al.*, 1988). Greither PPK is caused by a *KRT1* mutation whereas, gene for Sybert PPK is unknown (Gach *et al.*, 2005).

Focal Palmoplantar Keratoderma

This disorder is inherited in an autosomal dominant manner. Painful circumscribed hyperkeratosis like calluses is characteristic of focal PPK on the body weight bearing area of the soles. Their onset occurs within the first 2 years of life (Kubo *et al.*, 2013). Focal PPK is known for its major symptom of pachyonychia congenita (MIM 167200), caused by heterozygous mutations in *KRT6A*, *KRT6B*, *KRT16* or *KRT17* genes. Pathogenic variants in *KRT6C*, *KRT16* (MIM 613000) and *DSG1* (MIM 148700) cause focal PPK with or without minimal changes in the nails and other ectodermal tissues (Eliason *et al.*, 2012; Wilson *et al.*, 2010). Recently, a gain-of-function mutation in *TRPV3* was identified causing focal PPK (MIM 616400) in a Chinese family. *TRPV3* belongs to a large family of calcium permeable transient receptor potential ion channel membrane proteins. Mutation in *TRPV3* was suggested to disrupt the balance between keratinocyte proliferation and differentiation. Several mutations in *TRPV3* have also been reported causing Olmsted syndrome (MIM

614594), which shows mutilating diffuse PPK and perioral hyperkeratotic plaques, supporting the importance of *TRPV3* activity in keratinocyte differentiation (He *et al.*, 2015).

Striate Palmoplantar Hyperkeratosis

Striate PPK shows skin thickening prominent in a linear pattern along the flexor aspects of the fingers and over pressure points on the soles. It is inherited in autosomal dominant pattern and is divided into 3 subtypes based on responsible gene: PPKS I (*DSG1*), PPKS II (*DSP*) and PPKS III (*KRT1*) (Whittock *et al.*, 2002).

Heterozygous mutation in *DSG1* (MIM 148700), causes striate, diffuse and focal PPK depending on site of mutation. A homozygous mutation in *DSG1* is reported causing striate PPK and SAM (MIM 615508) syndrome (Samuelov *et al.*, 2013). Recently, milder cases of SAM syndrome along with striate PPK were also reported (Has *et al.*, 2015; Schlipf *et al.*, 2016). *DSG1* is a major component of desmosomes in the upper layer of the epidermis. Loss of *DSG1* expression on the cell membrane leads to weakened intercellular adhesion. Thus, histo-pathological features of PPK with *DSG1* mutations show characteristic clues of varying degrees of intercellular space enlargement and partial separation of keratinocytes in the spinous and granular cell layers (Bergman *et al.*, 2010).

Striate PPK II develops in first two decades of life. Lesions over the palms are linear and focally distributed. Striate PPK II is due to mono-allelic mutations in *DSP* (MIM 612908) gene, a protein responsible for anchorage of keratin intermediate filament and formation of desmosomal plaque. Their phenotypes appear due to haplo-insufficiency of desmoplakin, (Has and Technau-Hafsi, 2016). Striate PPK III is seen during early childhood due to frame shift mutation in *KRT1* (MIM 607654) gene. The keratin filament in suprabasal keratinocytes and inner plaques of hemidesmosomes are impaired (Whittock *et al.*, 1999).

Punctate Palmoplantar Keratoderma

Clinico-genetically three types of punctate palmoplantar hyperkeratosis can be distinguish: PPKP I or Buschke–Fischer–Brauer type (MIM 148600, 614936), PPKP II also called porokeratotic PPK (MIM 175860) and PPKP III also called acrokeratoelastoidosis (MIM 101850) (Brown, 1971; Has *et al.*, 2016). All these types

show autosomal dominant inheritance, however each type displays its own clinical characteristic.

PPKP I type appears during late childhood to adolescence and shows multiple tiny punctate keratosis on the palms and soles (Brauer, 1913). The lesions increase in number and size with advancing age. Heterozygous mutations in *AAGAB*, encoding alpha and gama-adaptin binding protein p34 (Has *et al.*, 2016; Giehl *et al.*, 2012), and *COL14A1* (Guo *et al.*, 2012), were identified for PPKP I.

In PPKP II, tiny keratotic spines on palmoplantar surface and columnar para-keratosis are seen during an individual's early 20s (Brown, 1971). PPKP III (MIM 101850) is characterized by small keratotic papules that primarily involve the margins of the hands and feet, appearing during adolescence or adult life. Histological findings reveal degeneration of elastic fibers. It is inherited possibly in autosomal dominant pattern with a suspected gene defect on chromosome 2. Overexpression of filaggrin is also seen in this type (Braun-Falco, 2009) however, very little is known about the genes responsible for PPKP II and acrokeratoelastoidosis (Has *et al.*, 2016).

Ichthyosis

The term ichthyosis is derived from Greek "ichthys" meaning fish. It was first used by Robert Willan in 1808 to signify generalized cornification disorders. The clinical features of ichthyosis include dry scales, cracking, blistering and shedding of the skin. Majority of ichthyosis are inherited, but acquired forms can develop in setting of malignancy, infectious, autoimmune diseases or some hormonal causes as well. On the basis of mode of inheritance ichthyosis can be X-linked, autosomal semi-dominant, autosomal dominant and recessive.

Mechanisms of pathogenesis of ichthyosis are thought to be related to at least one of the three components of stratum corneum i.e. intercellular lipid layer, cornified cell envelope (CCE) and keratin-filaggrin degradation products filling the cytoplasm of cornified cell. (Williams *et al.*, 2005). Approximately, 40 genes have been identified responsible for at least 20 varieties including inherited and how gene cause an acquired ichthyosis (Oji *et al.*, 2010).

Six major distinct subtypes are known in hereditary non-syndromic ichthyosis, starting with the most severe form, they are; harlequin ichthyosis (MIM 242500),

lamellar ichthyosis (MIM 242300), congenital ichthyosiform erythroderma (MIM 242100), epidermolytic ichthyosis (MIM 113800), recessive X-linked ichthyosis (MIM 308100) and ichthyosis vulgaris (IV, MIM 146700) (Oji *et al.*, 2010). Superficial epidermolytic ichthyosis (MIM 146800) is an additional subtype similar to epidermolytic ichthyosis. To date, 13 genes have been identified and demonstrated to cause isolated ichthyosis in human patients, including *FLG*, *KRT1*, *KRT2*, *KRT10*, *TGM1*, *ABCA12*, *ALOXE3*, *ALOX12B*, *NIPAL4*, *STS* and *FLJ3950* (Takeichi *et al.*, 2016).

Classification of Inherited Ichthyosis

The nonspecific term ''inherited ichthyosis'' refers to mendellian disorders of cornification (MEDOC) affecting all or most of the integument. Inherited ichthyosis is broadly divided into two types (Oji *et al.*, 2010).

- Non-syndromic/Isolated Ichthyosis
- Syndromic type of Ichthyosis

Non-Syndromic/Isolated Types of Ichthyosis

a. Common Ichthyosis

Based on reputation, ichthyosis vulgaris (IV) and recessive X-linked ichthyosis are categorized under common non-syndromic ichthyosis (Oji *et al.*, 2010).

1. Ichthyosis Vulgaris (IV)

Ichthyosis vulgaris (MIM 146700) is the mildest and most common form (95%) of hereditary non-syndromic ichthyosis, with an incidence of 1:250-1:1000. It is characterized by xerosis, scaling, pruritus and eczema (Oji *et al.*, 2010). The phenotypic manifestations tend to appear at the age of 2 months. Characteristically, the extensor sides of the lower legs and the back are most commonly affected with Keratosis pilaris and palmoplantar hyper-linearity sometimes. Ichthyosis vulgaris is inherited in autosomal semi-dominant pattern where both paternal and maternal alleles contribute equally (Hernandez *et al.*, 1999). Loss of function mutation in the filaggrin (*FLG*) gene is major culprit of ichthyosis vulgaris where a number of frame shift and null mutations leads to ichthyosis vulgaris phenotypes (Segre, 2006; Smith

et al., 2006). Loss or reduction in filaggrin expression results in excessively dry skin and impaired barrier function, causing IV (Akiyama, 2011).

Normally, the degradation products of keratohyalin granules occupy the cytoplasm of keratinized cells in the SC for proper skin barrier function. The epidermis of IV patients shows decrease in size and numbers or complete absence of keratohyalin granules. Keratohyalin granules in the granular layer of the epidermis are composed of large (400 kDa) pro-filaggrin polyproteins. Upon the terminal differentiation of keratinocytes, pro-filaggrin is cleaved into identical (37 kDa) filaggrin peptides. The liberated filaggrin aggregates the keratin filaments (Steinert *et al.*, 1981), causing a collapse of the granular cells into a flattened squame-shape. In addition, the degradation products of filaggrin contribute to moisture retention in the cornified layers. Thus, filaggrin is a major component of keratohyalin granules indispensable to the normal, intact, skin barrier function.

2. Recessive X-linked Ichthyosis (RXLI)

Recessive X-linked ichthyosis (MIM 308100) is the second most common ichthyosis, with a prevalence of 1:2000-1:6000 (Oji *et al.*, 2010). It is clinically severe than IV characterized by widespread, dark brown polygonal scales and generalized dryness. Cutaneous manifestations appear soon after birth and tend not to improve with age. The histopathology of RXLI typically shows compact hyperkeratosis and slight acanthosis with a normal granular layer.

Roughly, 90% of RXLI patients have large deletions in steroid sulfatase (*STS*: Xp22.3) gene leading to loss of enzymatic activity (Palmer *et al.*, 2006). With respect to the phenotypic spectrum of RXLI, *STS* gross deletions may lack polygonal dirty scales and cause milder phenotypes with skin peeling and eczema than most classic forms of RXLI. In classic RXLI cases *STS* deletion by whole-genome chromosomal microarray (CMA) are found with dirty polygonal scales over the skin (Hand *et al.*, 2015). Steroid sulfatase is concentrated in lamellar granules and then secreted into the intercellular spaces of the stratum corneum, along with other lipid hydrolases. In those spaces, steroid sulfatase degrades cholesterol sulfate, generating some cholesterol for the skin barrier. Likewise, the progressive decline in cholesterol sulfate permits corneodesmosome degradation leading to intact desquamation. Thus, two molecular pathways contribute to disease pathogenesis in RXLI. Steroid sulfatase deficiency

leads to both malformation of the intercellular lipid barrier, and a delay in corneodesmosome degradation which, results in corneocytes retention (Elias *et al.*, 2004).

b. Autosomal Recessive Congenital Ichthyosis (ARCI)

Autosomal recessive congenital ichthyosis (ARCIs) is a heterogeneous group of genodermatosis seen at birth and characterized by scaling and hyperkeratosis. ARCI embraces three different types of ichthyosis, harlequin ichthyosis (MIM 242500), congenital ichthyosiform erythroderma (MIM 242100) and lamellar ichthyosis (MIM 242300). Mutations in eight genes have been pronounced in ARCI to date, including *ABCA12*, *NIPAL4*, *CYP4F22*, *PNPLA1*, *CERS3*, *ALOX12B* and *ALOXE3* and the most dominant *TGM1* (Fischer, 2009; Herman *et al.*, 2009; Ahmed and O'Toole, 2014).

1. Harlequin Ichthyosis (HI)

Harlequin ichthyosis (MIM 242500) is the most devastating congenital skin disorder. The affected newborns show large, thick and plate-like scales over the whole body with severe ectropion, eclabium and flattened ears (Akiyama, 2006a). Harlequin ichthyosis patients often die in the first two weeks however, once they survive beyond the neonatal stage, the phenotypes improve within several weeks after birth. Other symptoms include dehydration, temperature instability, electrolyte imbalance, feeding problems and bacterial infections. An incidence of approximately 1:300,000 births have been estimated with 200 cases reported internationally (Belengeanu *et al.*, 2009). High death rate in early age is due to rigid adherent scales on the thorax or *ABCA12* malfunction in type II alveolar epithelial cells which causes defective alveolar surfactant secretion and respiratory distress (Lefevre *et al.*, 2003; Yanagi *et al.*, 2008). In HI, lamellar granules are distorted, reduced or absent resulting in disruption of intercellular lipid deposition in the stratum corneum and hence impaired desquamation and permeability leading to complications of harlequin ichthyosis.

In HI cases, only *ABCA12* (MIM 607800) have been reported as underlying genetic defects (Akiyama and Shimizu, 2008). More than 70 mutations in *ABCA12* associated with ARCI have been described, with 51 of these resulting in HI phenotypes (Peterson *et al.*, 2013). The *ABCA12* codes for a membrane associated ATP-binding cassette

transporter A12 (*ABCA12*) protein which functions to transport lipid glucosylceramides to the extracellular space through lamellar granules. Lamellar granules secrete lipid glucosylceramides and hydrolytic enzymes (proteases, lipases) and proteins (corneodesmosin) which are required for desquamation (skin shedding). The lipid glucosylceramides are hydrolyzed to form hydroxyl-ceramides, which are covalently linked to cornified envelope proteins to form the extracellular lamellar membrane, needed to keep the skin barrier permeable (Scott *et al.*, 2013).

2. Lamellar Ichthyosis (LI)

Lamellar ichthyosis (MIM 242304) is a rare form of autosomal recessive ichthyosis having milder phenotypes than HI with prevalence of 1:200,000 to 1:300,000 patients. The characteristic scales covering most of body surface are; large, thick and dark brown or gray in colour. Neonates are frequently born with collodion membrane. Scarring alopecia and Palmoplantar Keratoderma are frequently seen in LI cases (Akiyama *et al.*, 2003; Griffiths *et al.*, 1998).

To date, eight genes; *ABCA12*, *ALOXE3*, *ALOX12B*, *CERS3*, *CYP4F22*, *NIPAL4*, *PNPLA1* and *TGM1* have been reported in lamellar ichthyosis of these, *TGM1* and *ABCA12* are most frequently reported in Asian populations (Takeichi and Akiyama, 2016).

TGM1 gene located on chromosome 14q12 is known to cause lamellar ichthyosis (Huber *et al.*, 1995). Homozygous or compound heterozygous mutations are mostly observed in *TGM1* for LI phenotypes. Genetic variations in *TGM1* cause deficiency of keratinocyte transglutaminase, a Ca^{+2} dependent enzyme taking part in assembly of cornified cell envelope (Nemes *et al.*, 1999). Mutations in *ABCA12* have been associated with lamellar ichthyosis. Two more loci on chromosome 19p12-q12 contributes for lamellar ichthyosis along with mutated cytochrome P-450 oxidase (*CYP4F22*) gene (Lefevre *et al.*, 2006).

3. Congenital Ichthyosiform Erythroderma (CIE)

Congenital ichthyosiform erythroderma or non-bullous congenital ichthyosiform erythroderma (NBCIE: MIM 242100) is another subclass of ARCI. It is very rare form of ichthyosis and inherited in autosomal recessive manner. CIE possess clinical characteristics similar to lamellar ichthyosis but comparatively milder. As much as

90% of infants born with CIE are collodion babies, which gradually develops erythroderma with fine semi-adherent white scales. They also have palmoplantar keratoderma in association with ectropion, eclabium, scalp alopecia, decreased sweating with heat intolerance, nail dystrophies, painful fissures and digital contractures (Fischer *et al.*, 2000; Akiyama *et al.*, 2003).

Mutations in a number of genes have been identified for CIE including *ABCA12*, *ALOX12B*, *ALOXE3*, *CERS3*, *CYP4F22*, *LIPN*, *NIPAL4/ICHTHYIN*, *PNPLA1* and *TGM1* (Sugiura and Akiyama, 2015). Of these, *CERS3* was identified most recently as the ninth causative gene of CIE (Eckl *et al.*, 2013). All these genes contribute in formation of cornified cell envelop and stratum corneum. If any of these genes is mutated it leads to defective skin barrier function. Mutations in the genes *ALOX12B* and *ALOXE3* in patients with non-bullous congenital Ichthyosiform erythroderma were identified from the Mediterranean zone (Jobard *et al.*, 2002). Likewise, if *ABCA12* transporter activity is defective due to missense mutations, this will also be a leading cause of NBCIE (Sakai *et al.*, 2009).

c. Keratinopathic Ichthyosis

Keratinopathic ichthyosis is an umbrella term used for ichthyosis associated with keratin gene. Keratinopathic ichthyosis comprise epidermolytic ichthyosis (EI), superficial epidermolytic ichthyosis (SEI), annular epidermolytic ichthyosis (AEI), ichthyosis Curth Macklin (ICM), autosomal recessive epidermolytic ichthyosis (AREI), epidermolytic nevi (EN) and congenital reticular ichthyosiform erythroderma (CRIE).

All these types of keratinopathic ichthyosis are associated with genetic variations in the keratin family genes *KRT1*, *KRT2* and *KRT10* (Takeichi and Akiyama, 2016). There are 54 keratin genes in Homo sapiens, about 21 different genes including hair and hair follicle specific keratins have been associated with diverse hereditary disorders (Szeverenyi *et al.*, 2007).

1. Epidermolytic Ichthyosis (EI)

Epidermolytic hyperkeratosis or bullous congenital ichthyosiform erythroderma (BCIE: OMIM 113800) is the most predominant keratinopathic ichthyosis characterized by generalized blisters formation and multiple erosions with

erythroderma. The patients show blistering and erythema at birth which diminishes with age, and generalized epidermolytic hyperkeratosis in adulthood (Braverman *et al.*, 1902). It is frequently inherited in autosomal dominant manner.

Heterozygous mutations in *KRT1* (OMIM 139350) and *KRT10* (OMIM 148080) genes trigger keratinopathic ichthyosis. Palmoplantar keratoderma (PPK) is a more prominent in EI patients underlying mutations in *KRT1* than those having mutations in *KRT10* (DiGiovanna and Bale, 1994).

Affected individuals have mutations in highly conserved carbonyl terminal of rod domain of keratin 1 or in highly conserved amino-terminal of the rod domain of keratin 10. Consequently, filament assembly and elongation would be severely compromised whereas heterodimer formation would remain unaffected. Hence, the structural stability and flexibility of epidermal cells would be compromised (Compton *et al.*, 1992; Greenhalgh *et al.*, 1992)

2. Superficial Epidermolytic Ichthyosis (SEI)

Superficial epidermolytic ichthyosis also known as ichthyosis bullosa of Siemens (IBS: MIM 146800) is an autosomal dominant genodermatosis. Clinically it resembles epidermolytic ichthyosis but phenotypes are generally milder. Generalized erythematic and extensive blistering can be observed at births which are more prominent during summer months. Later in life, erythroderma reverts almost completely and keratotic lichenification may concentrate on the joint areas of arms and legs typically showing the so called "Mauserung phenomenon" (Whittock *et al.*, 2001). Superficial epidermolytic ichthyosis is due to heterozygous mutations in *KRT2* (MIM 600194) gene. To date, 15 pathogenic missense mutations in *KRT2* have been reported causing superficial epidermolytic ichthyosis (Takeichi and Akiyama, 2016).

Syndromic Forms of Ichthyosis

In "syndromic ichthyosis" affected individuals possess defects in other organs of embryonic origin other than skin (Oji *et al.*, 2010). Syndromic ichthyosis could be;

- X-linked syndromic ichthyosis
- Autosomal syndromic ichthyosis

1. X-linked Syndromic Ichthyosis

Dominant X-linked ichthyosis accounts for more than 95% of all forms of ichthyosis affecting approximately 1 in 6000 males (Wells *et al.*, 1966). One prominent example of X-linked ichthyosis is ichthyosis follicularis, atrichia and photophobia (IFAP: MIM 308205). It is characterized by respiratory infections or progressive deteriorating neurologic symptoms like, generalized seizures, temporal lobe malformation and other cerebellar abnormalities (KoniG and Happle, 1999). The complete IFAP phenotypes could be seen in male patients only while, female carriers may be present with linear pattern of follicular ichthyosis, mild atrophoderma, hypotrichosis and hypohidrosis (Konig *et al.*, 1999). Main cause of IFAP is not exactly known but it could be related to follicular keratosis within the eyelids. IFAP syndrome is caused by functional deficiency of membrane-bound transcription factor protease, site 2 (*MBTPS2*), a membrane embedded zinc metallo-protease that activates signaling proteins involved in sterol control of transcription and ER stress response (Oeffner *et al.*, 2009).

Conradi-Hunermann-Happle syndrome or chondrodysplasia punctata type 2 (CDPX2) is an X-linked dominant syndrome. This syndrome is fatal to male embryos and may be observed in female patients as well (Happle *et al.*, 1977; Braverman *et al.*, 1999). Congenitally affected females show severe ichthyosiform erythroderma which later change into striated hyperkeratosis following ichthyosis linearis. Short stature, hypo or hyperpigmentation in mild state can be observed in few individuals. Mutation in *EBF* gene results in this disease (Has *et al.*, 2002). X chromosome inactivation regulation in individuals is the process by which expression varies within families. This *EBF* gene play role in neuronal differentiation in spinal cord and hindbrain regions to enabled their precise positioning along the neurogenic cascade (Garcia-Dominguez *et al.*, 2003).

2. Autosomal Syndromic Ichthyosis (ASI)

Autosomal syndromic ichthyosis (ASI) is caused by genes located on autosomes, where chances of affected males and females are same. Some examples of autosomal syndromic ichthyosis are given here.

Trichothiodystrophy (TTD: MIM 601675) is characterized by sulphur deficient brittle hairs as result of reduction in cysteine rich matrix protein level. Other features include

small stature, mental retardation, ichthyotic skin, unusual facial features, and photosensitivity (Bergmann and Egly, 2001; Itin *et al.*, 2001). Most of the photosensitive patients have defective *XPD* gene, but mutation in *XPB* and *MPLKIP* has also been reported (Lehmann, 2003; Shah *et al.*, 2016). Both *XPB* and *XPD* are component of TFIIH core having ATP-dependent DNA helicase activity needed for nucleotide excision repair (NER) during transcription. Any defect in these genes can cause trichothiodystrophy (Coin *et al.*, 1999).

Sjogren-Larsson syndrome (SLS) is an inborn disease of lipid metabolism characterized by triad of congenital ichthyosis, intellectual disability, and spasticity. It is a rare syndrome affecting 1 in 100,000 new born (Raghuveer *et al.*, 2006; Subramanian *et al.*, 2016). *ALDH3A2* encodes for fatty alcohol: NAD+ oxidoreductase, leading to failure of fatty aldehyde degradation (Rizzo and Craft, 1991). Around 99 mutations have been reported in *ALDH3A2* gene with highest percentage of missense mutations. Redundant fatty aldehydes in SLS patients creates adducts with myelin and accumulate in stratum corneum and granular layer causing SLS manifestations (Rizzo and Carney, 2005; Subramanian *et al.*, 2016).

Refsum syndrome (RS: MIM 266500), an autosomal recessive syndromic ichthyosis characterized by neurological signs like, polyneuropathy and cerebellar ataxia associated with tissue storage of phytanic acid (Dieterich and Brandt, 1993). Mutation in *PHYH* gene is responsible for Refsum syndrome which encodes phytanoyl-CoA hydroxylase (PhyH). Phytanic acid accumulation occurs due to the abnormal function of phytanoyl-CoA hydroxylase (Jansen *et al.*, 1997).

Netherton syndrome (NS: MIM 256500) is an autosomal recessive congenital ichthyosis in association with chronic inflammation of skin, hair anomalies, failure to thrive epidermal hyperplasia with defective epidermal barrier function and atopic dermatitis. It is caused by splice site or indel mutations in *SPINK5* (MIM 605010) encoding a serine protease inhibitor (lympho-epithelial Kazal-type inhibitor: LEKTI) (Raghunath *et al.*, 2004). This protein is expressed in epithelial cells having 15 domains two of which are cysteine rich helping in disulfide bonds, characteristic of Kazal-type inhibitors (Furio *et al.*, 2011). In the epidermis LEKTI regulate desquamation by inhibiting Kallikrein-related peptidase 5 family proteins, present in epidermis and degrade proteins among cell junctions (Raghunath *et al.*, 2004).

Epidermolysis Bullosa (EB)

The term epidermolysis bullosa was first used by H. Koebner in 1886. These mechano-bullos (MB) diseases are heterogeneous group of disorders of the skin and mucus membrane. It is characterized by blister formation and erosions in response to mechanical traumas, commonly seen in areas subjected to frictional stress like, oral cavity and limns (Bruckner *et al.*, 2010).

The fragility of skin and mucus membrane is due to aberrations either in cytoskeleton of basal keratinocyte or basement membrane (Masunaga, 2006). Besides humans, it is also perceived in other mammals like calves and dogs (Spirito *et al.*, 2006; Foster *et al.*, 2010).

Classification and Types of Epidermolysis Bullosa

The epidermolysis bullosa is broadly divided into inherited and acquired types. The inherited blistering complications were diversified into different groups in international consensus meetings held from 1962 till 2008. In 2005 EB classification was based on molecular genetics, which described approximately 30 EB subtypes (Uitto and Richard, 2005). In the third consensus meeting held in Vienna in 2007, kindler syndrome (KS) was included as forth type of EB along with epidermolysis bullosa simplex (EBS), junctional epidermolysis bullosa (JEB) and dystrophic epidermolysis bullosa (DEB) (Daisuke Sawamura *et al.*, 2010).

Epidermolysis bullosa simplex (EBS) is inherited in autosomal dominant as well as recessive inheritance, characterized by blister formation in the basal keratinocytes or intra epidermal region. It accounts for 92% of all EB types (Fine *et al.*, 1999). It has 3 subtypes; the Dowling-Meara , Weber–Cockayne, and a severe form Koebner. Total 7 genes are reported causing EBS. In most cases *KRT5* and *KRT14* genes are affected (Lane *et al.*, 1992; Chan *et al.*, 1993). *PLEC1* gene has also been reported in autosomal recessive EBS with cutaneous blistering and pyloric aresia (Pfendner *et al.*, 2005; Sawamura *et al.*, 2007).

Junctional epidermolysis bullosa an autosomal recessive syndrome, accounts for 2% of EB types. It is characterized by blister in lamina lucida and stubborn erosions. It has three subcategories; the severe Herlitz type, milder non-Herlitz and lethal pyloric atresia (Sawaruma *et al.*, 2010). These three types are triggered either by missense

mutations in *LAMA3*, *LAMB3* *LAMC2* or *BP180* genes. *LAMB3* is responsible for 70% of all JEB (Pulkkinen *et al.*, 1994; Pfendner and Lucky, 2008; Nishie *et al.*, 2007). Geneticists have identified premature termination codons (PTC) in lethal pyloreic atresia whereas: missense mutations accompany non-lethal variants (Abe, 2007).

Dystrophic EB manifests generalized blistering, scarring and milia formation, accounting for 5% of all EBs. Dystrophic EB might be autosomal dominant (MIM 131750) or recessive (MIM 226600). The phenotypes of dominant DEB are milder than recessive DEB. It is caused by faulty *COL7A1* (MIM 120120) gene, which encodes type VII collagen, a major component of anchoring fibrils expressed in basement membrane (Ryynanen *et al.*, 1992). Mostly glycine residue gets changed which apply dominant negative effect on formation and assembly of type VII collagen. In recessive DEB, premature termination codon on both alleles triggers low or no protein expression contributing to severe phenotypes whereas, one defective allele underlie mild phenotypes (Christiano *et al.*, 1993; Latifa Hilal *et al.*, 1993). So, the degree of severity is related to collagen VII protein level.

Kindler syndrome (OMIM 173650) is an autosomal recessive syndrome characterized by trauma-induced blistering, poikiloderma, skin atrophy, mucosal inflammation and photosensitivity. The pathogenesis of Kindler syndrome underlie mutations in actin cytoskeleton-associated protein, fermitin family homolog 1, encoded by *FERMT1* gene (Jobard *et al.*, 2003). This protein mediates anchorage between the actin cytoskeleton and the extracellular matrix through focal adhesion. Whenever, *FERMT1* gets mutated it leads to imperfect focal adhesion.

To investigate about the molecular pathogenesis of hereditary skin disorders, three consanguineous Pakistani families, two with autosomal recessive ichthyosis and one with palmoplantar keratoderma were selected. Linkage analysis and homozygosity mapping was carried out by use of microsatellite markers. Of the three families, one showed linkage to *FLG*, second with *ABCA12* while the third one presented no linkage. Sanger DNA sequencing was performed which failed to reveal any sequence variant. This study will help to provide further insight into molecular characterization of hereditary skin disorders.

Chapter 2

Materials and Methods

MATERIALS AND METHODS

Study Subjects

In present study three Pakistani families (A, B, C) were included suffering with hereditary skin disorders. Families A, B and C belong to rural area of Khyber Pukhtunkhwa, Pakistan. The study was proceeded in appreciation with "institutional review board" of Quaid-I-Azam University, Islamabad, Pakistan. These families were first located and visited at their home town to collect the blood samples. Additionally, thorough family histories of all the three families were obtained. Photographs of all the affected members of each family were taken in consensus with elders of these families.

Pedigrees/Family Charts

Thorough family history and clinical information were obtained by visiting their settled areas. In order to approve the mendellian inheritance pattern in families under study, their pedigree sketches were drawn. The elder members of each family were questioned about the disease onset, severity, relationship among the couple and parents of the partners. Pedigrees were designed following standards defined by Bennett *et al.* (1995). In constructing pedigrees double lines were used to represent consanguineous marriages. Squares and circles denote males and females respectively. Correspondingly, the shaded squares and circles symbolize affected male and female members whereas; colourless squares and circles represent the normal individuals. Inclined crossed lines over squares and circles indicate deceased members. Each generation is indicated by roman numerals from top to bottom, while members and their position are denoted by Arabic numerals within each generation.

Collecting Blood Specimens

Venus blood samples were collected from all available affected and unaffected members of each family. Basic precautions were kept in mind during blood collection. Blood was drawn using 10 ml sterilized syringes (BD 0.8mm x 38mm 21G x 1 ½ TW, Franklin lakes, USA) and immediately transferred to the ethylene di-amine tetra acetate (EDTA) vacutainer tubes (BD vacutainer K3-EDTA, Franklin Lakes, USA). The collected blood samples were kept at 4°C in Human Molecular Genetics

Laboratory (HMGL) in the Department of Biochemistry, Quaid-i-Azam University Islamabad, Pakistan till DNA isolation.

DNA Extraction by Phenol-Chloroform Method

Collected blood of all members stored previously at 4°C was taken out and incubated for about one hour at room temperature. Genomic DNA was extracted from whole blood using standard phenol chloroform method (Sambrook *et al.*, 1989).

Steps of Phenol-Chloroform DNA Extraction Method

- 750 µl of blood was taken from EDTA tube and inverted 3-6 times with an equal volume of solution A in a 1.5 ml micro-centrifuge tube (Oxygen, Union, USA). An incubation time of 20-30 minutes at room temperature was given to it.
- The eppendorf tubes were centrifuged in a micro-centrifuge at 13,000 rpm (Rotation per minute) for 1 min.
- Supernatant was discarded carefully. Pellet was re-suspended in 450µl of solution A and again centrifuged at 13,000 rpm for 60 seconds.
- Unwanted supernatant was again discarded and this time pellet was suspended in 450 µl solution B, 20 µl of 20% SDS and 15 µl of proteinase K (20 mg/ml). Samples were kept for incubation overnight at 37°C.
- On day second, 500 µl of fresh mixture of equal volume of phenol/solution C and D was added to it, mixed it thoroughly and centrifuged at 13,000 rpm for 10 minutes.
- Three prominent layers were visible; the uppermost aqueous phase was cleverly shifted to new eppendorf tube. The middle protein layer and lower phenol-Chloroform layer was discarded.
- This time 0.5 ml of fresh solution D was gently mixed with aqueous phase and centrifuged again at 13,000 rpm for 10 min.
- The aqueous layer containing DNA was collected again in autoclaved eppendorf tube. Precipitation of total genomic DNA was done by adding 55 µl of 3 M sodium acetate (pH 6) and 500 µl of chilled (stored at -20°C) isopropanol. DNA thread was clearly seen by inverting the tubes gently.

- DNA containing tubes were centrifuged for 10 min at 13.000 rpm. The supernatant was discarded again and remaining DNA pellet was washed with 200 ul chilled 70% ethanol (BDH, Poole, England) and centrifuged at 13,000 rpm for 7 min this time.
- The ethanol was poured down carefully and remaining ethanol was evaporated from intact DNA in vacuum concentrator 5301 (Eppendorf, Hamburg, Germany) for 7 min at 45°C.
- After evaporating residual ethanol DNA pellet was kept in 180 µl of Tris-EDTA (TE) buffer (Sigma-Aldrich, St Louis, MO, USA) at 37°C for 24 hours in order to completely suspend the DNA pellet and protect from DNase or RNase.
- On third day the extracted DNA was checked on 1% Agarose gel and visualized by UV Transilluminator (Biometra, Gottingen, Germany). This stock DNA solution was stored at 4°C for further use.

Composition of Solutions Used in Genomic DNA Extraction

Compositions of solutions used are given below.

Solution	Composition
Solution A:	Sucrose 0.32 M + MgCl2 5 mM + Tris (pH 7.5) 10 mM + 1 % (v/v) TritonX-100
Solution B:	EDTA (pH 8.8) 2 mM + NaCl 400 mM + Tris (pH 7.5) 10 mM
Solution C:	Saturated phenol
Solution D:	Chloroform : Isoamylalcohol (24:1)
20% SDS:	10 g in 50 ml water
DNA Dissolving Buffer:	10 mM Tris (pH 8.0) + 0.1 mM EDTA
Bromophenol Blue:	Sucrose 40 g + Bromophenol Blue 0.25 g

Agarose Gel Electrophoresis

In order to analyze the extracted DNA qualitatively, 1% agarose gel was used to resolve the DNA. Gel was prepared by mixing 45 ml of distilled water and 5 ml of 10-X TBE (0.89 M Tris-Borate, 0.032 M EDTA) in the conical flask. 0.5 g of agarose was weighed with the help of electric balance and added to it. Agarose was dissolved by heating the conical flask in microwave oven for 5 minutes. As soon as the transparent solution was obtained, 5 µl of ethidium bromide was added to the flask for DNA staining. The dye was thoroughly mixed in the solution and then the solution was carefully poured into the gel tank. A comb was placed to create wells for DNA loading later on. Solidification of gel was attained at room temperature by leaving for 20-30 minutes. 4 µl of extracted DNA was mixed with 4 µl of loading dye (0.25% bromophenol blue with 40% sucrose) and loaded into the gel wells. 1-X TBE buffer was poured into the gel tank and gel was place in it. Electrophoresis was performed for 40-50 minutes at 120 volts. DNA bands were visualized under U.V Transilluminator (Biometra, Gottingen, Germany). Photographs were taken with the help of Digital Camera EDAS 290 (Kodak, New York, USA) to properly analyze the DNA presence and quality.

Composition of Solutions Used in Agarose Gel Preparation

Compositions of solutions used in agarose gel are given below.

Solutions	Composition
Loading Dye:	40 g Sucrose + 0.25 g Bromophenol Blue
10X-TBE:	0.89 M Tris + 0.025 M Boric Acid + EDTA 0.5 M (pH 8.3)
Distilled Water:	45 ml

Genotyping and Linkage Analysis/Homozygosity Mapping

Homozygosity mapping is employed in rare recessive diseases as a useful tool due to the fact that recessive disease alleles appear homozygous (identical by descent) in consanguineous families. To find Homozygosity of any known gene for hereditary skin disorders in all the selected three families, linkage analysis was performed with highly polymorphic microsatellite markers (Invitrogen Genelink, USA) flanking the known autosomal recessive genes/loci. At least five microsatellite markers were used for each loci associated with gene responsible for hereditary skin disorders. From UCSC Genome browser and Rutgers's Combined Linkage-Physical Map (Matise *et al.*, 2007), the genetic map distance of microsatellite markers was obtained. Table 2.1 summarizes microsatellite markers flanking the region of known candidate genes.

Polymerase Chain Reaction (PCR)

Amplification of desired genomic DNA region was accomplished by using site specific microsatellite markers in polymerase chain reaction (PCR). A 25 µl PCR mixture was prepared as a whole in 200 ul PCR tubes (Axygen, California , USA). The amount and concentrations of chemicals used are given below;

Chemicals used in PCR reaction are;

Chemicals Used	**Concentration of Chemicals**	**Amount Used in PCR**
PCR Buffer:	10-X (200 Mm (NH4)2SO4 + 750 mM Tris-HCl (pH 8.8) + 0.1 % Tween 20)	2.5 ul
$MgCl_2$:	25 mM	2.0 ul
dNTPs:	10 mM	0.5 ul
Forward and Reverse Marker:	20 ng/ul	0.4 ul
Taq Polymerase:	0.5 U/ul	0.3 ul
DNA Dilution:	-	2.0 ul
PCR Water:	-	17.3 ul

Polyacrylamide Gel Electrophoresis (PAGE)

8% non-denaturing polyacrylamide gel was used to resolve the PCR amplified product. Preparation of gel solution was carried out in 500 ml graduated cylinder. For a single gel a total volume of 50 ml was prepared and poured in between the two glass plates with a separation distance of 1.5 mm. After placing the comb, gel was kept at room temperature for 45-60 minutes to allow polymerization. Before loading, the sample was mixed with 6 µl loading dye (0.25% bromophenol blue with 40% sucrose). The sample was picked with micropipette and loaded into the wells. Vertical gel tank (Whatman, Biometra, Gottingen, Germany) having 1-X TBE buffer was used for electrophoresis. Electrophoresis was carried out at 120 volts for 120-150 minutes. The gel was stained with ethidium bromide (0.5 µg/ml final concentration) and visualized by placing it under UV transilluminator (Biometra, Gottingen, Germany) and photograph was taken with the help of Digital camera DC 290 (Kodak, Digital Sciences, New York, USA).

Polyacrylamide Gel Composition

Chemicals used for polyacrylamide gel preparation:

Chemicals:	**Concentrations and Composition**	**Amount Used for 1 Gel**
30% Acrylamide:	Acrylamide (MERCK Darmstadt, Germany)+ N,N Methylene -bisacrylamide (29:1).	13.5 ml
10-X TBE:	Tris 0.89 M + Borate 0.89 M + EDTA 0.02 M.	5 ml
10% APS:	Ammonium per sulphate (5 g/45 ml distill water).	400 ul
TEMED:	N, N, N', N'-Tetra methyl ethylene diamine (Sigma-Aldrich, USA).	25 ul
Distilled Water:	-	Raised to 50 ml

DNA Sequencing and Mutation Screening

Family B and C were found linked to *ABCA12* and *FLG* genes on chromosome 2q35 and 1q21.3 respectively. In order find any pathogenic alterations exon 32 and 49 of *ABCA12* and all of coding exonic region of *FLG* gene were PCR amplified from genomic DNA of affected members of both families. For designing primers, online primer 3 software (Rozen and Skaletsky, 2000) was used. The primer sequence, their product size and annealing temperatures are given in the Table 2.2 and Table 2.3.

1st Sequencing PCR

For DNA sequence analysis of affected individuals, exon number 32 and 49 of *ABCA12* and all three exons of *FLG* gene were amplified in a 50 µl PCR reaction. The 50 ul reaction mixture contained 2.0 µl Genomic DNA dilution, 2.0 µl of forward and reverse primers each, 5.0 µl PCR buffer, 4.0 µl (25 mM) MgCl2, 1.0 µl (10 mM) dNTPs 0.7 µl Taq Polymerase (one unit) (MBI Fermentas, UK) and 33.3 µl PCR water. Thermo-cycling conditions were the same as described for linkage analysis. Amplified PCR products of DNA samples were analyzed on 2% horizontal agarose gel. Four microliter amplified samples were mixed with 4 ul loading dye and loaded into the wells. Electrophoresis was performed at 120 volts (80mA) for 30 minutes in 1X-TBE buffer. Amplified products were visualized by placing the gel on UV transilluminator (Biometra, Gottingen, Germany) to check amplification of desired product.

1st Purification of Amplified Product

In order to purify amplified PCR product Axygen Biosciences purification kit (Axygen scientific, Central Avenue, Union City, USA) was used. Series of purification steps are given below.

- 50 ul PCR product and 120 ul binding solution (buffer PCR-A) were mixed by tabbing in reaction tube.
- PCR product along with added binding buffer was transferred to the column having silica based membrane, placed in collection tube and followed by centrifugation at 13,000 rpm for 1 minute.
- Flow through was discarded from the collection tubes and 600 µl W2 buffer (ethanol containing washing buffer NaCl, EDTA, Tris-HCl) was added to the

column. Again it was centrifuged at 13,000 rpm for 1 minute and flow through was discarded again.

- Again 400 µl of buffer W2 was added to the column and centrifuged for 1 min at 13,000 rpm.
- it was subjected to an empty spin at 13,000 rpm for 2 min to remove any residual buffer from the column after the flow through was discarded.
- Column having purified amplified DNA product was transferred to 1.5 ml microfuge tubes and 20-30 µl of warm elution buffer was added and left for 5 minutes.
- DNA was eluted down by centrifugation at 13,000 rpm for 2-3 minutes. The filtrate containing purified DNA was tested on 2% agarose gel electrophoresis. This purified amplified product was further subjected to second sequencing PCR.

2nd Sequencing PCR

Sequencing reaction of purified PCR products was carried out in 0.2 ml PCR tubes by adding 1 µl Big Dye terminator V 3.1 ready reaction mixture, 1 µl sequencing buffer (PE Applied Biosystems, Foster city, CA, USA), 1-2 µl (20 ng) forward/reverse primer (depending on the requirement whether forward or reverse strand was to be sequenced), 2 µl (20ng) purified PCR product and 5-6 µl PCR water, making the final volume to 10 µl. The reaction mixture was taken through thermo-cycling conditions consisting of an initial DNA denaturation at 96°C for 5 minutes. Total of 30 cycles amplification was performed each consisting of 3 steps: 20 seconds at 96°C for DNA denaturation into single strand, 20 seconds for annealing at a temperature (55°C-60°C) at which the primer hybridize/anneal and 5 minutes at 65-70°C for primer extension. This was followed by a final 10 minutes extension at 70°C for Taq DNA polymerase to synthesize any partially extended DNA strands left.

2nd Purification of PCR Product

After second PCR, amplified product was purified following ethanol precipitation protocol (POP6 Protocol) in a series of steps listed below.

- First of all fresh stop solutions of 2 µl of 3 M sodium acetate (pH 5.2), 2 µl of 100 mM sodium EDTA (pH 8) and 1 µl of 20 mg/ml Glycogen) was prepared in 1.5 ml eppendorf tube.
- 10 ul amplified product was transfer from 200 µl PCR tube to 1.5 ml eppendorf tube. 5 µl of stop solution and 70 µl of 100% chilled ethanol were added to it. The mixture was vortexed and then centrifuged at 13,000 rpm for 20 minutes in refrigerated centrifuge (4°C).
- The supernatant was discarded and washed with 160 µl of 70% ethanol by centrifugation at 13,000 rpm for 8 minutes.
- Ethanol was poured out and the pellet was dried in vacuum concentrator dried at 45°C for few minutes to evaporate any residual ethanol.
- The pellet was resuspended in 15 µl sample loading solution (SLS). After vortexing and short spin at 4,000 rpm the sample was loaded into the sample loading tray.
- Sequencing of the purified product was done using DTCS Quick Start sequencing kit (Beckman Coulter, Fullerton, CA, USA) in CEQ8800 DNA sequencer (Beckman Coulter, USA).

Sequenced Data Analysis

Chromatograms of affected individuals both of families B and C obtained from CEQ8800 DNA sequencer (Beckman Coulter, USA) were compared with corresponding control gene sequences obtained through Ensemble Genome Browser database (http://www.ensembl.org/index.html) to find any sequence variation in affected individuals. BioEdit sequence alignment editor version 7.1.3.0 was used to identify any sequence variation among the affected individuals and normal gene sequence.

Table 2.1: List of microsatellite markers used for linkage analysis of candidate genes/loci:

S.No	Gene/Loci	Chromosomal Position	Markers Used	cM
1	Cathepsin C (*CTSC*)	11q14.2	D11S2002	91.48
			D11S901	93.01
			D11S1306	94.14
			D11S1367	98.13
			D11S873	99.78
			D11S4182	100.6
2	Desmoplakin (*DSP*)	6p24.3	D6S1617	12.99
			D6S1574	17.02
			D6S1668	18.14
			D6S1547	20.85
			D6S309	22.28
			D6S410	23.33
			D6S940	26.02
3	Plakophilin 1 (*PKP1*)	1q32.1	D1S1171	209.59
			D1S2655	211.52
			D1S1678	213.76
			D1S510	214.36
			D1S2872	216.24
			D1S2636	218.42
			D1S2782	219.68
4	Secreted LY6/PLAUR Domain Containing 1 (*SLURP1*)	8q24.3	D8S1741	164.27
			D8S1729	165.8
			D8S1520	169.79
			D8S1925	171.32
			D8S1926	172.62

Continue...

5	Tyrosine Aminotransferase (*TAT*)	16q22.2	D16S186 D16S3085 D16S3141 D16S3139 D16S2642 D16S512 D16S3051	85.34 86.57 87.07 88.47 89.91 90.92 92.75
6	Junction Plakoglobin (*JUP*) and Keratin genes (*KRT9, KRT10,KRT16*)	17q21.2	D17S702 D17S967 D17S1801 D17S930 D17S920 D17S2180 D17S797	67.26 67.94 68.54 69.99 70.65 71.81 72.85
7	Gap junction protein beta 2 and 6 (*GJB2, GJB6*)	13q12.11	D13S175 D13S250 D13S1236 D13S1275 D13S232 D13S1243	0.55 3.1 5.41 6.97 8.31 10.06
8	Gap junction protein beta 3 and beta 4 (*GJB3* and *GJB4*)	1p34.3	D1S2781 D1S233 D1S513 D1S2613 D1S472 D1S186 D1S432	60.04 61.5 62.23 65.97 68.26 70.05 72.65

Continue...

9	Keratin 6C, Type II (*KRT6C*)	12q13.13	D12S1590	64.2
			D12S347	65.92
			D12S1677	66.17
			D12S325	69.56
			D12S1632	72.58
			D12S305	74.31
10	Locus on chromosome# 3		D3S3699	188
			D3S1232	189
			D3S1618	192.67
			D3S3609	193.97
			D3S3592	195.04
			D3S1617	196.4
			D3S1262	198.2
			D3S2436	201.38
			D3S3651	202.71
			D3S3596	204.1
			D3S3530	207.05
			D3S2747	208.84
			D3S3054	211.61
			D3S3043	214.45
			D3S3642	215.44
			D3s2305	216.38
			D3S240	218.62

Continue...

11	Locus on chromosome # 15		D15S643	57.67
			D15S527	58.52
			D15S1036	62.13
			D15S1507	64.61
			D15S651	65.2
			D15S153	66.12
			D15S988	68.64
			D15S983	69.72
			D15S216	72.29
			D15S977	73.47
			D15S131	74.43
			D15S192	76.75
			D15S818	77.29
			D15S114	78.41
12	ATP-binding cassette, sub-family A, member 12 (*ABCA12*)	2q35	D2S371	209.43
			D2S317	211.09
			D2S322	212.06
			D2S2944	212.85
			D2S2319	212.85
			D2S143	212.85
			D2S1345	213.02
			D2S137	215.32
			D2S3282	216.19
13	Filaggrin (*FLG*)	1q21.3	D1S498	156.54
			D1S2345	157.08
			D1S2858	159.08
			D1S2777	160.41
			D1S1595	160.41
			D1S1653	163.77
			D1S2635	165.74

Table 2.2: List of primers used for *ABCA12* gene sequencing

Exon No	Forward /Reverse Primer Sequence	Product Size (bp)	Tm°C
32 Forward	CCCTCTGATTAGGGCCTGGA	500	59.5
32 Reverse	AGATACGTGCTGTCTAGCTGGG		60
49 Forward	CAAGTTAGTCACAGGACCTC	480	52
49 Reverse	ATTCTCACCATGAAACCTG		55

Table 2.3: List of primers used for *FLG* gene sequencing

Exon No	Forward /Reverse Primer Sequence	Product Size (bp)	Tm°C
1-F-NEW	AGCTGTAAGTGGGCACAAG	565	54.89
1-R-NEW	CATCCAGGTATAGGGGAAGA		55.0
2-F	ACAGAATCCAGATGACCCAGAT	581	58.33
2-R	CTTAGCCTGTTCCTATTGTCTCC		57.13
3-F	AAGAGCCCAAGAGAAACAGG	660	57.03
3-R	CTGGAGCCATCTCTTGACTG		56.94
4-F	GAAGCCTATGACACCACTGA	714	54.60
4-R	CTGTTCATGAGTGCTCACCT		57.25
5-F	CAAGCTTCATCTGCAGTCAG	801	55.66
5-R	ATGACCAGCTCTGTCTTCGT		55.86
6-F	CAGACAAACACGAAATGAGG	684	55.15
6-R	TGGTAGAGGAAAGACCCTGA		55.74
7-F	GGACAGGCTGGTCACCATC	478	59.90
7-R	GTGACGTGACCCTGAGTGC	478	58.61

Continue…

8-F-NEW	ACCACGAGCAATCGGTAGAT	501	58.07
8-R-NEW	TGTGTGTCTGACTCCTCTGAATG		58.80
9-F	AACTCCTCACGCAGAGACTT	535	55.11
9-R	CCGTCTCCTGATTGTTTGT		54.41
10-F	ATCCCACCATGAGCAATCG	406	60.27
10-R	CCATGTCTTTCTCCTGGACT		55.41
11-F	AGTGTTAGCCAGGACAGTGA	723	54.24
11-R	TGAGTGCCTAGAGCTGTTTC		54.22
12-F	TTCATCTGCAGTCAGAGACAG	710	55.01
12-R	TTTCTGGAAGCCGACTCA		56.30
13-F.NEW	GATCCTACCACGAGCAATCA	699	57.10
13-R.NEW	TTCCACTGTCTCCGACTACAGA		58.38
14F	TCGTCATGCAGAGACTTGGT	633	57.79
14R	TGTCTGCTGACACTTCTGGA		56.35
15-F	CAGCACTGGAGGAAGACAAA	673	57.43
15-R	GTGGTGGGATCCATGTCTTT		58.07
16-F	TGGGTCTGCTTCCAGAAA	676	56.13
16R	CCTATCTACCGATTGCTCTTG		55.20
17F	AGTGTCAGCACAGGGAAAAG	600	56.37
17R	AGATCCATGATGGTTTCTGG		55.79
18-F	CAGAAGTGCAAGCAGAAAAA	690	55.11
18-R	TAGAGGAAAGACCCTGAACG		54.41
19-F-NEW	GGCACTCATCATGCAGAGAA	570	57.67
19-R-NEW	GTCCATGAATGGTGTCCTGA	653	59.33

Continue...

20-F	CGTTCAGGGTCTTTCCTCTA	676	55.48
20-R	AGGAAGTCTGTGTGTGACGA		55.03
21-F	TCACCATGAAGCTTCCTCTC	720	56.35
21R	TGTCTTGTGCCTGCTCAT		54.41
22-F	GTCCATGCGATTAATGGACGT	753	60.55
22-R	ACGTCCATTAGCTAGCAGACGT		58.93
23-F	ACCATGAAGCTTCCACTCA	656	55.03
23-R	ATGGGAGGACTCAGACTGTT		54.51
24-F	GTAGTCAGGCCAGTGACAAT	697	53.54
24-R	TCCTGAACAGATCCACGAT		54.75
25-F	AGGAACAATCAGGAGACAGC	956	55.29
25-R	TCATGAGTGCTCACCTGGTA		56.65
26-F	ACGTATGTACGTAGCTACG	690	59.31
26-R	TGCTAGTGCAATGTGTGATTC		55.79
27-F	CGATCGTACGATCGTCGATGT	304	61.79
27-R	ACAGATGCTATGTTCATCGT		56.46
28-F	TCAAGTGCAGGAGAGAGACAT	480	60.55
28-R	AGTGGAAGCTTCATGGTGAC		58.93
29-F	ACGAGCAATCGGTAGATAGG	768	55.46
29-R	AGTGCCCTCAGATTGATAATG		58.28
30-F	GACAGTGACAGTGAGGCATA	735	52.84
30-R	GCTACCACCAAACTAATGAAA		54.46

Chapter 3
Results

RESULTS

Family A

Family A, identified with palmoplantar keratoderma was sampled from rural area of district Lucky Marwat, Khyber Pakhtunkhwa, Pakistan. This is a four generation pedigree (Figure 3.1) with consanguineous marriages. It contains two affected males in second generation (II-5, II-8) and seven normal individuals, nine affected in third generation including one female (III-9) and eight males along with five normal individuals. Fourth generation has four affected males (IV-2, IV-3, IV-4, and IV-6) and four other normal members. All affected individuals were identified with palmar and plantar keratosis on their palms and soles. No other deformity was identified in the affected members as evident in Figure 3.2. Analysis of pedigree depicted autosomal recessive mood of inheritance. From family A blood of three affected (III-7, III-10, IV-6) and one normal (III-5) individual were collected and tested for homozygosity mapping. Those members whose blood were collected are represented with asterisks (*) in the pedigree.

Family B

Family B is a native inhabitant of the Swat region of Khyber Pukhtunkhwa, Pakistan. It is identified with autosomal recessive form of congenital ichthyosis (ARCI) on the basis of clinical features. A five generation pedigree (Figure 3.3) was constructed for this family. The pedigree depicted consanguineous pattern of marriages and autosomal recessive mode of inheritance. The affected individuals arise from two normal carrier parents in fifth generation. Out of six individuals in fifth generation one male and one female (Figure 3.4) was found with ARCI. The affected female has dry and thick dark brown scales on their thoracic region, forehead and arms. While male have mild exfoliating scales or peeling skin on face. Blood samples were collected from five members including two affected (V-2, V-4) and three normal (III-2, IV-1, V-3) were collected. Their DNA was subjected to molecular analysis.

Family C

Family C belongs to district Mardan of Khyber Pukhtunkhwa province, Pakistan. A four generation pedigree (Figure 3.5) was constructed after getting information from

elders of the family. The pedigree consists of 19 individuals including three affected (IV-4, IV-5, and IV-6) members. Pedigree analysis suggests autosomal recessive mode of inheritance. The clinical features of affected individuals include ichthyosis with moles on their skin. These moles were more prominent over facial skin while less over hands. Blood samples were collected from eight members including five normal (III-4, III-5, IV-1, IV-2, IV-3) and three affected (IV 4, VI-5, VI-6) individuals.

Mapping Candidate Genes Involved in Skin Disorders

In the present study, all the three families (A, B, C) were analyzed for linkage to known loci for ichthyosis and palmoplantar keratoderma through microsatellite markers mapped within respective candidate regions. Microsatellite markers for genotyping were selected from the regions of respective candidate genes. The candidate genes analyzed for linkage, include *FLG* (1q21-22), *ABCA12* (2q34) *NIPAL4* (5q33), *KRT1* (12q13), *TGM1* (14q11), *ALOXE3* and *ALOX12B* (17p13), *CYP4F22* (19p12-q12), *PNPLA1* (6p21.31), *CTSC* (11q14.2), *DSP* (6p24.3), *PKP1* (1q32.1), *SLURP1* (8p24.3), *TAT* (16q22.2), *JUP*, *KRT9*, *KRT10* and *KRT16* (17q21.2), *GJB2* (13q12.11), *GJB3* and *GJB4* (1p34.3), *KRT1*, *KRT6C* (12q13.13) and two locus on chromosome number 3 (188-218 cM) and 15 (57.68-78 cM). Analysis of microsatellite markers was performed using a standard PCR protocol and subsequently run on polyacrylamide gel (8% non-denaturing) for further analysis.

PCR amplified product was stained with ethidium bromide and visualized on UV Transilluminator (Biometra, Gottingen, Germany). Photographs were taken using digital camera EDAS 290 (Kodak, NY, USA) to illustrate an exclusion or linkage to any gene/loci. If the normal individuals exhibited heterozygous banding pattern and affected individuals were homozygous for the same microsatellite marker, the markers were consider to be linked to the disease gene.

In family A, DNA extraction of four members, one normal (III-5) and three affected (III-7, III-10, and IV-6) individuals were carried and subjected to genotyping study. The results obtained from polymorphic microsatellite markers for *CTSC*, *DSP*, *PKP1*, *SLURP1*, *TAT*, *JUP*, *KRT9*, *KRT10* and *KRT16*, *GJB2*, *GJB3* and *GJB4*, *KRT1*, *KRT6C* and two locus on chromosome number 3 (188-218 cM) and 15 (57.68-78 cM) are listed in Table 2.1. Genotyping results of microsatellite markers depicted

no linkage to any known autosomal recessive loci for family A with palmoplantar hyperkeratosis. Thus, suggesting the involvement of any other causative known or novel mysterious loci somewhere in the genome.

In family B, autozygosity was carried out in five DNA samples. Of five members three normal (III-3, IV-1, V-3) and two affected (V-2, V-4) individuals were screened for autozygosity mapping using highly polymorphic microsatellite markers. Genotyping analysis revealed linkage of the family to *ABCA12* gene with cytogenetic location 2q35. Markers in linkage interval were homozygous in both the affected and heterozygous in unaffected individuals of the family. The markers that were found linked are D2S2944 (212.85 cM) and D2S143 (212.85 cM). Haplotypes of family B are given in Figure 3.8.

In family C, DNA samples of six individuals containing three normal (III-4, III-5, IV-2) and three affected (IV-4, IV-5, IV-6) were selected for homozygosity mapping with highly polymorphic microsatellite markers. Analysis of the results showed linkage to filaggrin (*FLG*) gene on chromosome 1q21.3. Linkage interval is flanked by D1S498 (156.54 cM) and D1S2777 (160.41 cM) microsatellite markers (Figure 3.10).

Sequencing *ABCA12* Gene

Genotyping results illustrate linkage of family B to *ABCA12* gene on chromosome 2q35. Primers for selected exons (32 and 49) were designed using Primer3 software. Designed primers sequences are listed in Table 2.2. The sequenced data analysis exons 32 and 49 of V-4 member revealed no pathogenic sequence variation. Hence, it is clear that mutation could be present either in rest of the coding or regulatory region of this gene or it may be somewhere else in the genome.

Sequencing *FLG* Gene

Homozygosity mapping of family C displayed linkage to *FLG* gene (1q21.3). Primers for all the coding exons were designed with help of primer3 software. List of primers are given in Table 2.3. Sanger sequencing of all the coding exons of affected individual (IV-4) was performed using respected primers. Data analysis of sequenced *FLG* gene revealed no recurrent or novel mutation throughout the coding region.

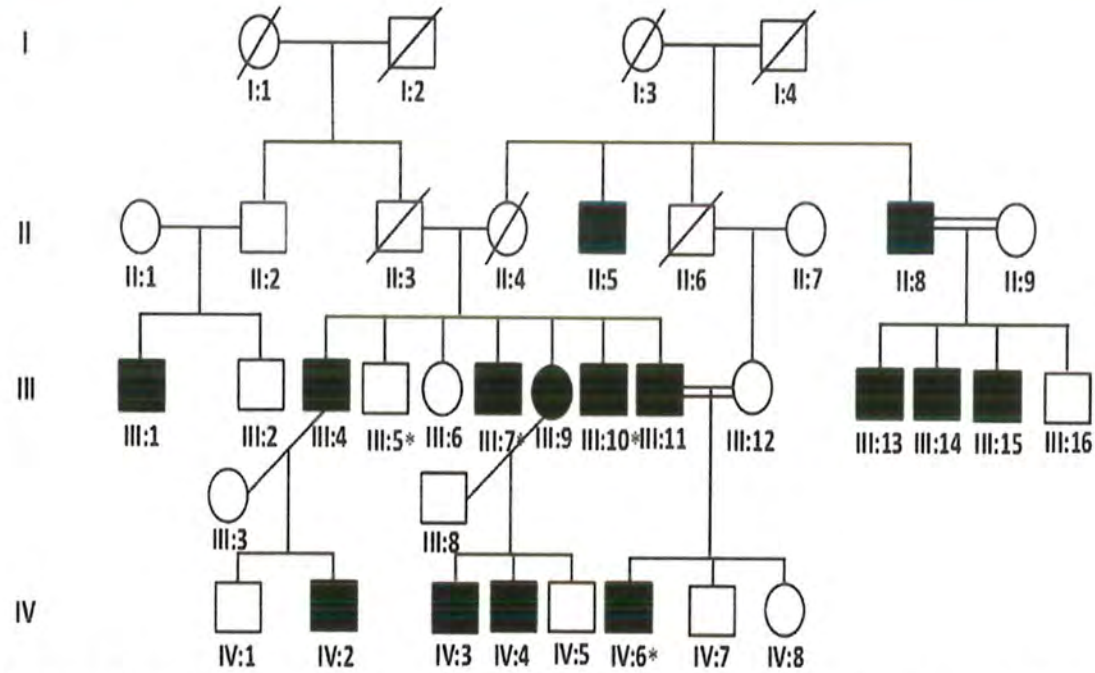


Figure 3.1: Pedigree of family A, segregating autosomal recessive type of diffuse palmoplantar hyperkeratosis. Circles and squares symbolize females and males respectively. A shaded circle or square represents an affected while unshaded symbol represents normal individuals. Double lines specify consanguineous marriages. Cross lines over the symbols represent deceased individuals. The roman numerals indicate the generation number of individuals within a pedigree while Arabic numerals indicate their position within a generation.

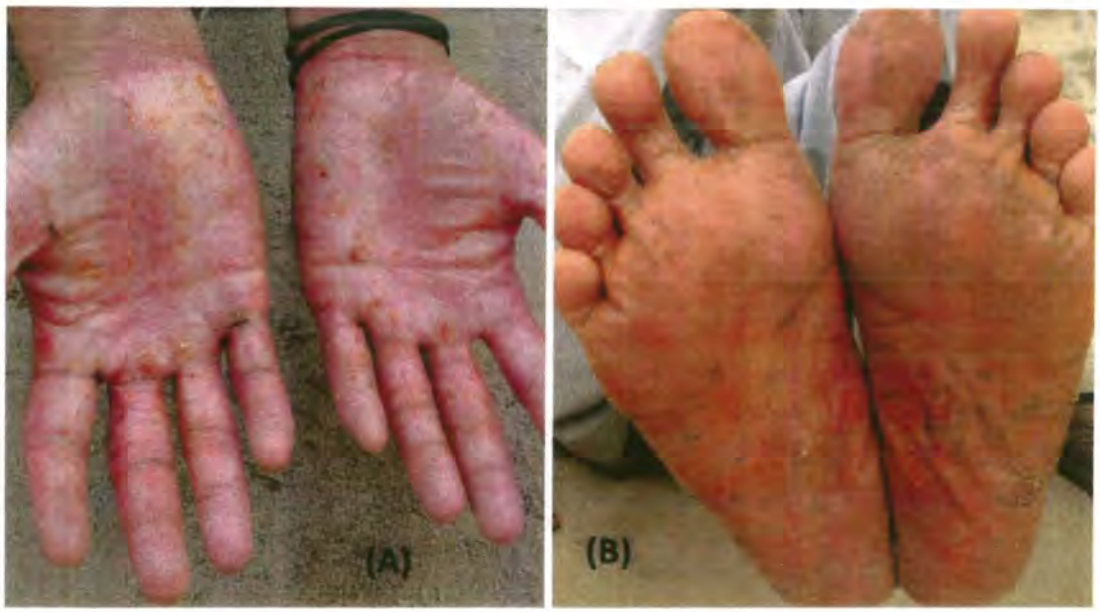


Figure 3.2: Affected individuals (III-7, III-10, IV-6) of family A having congenital diffuse palmoplantar keratoderma. Images show highly keratinized yellowish skin covering the palms and soles with clearly erythematous border with normal skin. No other associated abnormalities were found. Pictures were published with consent from family elders.

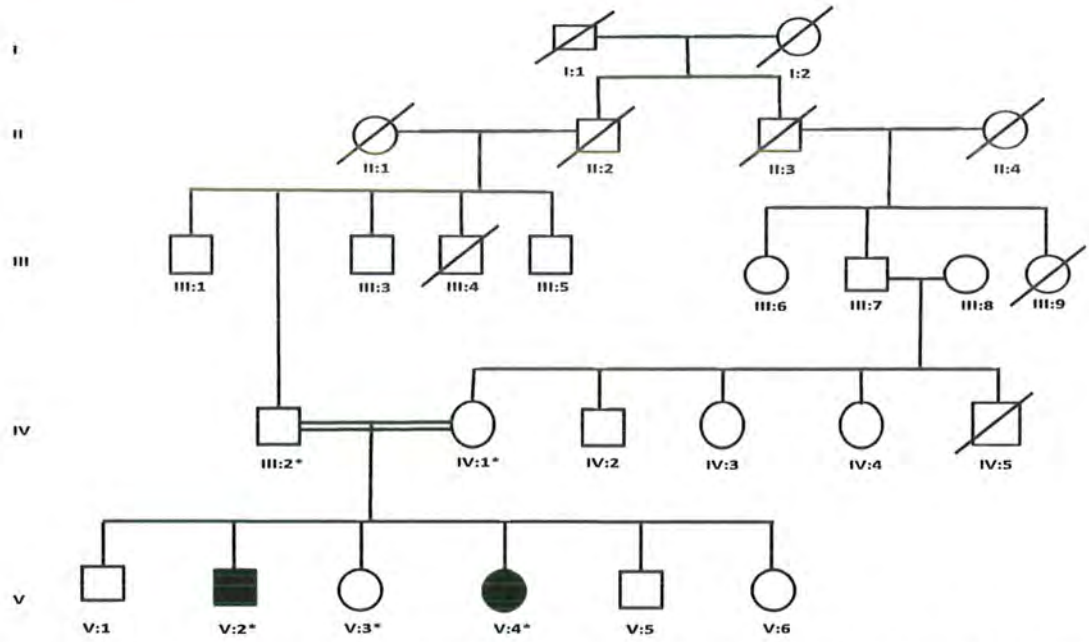


Figure 3.3: Pedigree sketch of family B segregating hereditary autosomal recessive congenital ichthyosis (ARCI). Squares and circles illustrating male and female individuals of the family respectively. Filled shapes indicate affected individuals and blank shapes indicate normal individuals. Consanguineous union is represented by double lines between two individuals. Deceased individuals are marked by crossed lines over square or circle. Roman numerals and Arabic numbers indicate generation number and members in the pedigree respectively. Shapes labeled with asterisk (*) are symbolizing the members whose blood samples were available for the present study.

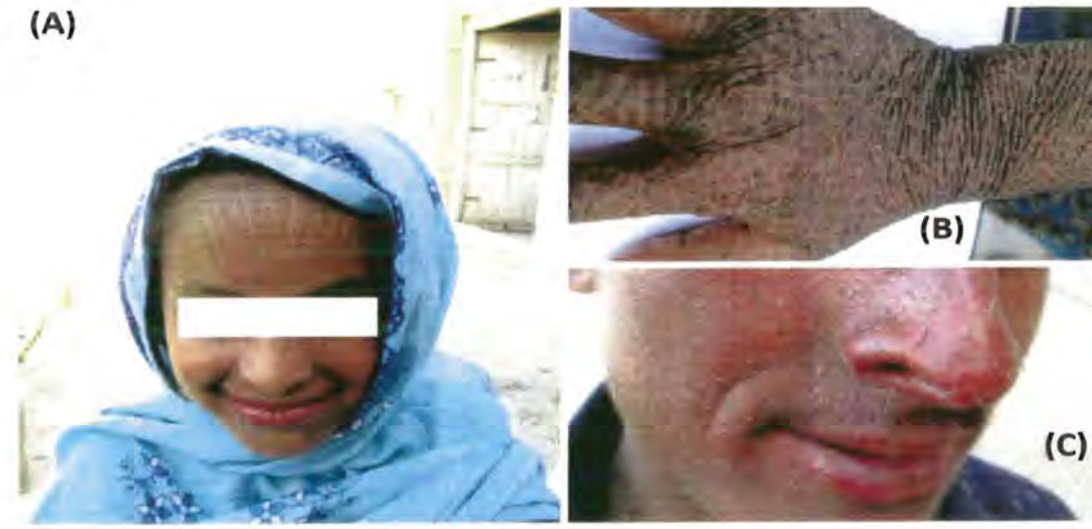


Figure 3.4: Affected sister and brother (V-2, V-4) of family B having lamellar ichthyosis phenotypes. The entire body surface of the affected female was covered with large thickened dark brown fish like scales and scanty eyebrows. Scales on the forehead and arm of female are shown in pictures. The male affected member has spiny peeling scales on face as shown above.

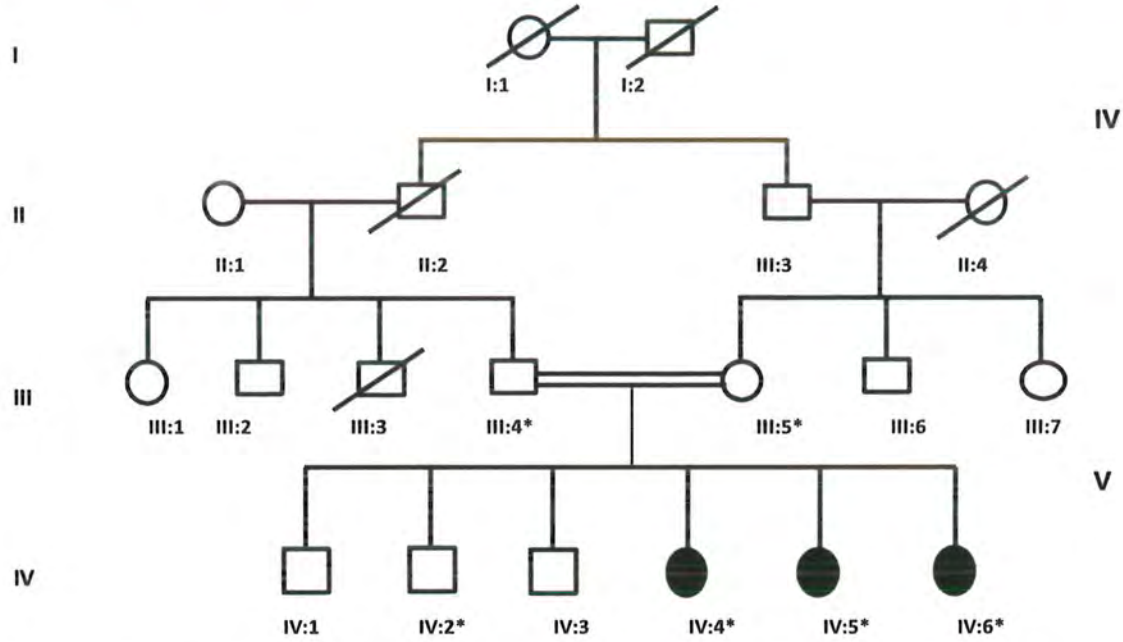


Figure 3.5: Pedigree drawings of family C with ichthyosis having autosomal recessive pattern of mendellian inheritance. The colourless symbols represent unaffected individuals while black colour symbols represent affected individuals. Symbols with crossed lines represent deceased members. The individual numbers labeled with asterisks (*) indicate the samples available for this study.

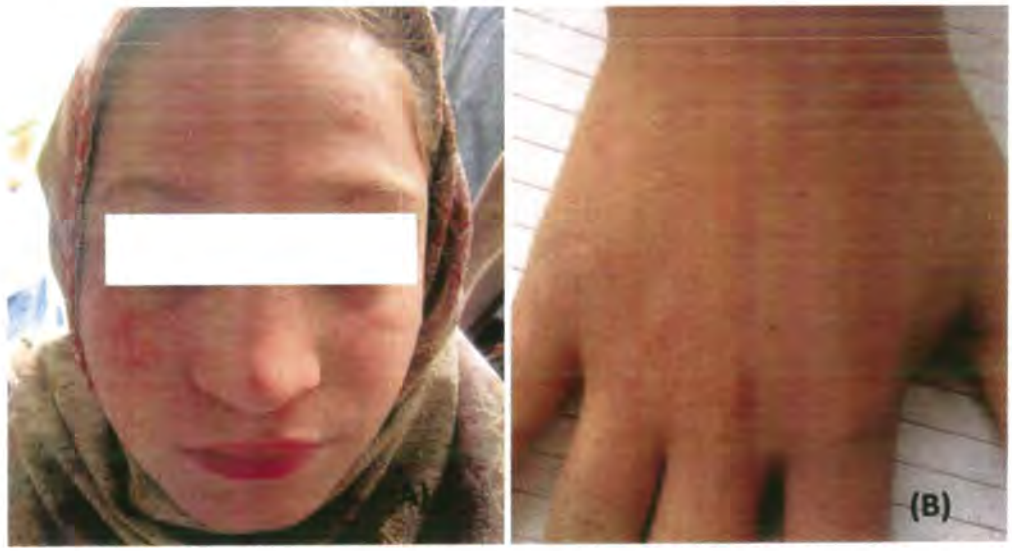


Figure 3.6: Affected individuals (IV-4, IV-5, IV-6) of family B with clinical features of ichthyosis vulgaris having spotted skin throughout body. The spots are more concentrated on face while these are less prominent over the arms and rest of the body. All the affected members observed were females only.

Family B: *ABCA12* gene

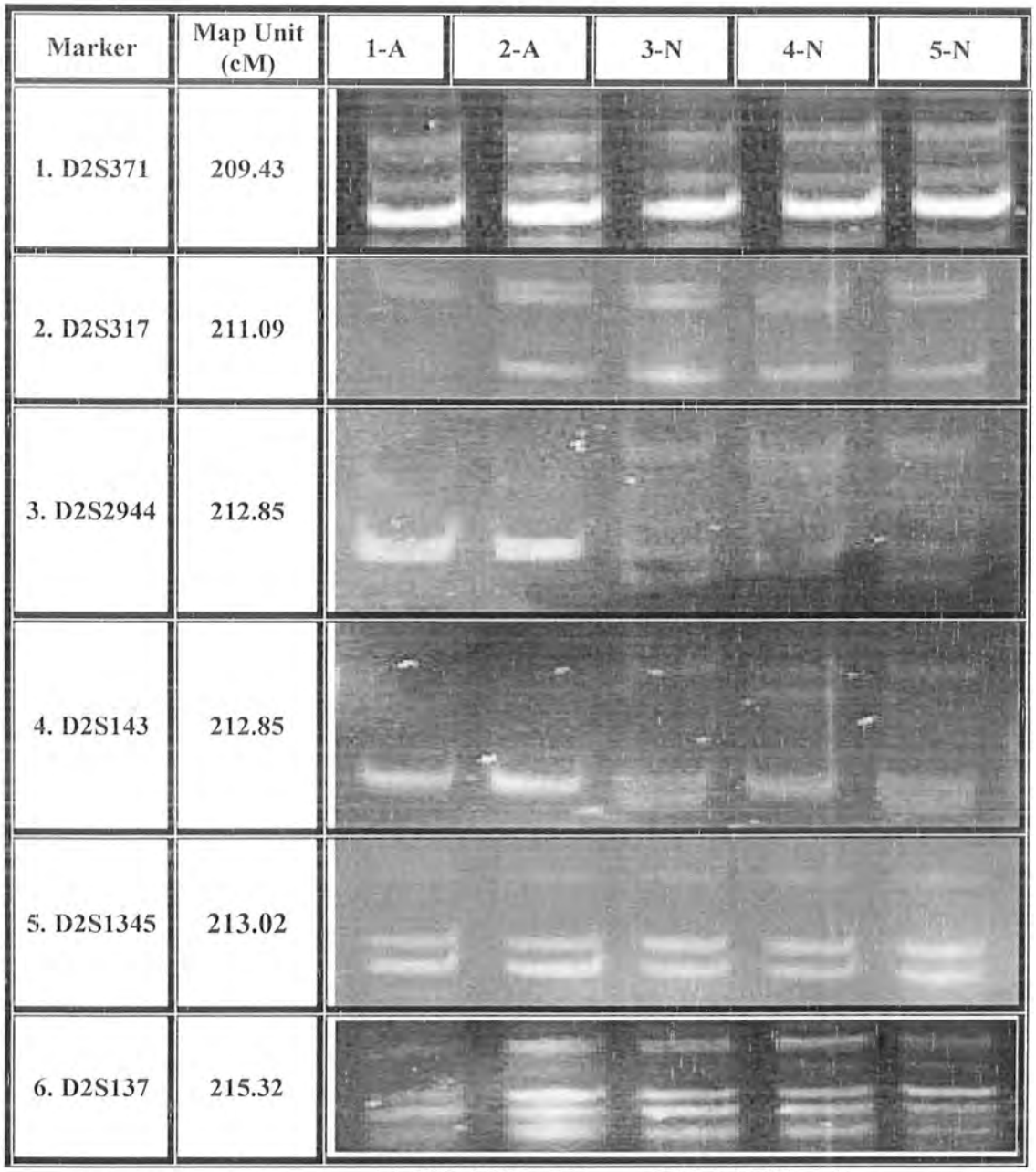


Figure 3.7: The electropherogram of 8% non-denaturing polyacrylamide gel stained with ethidium bromide (Et.Br), illustrating allelic pattern amplified with respective microsatellite markers flanking *ABCA12* gene on chromosome 2q34. Roman numerals and Arabic numbers ascertain the generation number and position of the individuals with in a generation in pedigree.

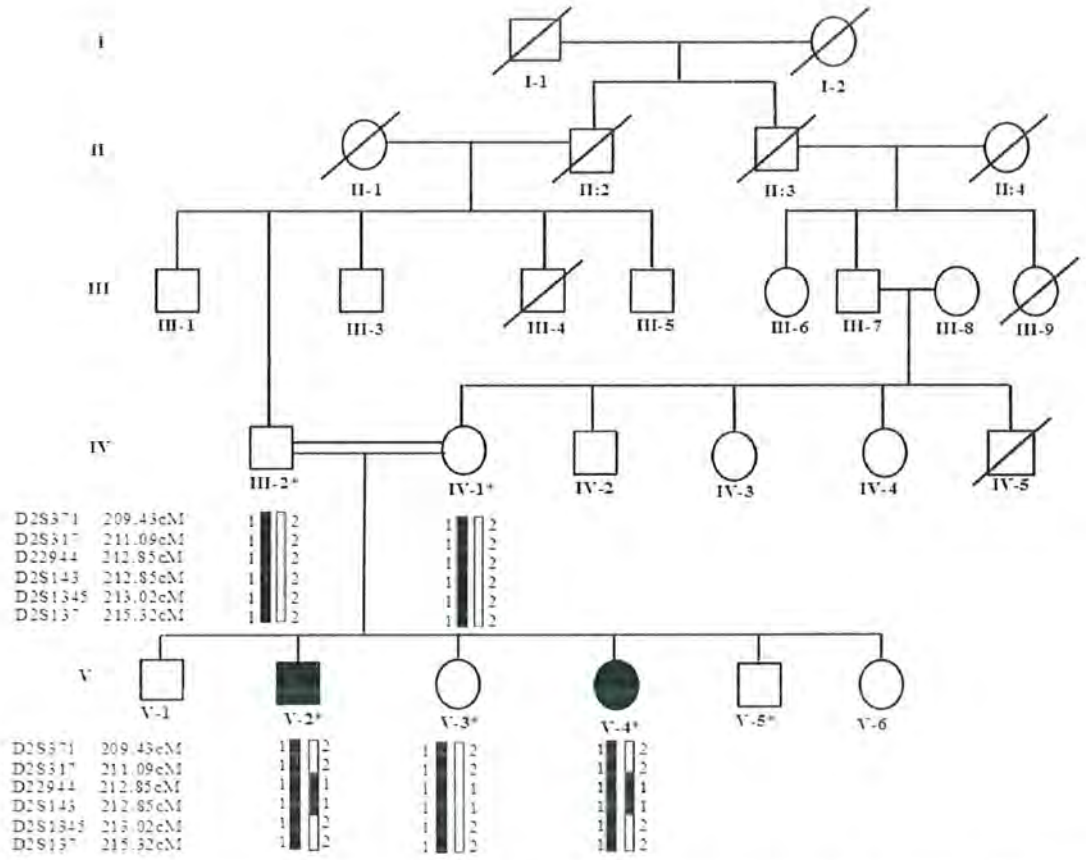


Figure 3.8: Pedigree illustrates family B; segregating autosomal recessive lamellar ichthyosis with haplotypes of the most closely linked locus specific microsatellite markers under each genotyped member and their cytogenetic position in terms of centimorgan (cM). Black symbols depict affected members whereas blank symbols represent normal members of the family.

Family C: *FLG* gene

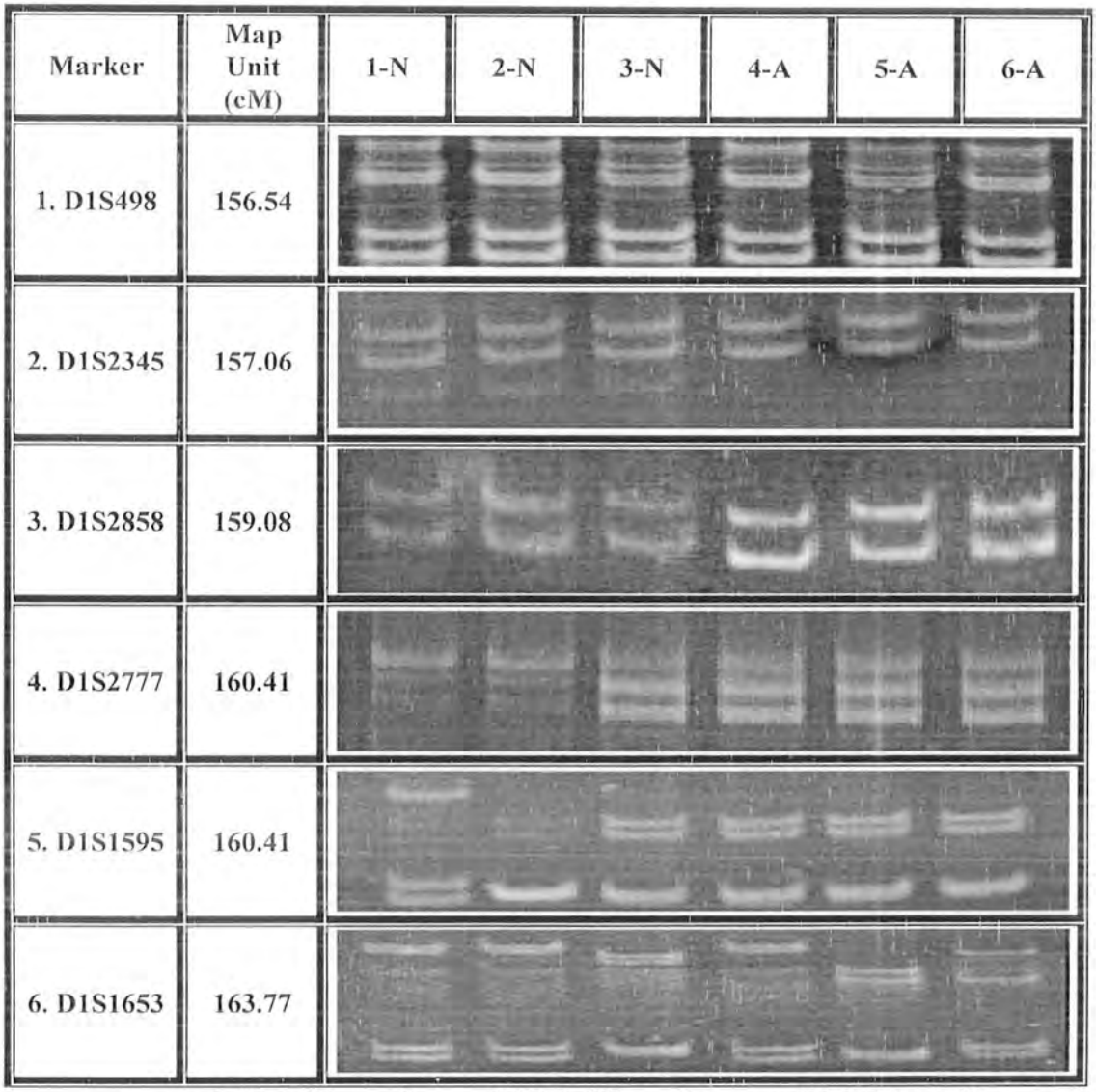


Figure 3.9: The electropherogram of 8% non-denaturing polyacrylamide gel stained with ethidium bromide (Et.Br), illustrating allelic pattern amplified with respective microsatellite markers flanking *FLG* gene on chromosome 1q21-22. Roman numerals and Arabic numbers ascertain the generation number and position of the individuals with in a generation in pedigree.

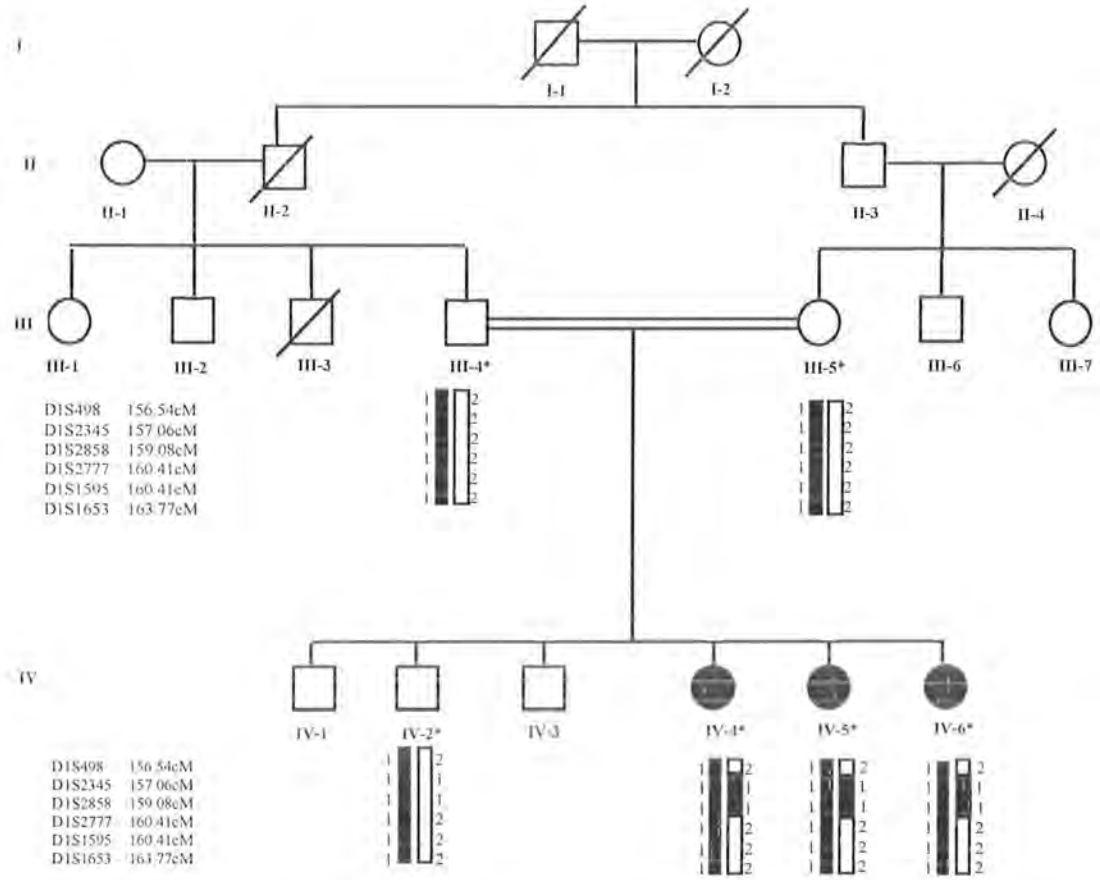


Figure 3.10: Pedigree illustrates family C, segregating autosomal recessive ichthyosis vulgaris with haplotypes of the most closely linked locus specific microsatellite markers under each genotyped member and their cytogenetic position in terms of centimorgan (cM). Black symbols depict affected members whereas blank symbols represent normal members of the family.

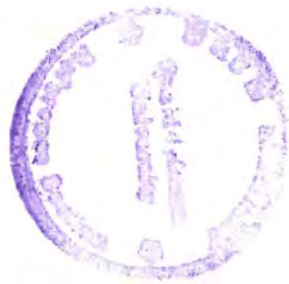
Family A: Cathepsin C (*CTSC*) gene

Marker	Map Unit (cM)	1-N	2-A	3-A	4-A
1.D11S2002	91.48				
2. D11S901	93.01				
3.D11S1306	94.14				
4.D11S1367	98.13				
5. D11S873	99.78				
6.D11S4182	100.6				

1-III-5 (Normal) 2-III-7 (Affected)

3-III-10 (Affected) 4-IV-6 (Affected)

Figure 3.11: The electropherogram of 8% non-denaturing polyacrylamide gel stained with ethidium bromide (Et.Br), illustrating allelic pattern amplified with respective microsatellite markers flanking *CTSC* gene on chromosome 11q14.2. Roman numerals and Arabic numbers ascertain the generation number and position of the individuals with in a generation in pedigree.



Family A: Desmoplakin (*DSP*) gene

Marker	Map Unit (cM)	1-N	2-A	3-A	4-A
1. D6S1617	12.99				
2. D6S1574	17.02				
3. D6S1668	18.14				
4. D6S1547	20.85				
5. D6S309	22.28				
6. D6S410	23.33				
7. D6S940	26.02				

1-III-5 (Normal) 2-III-7 (Affected)

3-III-10 (Affected) 4-IV-6 (Affected)

Figure 3.12: The electropherogram of 8% non-denaturing polyacrylamide gel stained with ethidium bromide (Et.Br), illustrating allelic pattern amplified with respective microsatellite markers flanking *DSP* gene on chromosome 6p24.3. Roman numerals and Arabic numbers ascertain the generation number and position of the individuals with in a generation in pedigree.

Family A: Plakophilin 1 (*PKP1*) gene

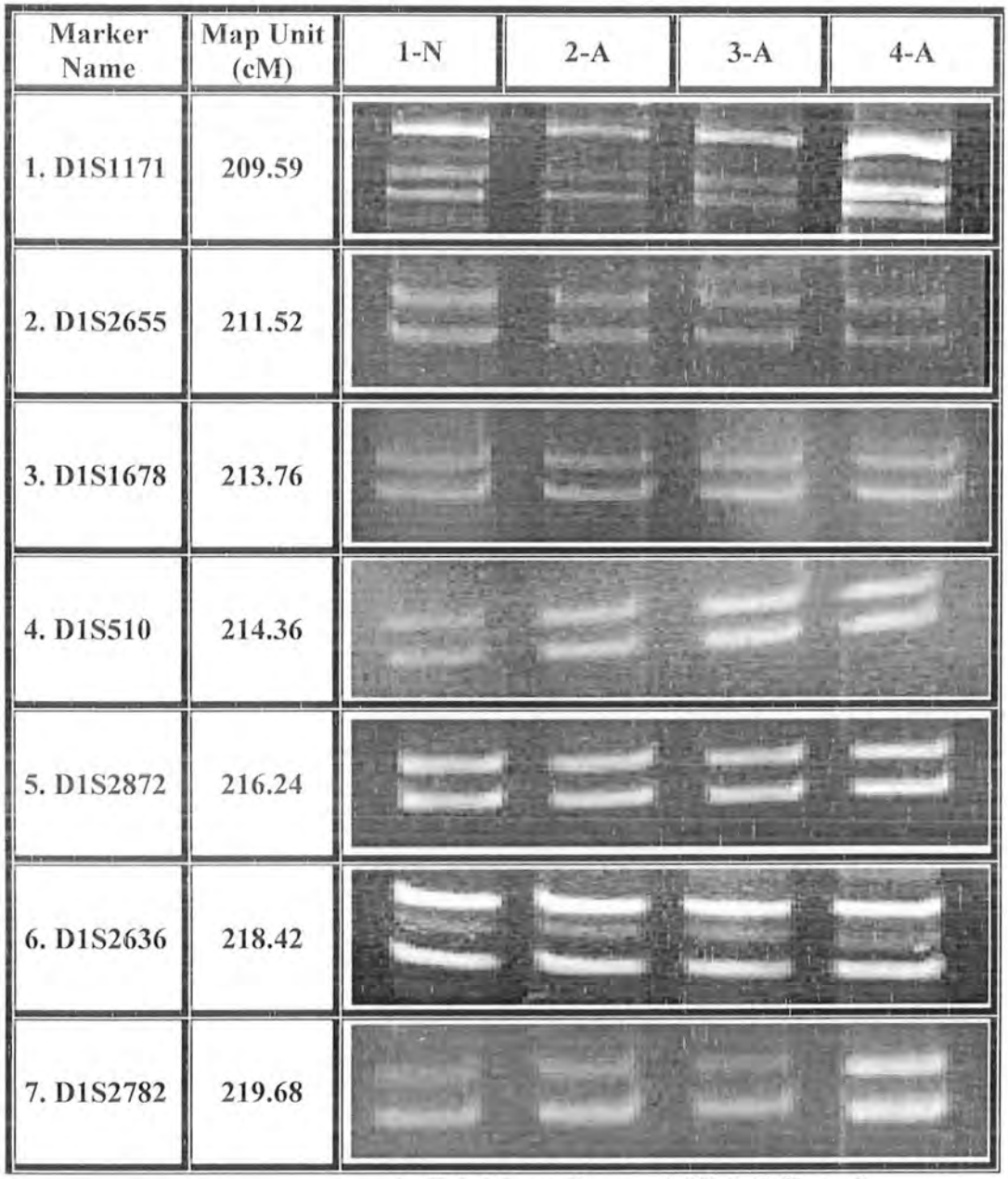


Figure 3.13: The electropherogram of 8% non-denaturing polyacrylamide gel stained with ethidium bromide (Et.Br), illustrating allelic pattern amplified with respective microsatellite markers flanking *PKP1* gene on chromosome 1q32.1. Roman numerals and Arabic numbers ascertain the generation number and position of the individuals with in a generation in pedigree.

Family A: Secreted LY6/PLAUR Domain Containing 1 (*SLURP1*) gene

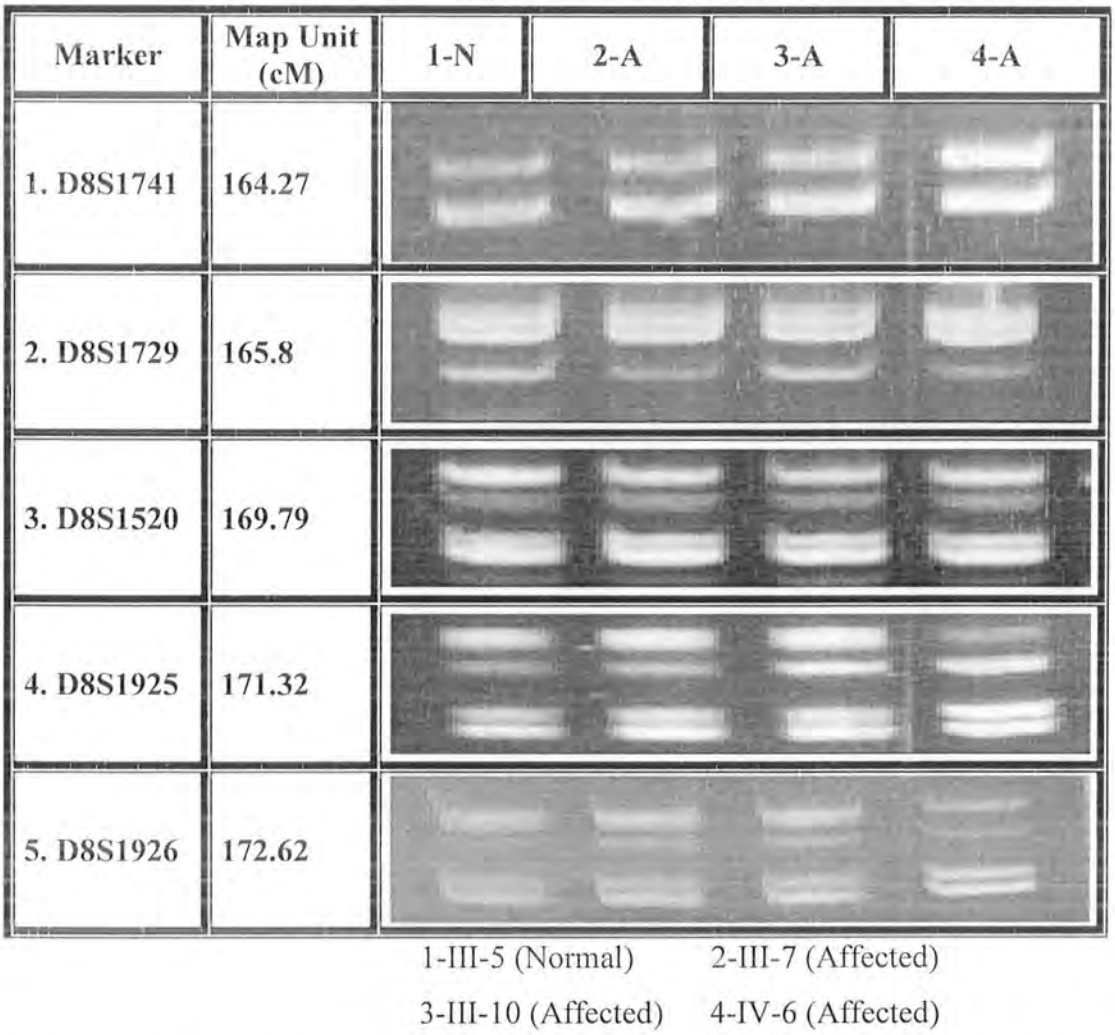


Figure 3.14: The electropherogram of 8% non-denaturing polyacrylamide gel stained with ethidium bromide (Et.Br), illustrating allelic pattern amplified with respective microsatellite markers flanking *SLURP1* gene on chromosome 8q24.3. Roman numerals and Arabic numbers ascertain the generation number and position of the individuals with in a generation in pedigree.

Family A: Tyrosine Aminotransferase (*TAT*) gene

Marker	Map Unit (cM)	1-N	2-A	3-A	4-A
1. D16S186	85.34				
2.D16S3085	86.57				
3.D16S3141	87.07				
4.D16S3139	88.47				
5.D16S2642	89.91				
6.D16S512	90.92				
7.D16S3051	92.75				

1-III-5 (Normal) 2-III-7 (Affected)

3-III-10 (Affected) 4-IV-6 (Affected)

Figure 3.15: The electropherogram of 8% non-denaturing polyacrylamide gel stained with ethidium bromide (Et.Br), illustrating allelic pattern amplified with respective microsatellite markers flanking *TAT* gene on chromosome 16q22.2. Roman numerals and Arabic numbers ascertain the generation number and position of the individuals with in a generation in pedigree.

Family A: Junction Plakoglobin (*JUP*) and Keratin (*KRT9*, *KRT10*, *KRT16*) genes

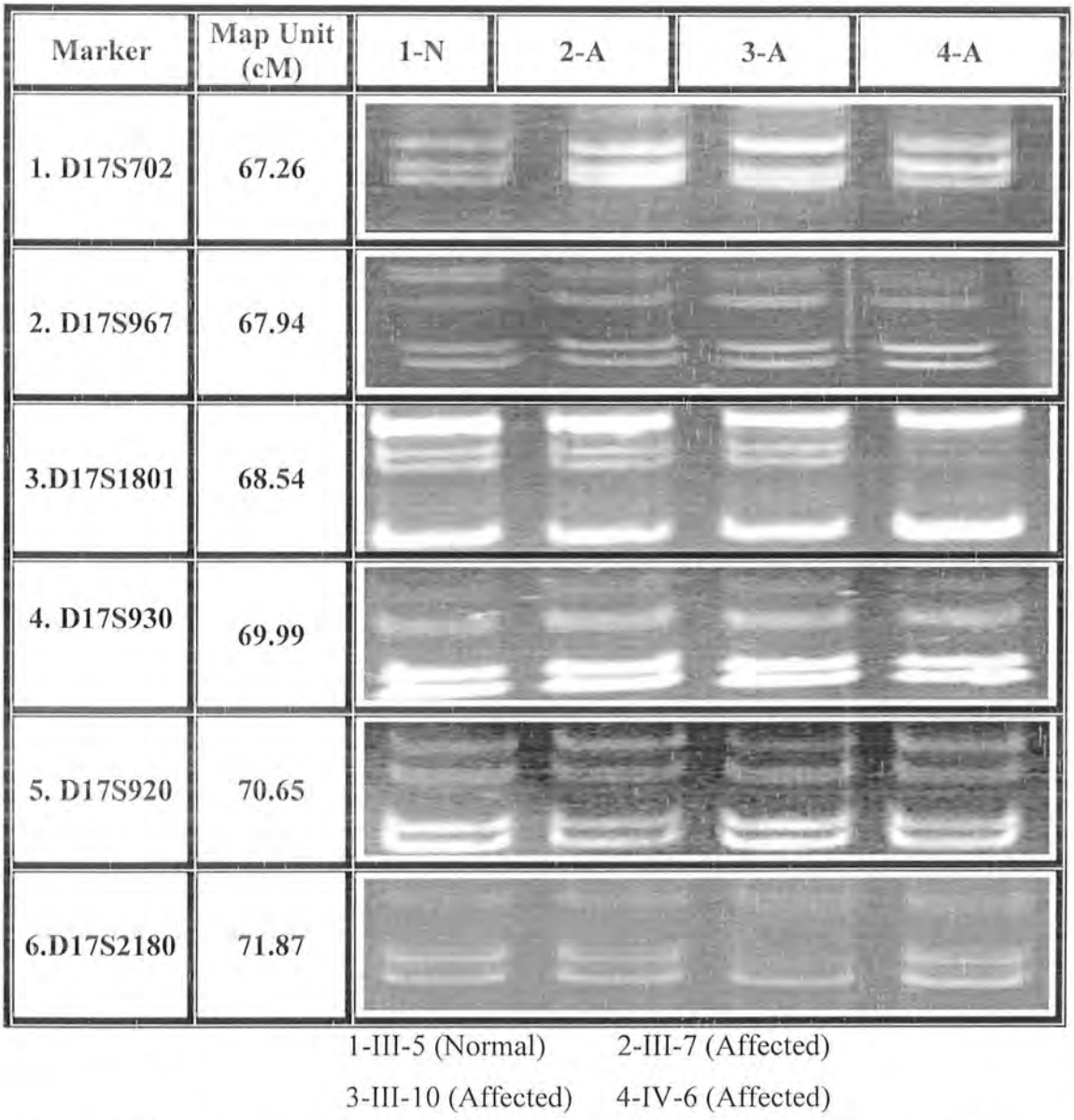


Figure 3.16: The electropherogram of 8% non-denaturing polyacrylamide gel stained with ethidium bromide (Et.Br), illustrating allelic pattern amplified with respective microsatellite markers flanking *JUP*, *KRT9*, *KRT10* and *KRT16* genes on chromosome 17q21.2. Roman numerals and Arabic numbers ascertain the generation number and position of the individuals with in a generation in pedigree.

Family A: Gap junction protein beta 2, and 6 (*GJB2, GJB6*) gene

Marker	Map Unit (cM)	1-N	2-A	3-A	4-A
1. D13S175	0.55				
2. D13S250	3.1				
3.D13S1236	5.41				
4.D13S1275	6.97				
5. D13S232	8.31				
6.D13S1243	10.06				

1-III-5 (Normal) 2-III-7 (Affected)

3-III-10 (Affected) 4-IV-6 (Affected)

Figure 3.17: The electropherogram of 8% non-denaturing polyacrylamide gel stained with ethidium bromide (Et.Br), illustrating allelic pattern amplified with respective microsatellite markers flanking *GJB2* gene on chromosome 13q12.11. Roman numerals and Arabic numbers ascertain the generation number and position of the individuals with in a generation in pedigree.

Family A: Gap junction protein beta 3 and beta 4(*GJB3* and *GJB4*) gene

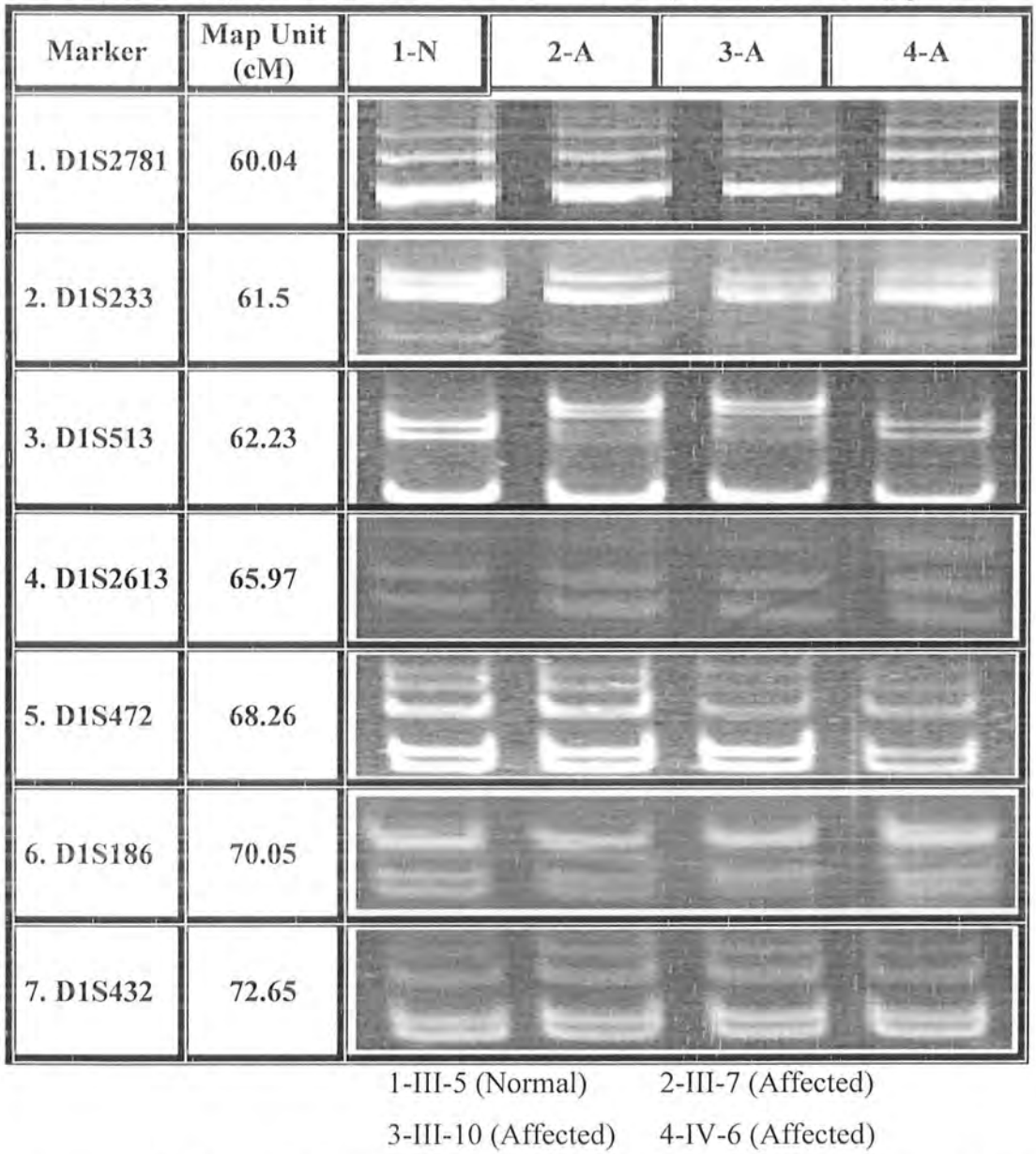


Figure 3.18: The electropherogram of 8% non-denaturing polyacrylamide gel stained with ethidium bromide (Et.Br), illustrating allelic pattern amplified with respective microsatellite markers flanking *GJB3* and *GJB4* gene on chromosome 1p34.3. Roman numerals and Arabic numbers ascertain the generation number and position of the individuals with in a generation in pedigree.

Family A: Keratin 6C Type II and KRT1 (*KRT6C, KRT1*) gene

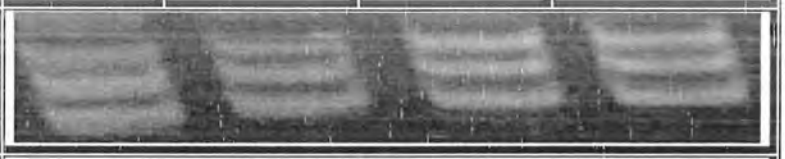
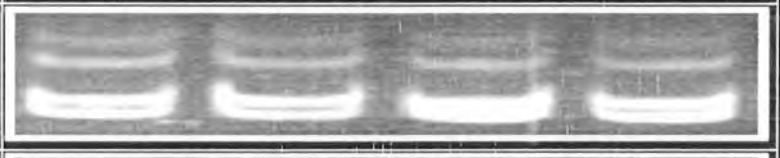
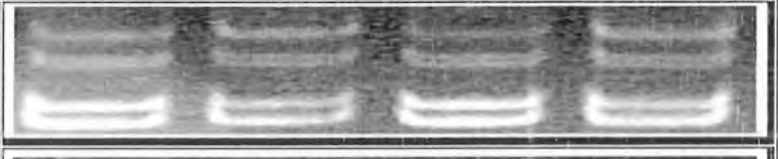
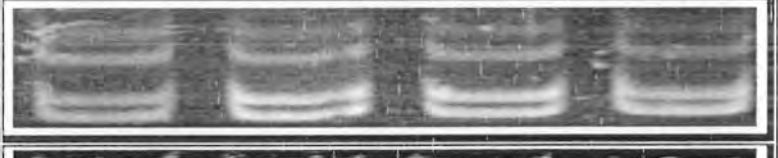
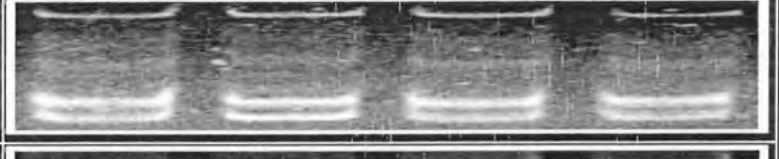
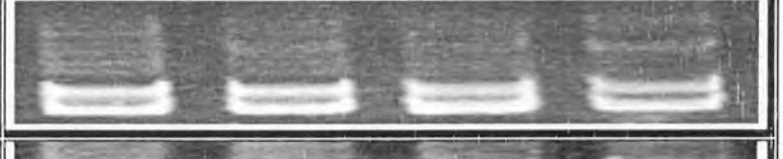
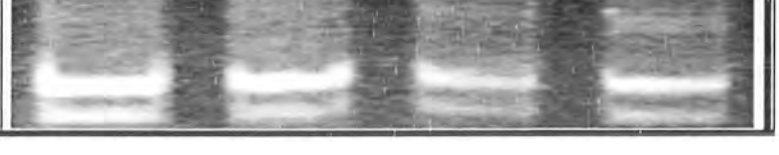
Marker	Map Unit (cM)	1-N	2-A	3-A	4-A
1.D12S1590	64.2				
2. D12S347	65.92				
3.D12S1677	66.17				
4. D12S325	69.56				
5.D12S1632	72.58				
6. D12S305	74.31				

1-III-5 (Normal) 2-III-7 (Affected)

3-III-10 (Affected) 4-IV-6 (Affected)

Figure 3.19: The electropherogram of 8% non-denaturing polyacrylamide gel stained with ethidium bromide (Et.Br), illustrating allelic pattern amplified with respective microsatellite markers flanking *KRT6C* gene on chromosome 12q13.13. Roman numerals and Arabic numbers ascertain the generation number and position of the individuals with in a generation in pedigree.

Locus on Chromosome # 3

Marker	Map Unit (cM)	1-N	2-A	3-A	4-A
1. D3S3699	188				
2. D3S1232	189				
3. D3S1618	192.67				
4. D3S3609	193.97				
5. D3S3592	195.04				
6. D3S1617	196.4				
7. D3S1262	198.2				
8. D3S2436	201.38				
9. D3S3651	202.71				
10.D3S3596	204.1				
11.D3S3530	207.05				

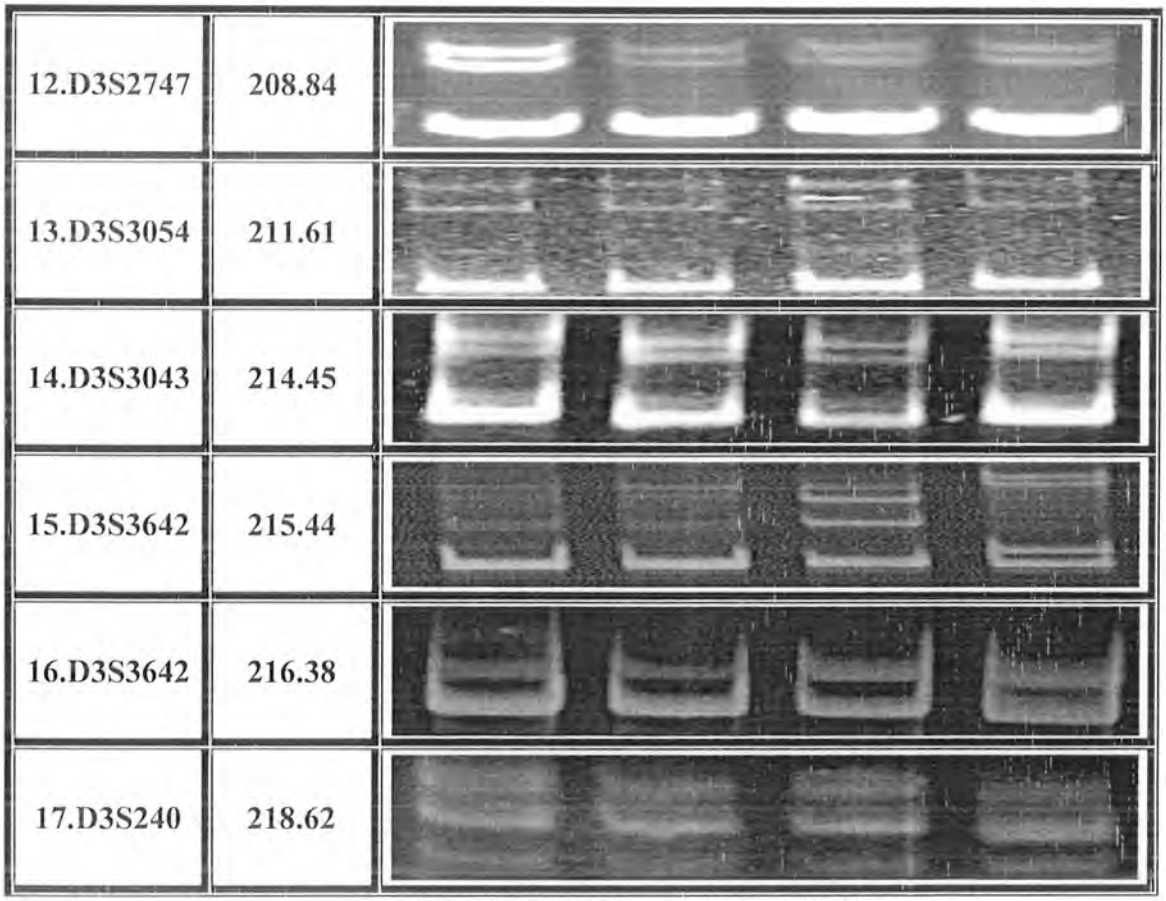


Figure 3.20: The electropherogram of 8% non-denaturing polyacrylamide gel stained with ethidium bromide (Et.Br), illustrating allelic pattern of 30 cM region flanked by D3S3699 (188 cM) and D3S240 (218 cM) microsatellite markers on chromosome# 3. Roman numerals and Arabic numbers ascertain the generation number and position of the individuals with in a generation in pedigree.

Locus on Chromosome # 15

Marker	Map Unit (cM)	1-N	2-A	3-A	4-A
1.D15S643	57.67				
2.D15S527	58.52				
3.D15S1036	62.13				
4.D15S1507	64.61				
5.D15S651	65.2				
6.D15S153	66.12				
7.D15S988	68.64				
8.D15S983	69.72				
9.D15S216	72.29				
10.D15S977	73.47				
11.D15S131	74.43				

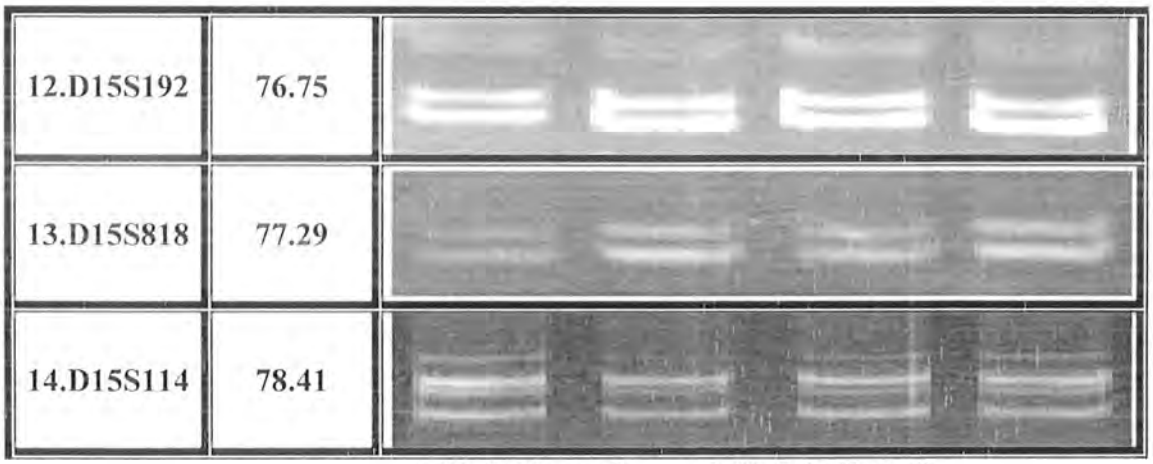


Figure 3.21: The electropherogram of 8% non-denaturing polyacrylamide gel stained with ethidium bromide (Et.Br), illustrating allelic pattern of 21 cM region flanked by D15S643 (57.67 cM) and D15S114 (78.41 cM) microsatellite markers on chromosome# 3. Roman numerals and Arabic numbers ascertain the generation number and position of the individuals with in a generation in pedigree.

Chapter 4
Discussion

DISCUSSION

The development of skin and their appendages (hair, nails, teeth, sweat glands) are under genetic control. Any defect in these developmental processes can lead to skin disorders. These skin disorders are directly or indirectly related to individual's genotype. Geneticists have identified more than 28 major hereditary skin disorders of cornification including ichthyosis and palmoplantar keratoderma primarily affecting stratum corneum of the skin epidermis (Richard, 2005).

Palmoplantar keratoderma are heterogeneous group of genodermatosis encompassing approximately 60 genetically distinct diseases characterized by abnormal thickening of the stratum corneum on the palms and soles (Wilson *et al.*, 2010). It is broadly classified into acquired and hereditary types. The later one is sub-classified into diffuse, striate, punctate and focal types (Sakiyama and Kubo, 2016). Palmoplantar keratoderma can be inherited in autosomal dominant, autosomal recessive, X-linked or mitochondrial inheritance affecting specifically keratin filaments, desmosomes, gap junctions and water channels (Has and Technau-Hafsi, 2016).

Ichthyosis is clinically and genetically heterogeneous group of mendellian disorders of cornification characterized dry, thick, rough scales and cracking of the skin surface. Hyperkeratinization and shedding of skin is caused by abnormal desquamation of stratum corneum. Nearly 40 genes held responsible for 20 different types of ichthyosis. It can be of inherited or acquired form. Based on inheritance pattern, hereditary ichthyosis can be X-linked, autosomal semi-dominant, dominant and recessive (Oji *et al.*, 2010). Ichthyosis can be syndromic and non-syndromic types depending upon involvement of extra cutaneous genetic anomalies. Syndromic type ichthyosis is found in association with defects in extra cutaneous tissues like hair abnormalities, prominent neurologic signs and fatal disease course while non-syndromic types exhibit cutaneous symptoms only. Syndromic types are further divided into X linked ichthyosis syndromes and autosomal ichthyosis syndromes while non-syndromic are divided into common ichthyosis, autosomal recessive congenital ichthyosis (ARCI) and keratinopathic ichthyosis (Oji *et al.*, 2010). Skin is the outermost layer; accounting for 12-15% of the body weight and provide protection against many external factor. Skin may either be glabrous or hairy skin which is present all over the body except palms and soles (Marks *et al.*, 2006; MacGrath *et al.*,

2004). It has three major layers the epidermis, dermis and hypodermis. Dermis support against mechanical injuries and provide nutrition to epidermis. Stratum corneum; part of the stratified epidermis contains enucleated corneocytes surrounded by lipid regions and lamellar sheets augmented with ceramides, free fatty acids and cholesterol (Elias *et al.*, 2005).Advances in the field of molecular biology have enabled the investigators to understand various complex mechanisms involved in pathogenesis of skin and its associated appendages.

Mechanism of pathogenesis of ichthyosis is thought to be related to at least one of the three major components of stratum corneum including intercellular lipid layer, cornified cell envelop (CCE) and keratin-filaggrin degradation products filling the cytoplasm of cornified cell (Williams *et al.*, 2005).

Owing to social and spiritual beliefs in Pakistan, consanguineous marriages are very common attributing to high prevalence of genetic abnormalities. Autozygosity mapping is an effective strategy for mapping genes causing recessive human disorders. This method follows the identification of disease locus homozygous by decent in descending generations. A single affected child of a first cousin marriage is shown to contain the same total information about linkage as a nuclear family with three affected children (Lander *et al.*, 1987).

In the present study, three consanguineous Pakistani families (A, B, C) with different types of hereditary skin disorders belonging to Khyber Pukhtunkha province of Pakistan were analyzed at clinical and molecular level. For this purpose their blood samples were collected and subjected to DNA extraction. To find out autozygous region these families DNA was amplified with microsatellite markers specific for pre-reported gene/loci. If linkage was established the candidate gene/loci in that region was subjected to sequencing to find out any pathogenic sequence variation. So, basically the research is carried out in two phases; homozygosity mapping with microsatellite markers and sequencing of putative gene (s) in the linkage interval.

Family A was identified with congenital diffuse type palmoplantar hyperkeratosis. The affected individuals have yellowish skin on their palmar and plantar surface having clear erythematous borders and normal skin over rest of the body. DNA of available affected and unaffected individuals were subjected to homozygosity mapping by typing microsatellite markers flanking the genes causing autosomal

recessive palmoplantar keratoderma. Minimum five or six microsatellite markers were selected for genotyping in the main region of known genes/loci. The genes/loci tested for linkage analysis include *CTSC* (11q14.2), *DSP* (6p24.3), *PKP1* (1q32.1), *SLURP1* (8p24.3), *TAT* (16q22.2), *JUP*, *KRT9*, *KRT10* and *KRT16* (17q21.2), *GJB2* (13q12.11), *GJB3* and *GJB4* (1p34.3), *KRT1*, *KRT6C* (12q13.13) and two locus on chromosome number 3 (188-218 cM) and 15 (57,68-78 cM). Results obtained for the mentioned genes/loci through highly polymorphic microsatellite markers revealed no linkage to the loci tested, suggesting the exclusion of the family from known genes, most probably a novel gene is involved in the pathogenesis of the family.

Affected individuals of family B presented phenotypes of ARCI type lamellar ichthyosis. The affected members showed large thick, dark brown fish like scales over thoracic region which extends to the forehead and possess scanty eyebrows. These scales were more severe at the upper and lower extremities of the body. Homozygosity mapping of all available individuals of the family was performed by at least 5 microsatellite markers for each candidate gene. These genes include *FLG* (1q21-22), *ABCA12* (2q34) *NIPAL4* (5q33), *KRT1* (12q13), *TGM1* (14q11), *ALOXE3* and *ALOX12B* (17p13), *CYP4F22* (19p12-q12), *PNPLA1* (6p21.31). Family B was linked to *ABCA12* gene. The gene sequencing was performed by designing primers for selected coding exons i.e. (32, 49). The selected exons were sequenced in one of the affected individual of the family. Sequence analysis failed to show any pathogenic variation suggesting that the mutation responsible for diseased phenotypes may be present in unsequenced coding exons or regulatory region of the gene. *ABCA12* contains 53 exons, and encodes for protein consisting of 2595 amino acids (Akiyama, 2010). Exon 32 and 49 were selected as most of the mutations in Pakistani families are reported in these exons. More than 70 mutations are reported in this gene (Peterson *et al.*, 2013) with most common mutation of c.7322delC (p. Val2442SerfsTer28) in exon 49 only reported Pakistani families (Kelsell *et al.*, 2005; Thomas *et al.*, 2006, 2008). *ABCA12* is a keratinocyte trans-membrane lipid transporter protein which involves the transport of lipids across membrane through lamellar granules. It is also involved in transport of ceramides to form extracellular lipid bilayer in stratum corneum of epidermis which plays an important role in skin barrier function (Shimizu, 2013). Mutation in *ABCA12* can lead to the defective lipid secretion in to lamellar granules which then become expelled from apical surface of

keratinocytes which can lead to lamellar ichthyosis or harlequin ichthyosis phenotypes (Levfre *et al.*, 2003; Akiyama *et al.*, 2005)

Family C presented the clinical features of common autosomal recessive ichthyosis vulgaris. The clinical features of affected individuals include ichthyotic moles on their skin. These moles were more prominent over facial skin while less over hands and other body parts. For autozygosity mapping, minimum 5-6 microsatellite markers were used for genotyping the candidate region of different genes in all available individuals. All the known genes for isolated autosomal recessive ichthyosis were genotyped. These include *FLG* (1q21-22), *ABCA12* (2q34) *NIPAL4* (5q33), *KRT1* (12q13), *TGM1* (14q11), *ALOXE3* and *ALOX12B* (17p13), *CYP4F22* (19p12-q12), *PNPLA1* (6p21.31). This family shows linkage to *FLG* gene. Its sequencing was performed by designing primers for all the coding exons. All exons were sequenced in one of the affected individual of the family. This family also failed to show any pathogenic sequence variant suggesting that the mutation responsible for diseased phenotypes may be present in the regulatory region of *FLG* gene.

FLG has three exons coding for profilaggrin precursor protein which is main component of keratohyalin granules in stratum granulosum of epidermis. It is important structural protein involved in aggregation of keratin intermediate filaments and provides compactness through epidermal differentiation (Mischke *et al.*, 1996). Profilaggrin proteolytic cleavage products have many functions like water retention, pH maintenance for proper skin barrier formation. A number of dominant, semi-dominant and recessive genetic alterations have been reported in *FLG* for ichthyosis vulgaris (Ervine *et al.*, 2011; Smith *et al.*, 2006) causing impaired skin barrier formation. It affect the keratin filament of the cytoskeleton and structure of SC. A decrease in pH, number of keratohyalin granules, corneodesmosome density and architecture of extracellular lipid matrix and tight intercellular junctions are observed with defective *FLG* gene.

This study was aimed to investigate the skin diseases pathogenesis at molecular level. Moreover, the study will help to provide further insight into the molecular characterization of hereditary ichthyosis and palmoplantar keratodermas. Over last decade, understanding of how various genes involved in causing hereditary skin disorders has increased enormously. Identification of genes causing these rare phenotypes is a difficult task. To establish a relationship between phenotypes and genotype is the aim of

genetic research. Once the genes causing these rare diseases are identified, gene therapy would be a useful approach towards the diseased treatment.

Chapter 5
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