Clinical and Genetic Characterization of Hereditary Epidermolysis Bullosa and Ichthyoses in Three Consanguineous Families

by

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In the Name of ALLAH, the Gracious, the Merciful

Lord of all Worlds

All Prays Belong to ALLAH

Master of the day of Judgement

Declaration

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

Hamadia Jan

I Dedicate This Thesis

To

My Loving

Parents and Family

Whose Love is Teaseless

Whose Affections are Limitless

Whose Compassions are Matchless

And Whose Prayers are Selfless

May They Live Long!

Ameen

CONTENTS

ACKNOWLEDGMENTS I

LIST OF FIGURES III

LIST OF TABLES VII

LIST OF ABBREVIATIONS VIII

ABSTRACT XII

Chapter 1 INTRODUCTION

Skin 1

Skin Types 1

Human Skin Morphology 1

Epidermis 1

Dermis 2

Hypodermis 3

Ectodermal Appendages 3

- **a. Nail** 3
- **b. Teeth** 3
- **c. Sweat Glands** 4
- **d. Hairs** 4

Genetic Skin Disorders 5

Ectodermal Dysplasia 5

Epidermolysis Bullosa 5

- **a. EB Simplex** 7
- **b. Junctional EB** 7
- **c. Dystrophic EB** 8
- **d. Kindler EB** 8

Ichthyosis 10

Classification of Ichthyoses 11

Inherited ichthyoses 11

Nonsyndromic Ichthyosis 11
Common Ichthyosis 11
a. Ichthyosis Vulgaris 11
b. Recessive X-linked ichthyosis 11
Autosomal Recessive Congenital Ichthyoses 12
a. Harlequin ichthyosis 12
b. Lamellar ichthyosis 14
c. Congenital ichthyosiform erythroderma 14
Keratinopathic ichthyoses 14
Syndromic Ichthyosis 15
a. Netherton Syndrome 15
b. Sjogren-Larsson Syndrome 15
c. Refsum syndrome 16
d. Trichothiodystrophy 16
e. X-Linked Dominant Ichthyosis 17
Acquired Ichthyosis 17
a. Ichthyosis with cancerous Diseases 17
b. Ichthyosis with Noncancerous Diseases 17
c. Medications Induced Ichthyosis 17

Chapter 2 MATERIALS AND METHODS

Ethical Approval 21
Recruitment Of Families 21
Extraction Of Genomic DNA 21
a. Phenol-Chloroform Method 21
b. Thermo Scientific DNA Extraction Kit 22
Agarose Gel Electrophoresis 23
DNA Quantification and Dilution 24
Genotyping and Linkage Analysis 24
Polymerase Chain Reaction 24
Polyacrylamide Gel Electrophoresis 24

Primer Designing 25
Pre sequencing PCR 25
Agarose gel electrophoresis (2%) 25
Purification of the amplified product 26
Sanger Sequencing 27

Chapter 3 RESULTS

Family A 38
Family B 38
Family C 38
Genotyping and Linkage Analysis 39
Sequencing *EXPH5* Gene 40
Sequencing *CERS3* Gene 40

Chapter 4 DISCUSSION 60

Chapter 5 REFERENCES 68

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LIST OF FIGURES

Figure No	Title	Page No
Figure 1.1	Epidermolysis Bullosa (EB) Types.	**6**
Figure 1.2	Ichthyosis classification.	**10**
Figure 3.1	Pedigree sketch of family A, segregating in the AR pattern of EB simplex. The circles symbolize females, and the squares males. A shaded circle or square represents an affected while an unshaded symbol represents normal individuals. Double lines specify consanguineous marriages. Roman and Arabic numbers define the generation position and number of the members in a family pedigree. Those members whose blood were collected are represented with asterisks (*) in the pedigree.	**42**
Figure 3.2	Clinical representation of affected individuals (IV-4, IV-5) in family A. **(A-C)** Extreme dryness and white scales on hands with nail dystrophy in IV-4. **(D-F)** Swelling and white patches on fingers joints, abnormal dome-shaped nails. Sole was more affected and had cuts on the heel side in IV-5.	**43**
Figure 3.3	Polyacrylamide electropherogram illustrating homozygosity among the affected members (IV-4 and IV-5) for microsatellite markers D11S898 and D11S2017 flanking the EXPH5 gene on chromosome 11q22.3. Roman and Arabic numbers define the generation position and number of the members in a family pedigree.	**44**
Figure 3.4	Pedigree illustrates of family A, segregating AR type of EB simplex with haplotypes of the closely linked locus-specific microsatellite markers under each genotyped member. Cytogenetic position presented in centimorgan (cM). Black symbols depict affected members whereas blank symbols represent normal members of the family. Genotypes with 1 1 represents homozygous while 1 2 heterozygous states of the marker.	**45**

Figure 3.5	Partial sequencing chromatogram of all six coding exons of *EXPH5* in an affected member (IV-4) of family A.	**46**
Figure 3.6	Pedigree of family B, segregating hereditary AR congenital ichthyosis (ARCI). The circles symbolize females and the squares males individuals of the family. A shaded circle or square represents an affected while an unshaded symbol represents normal individuals of family. Double lines specify consanguineous marriages. Roman and Arabic numbers define the generation position and number of the members within a generation in pedigree. Those members whose blood were collected are represented with asterisks (*) in the pedigree.	**47**
Figure 3.7	Clinical features of affected individuals (IV-4, IV-5) segregating autosomal recessive congenital ichthyosis (ARCI) in family B. **(A-B)** Stiff and hard skin at hands and hyperkeratosis over feet in an affected individual IV-4. **(C-F)** Affected individual IV-5 had skin dryness and black scales on the back of the neck, elbow, back, and legs.	**48**
Figure 3.8	Polyacrylamide electropherogram illustrating homozygosity among the affected members (IV-4 and IV-5) for microsatellite marker D15S87 flanking the *CERS3* gene on chromosome 15q26.3. Roman and Arabic numbers define the generation position and number of the members in a family pedigree.	**49**
Figure 3.9	Pedigree illustrates family B; segregating AR congenital ichthyosis haplotypes of the closely linked locus-specific microsatellite markers under each genotyped member. Cytogenetic position is represented in centimorgan (cM). Black symbols depict affected members whereas blank symbols represent normal members of the family.	**50**
Figure 3.10	Sequencing chromatogram illustrating sequencing of the coding exon of *CERS3* indicating a G to A transition at splice acceptor site (c.466-1G>A). **Panel A** (upper) shows the DNA sequence in the affected member (IV-4), **panel B** (middle) shows the DNA sequence in the heterozygous carrier (III-4) and **panel C**	**51**

	(bottom) shows the DNA partial sequence *CERS3* in the normal member (IV-6) of family B. The black arrow indicates the nucleotide change in the sequence. **Panel C, D** manifesting *CERS3* gene and protein structure.	
Figure 3.11	Pedigree of family C, segregating hereditary AR congenital ichthyosis. The circles symbolize females and the squares males individuals of the family. A shaded circle or square represents an affected while an unshaded symbol represents normal individuals of a family. Double lines specify consanguineous union. Cross lines over the symbols represent deceased individuals. Roman and Arabic numbers define the generation position and number of the members in a family pedigree. Those members whose blood were collected are represented with asterisks (*) in the pedigree.	**53**
Figure 3.12	Polyacrylamide electropherogram illustrating the pattern of alleles amplified with specific microsatellite markers flanking *ABCA12* gene in family C. Roman and Arabic numbers define the generation position and number of individuals in a family pedigree.	**54**
Figure 3.13	Polyacrylamide electropherogram illustrating the pattern of alleles amplified with specific microsatellite markers flanking *CERS3* gene in family C. Roman and Arabic numbers define the generation position and number of the individuals in a family pedigree.	**54**
Figure 3.14	Polyacrylamide electropherogram illustrating the pattern of alleles amplified with specific microsatellite markers flanking *LIPN* gene in family C. Roman and Arabic numbers define the generation position and number of the individuals in a family pedigree.	**55**
Figure 3.15	Polyacrylamide electropherogram illustrating the pattern of alleles amplified with specific microsatellite markers flanking *ST14* gene in family C. Roman and Arabic numbers define the generation position and number of the individuals in a family pedigree.	**55**

Figure 3.16	Polyacrylamide electropherogram illustrating the pattern of alleles amplified with specific microsatellite markers flanking the *PNPLA* gene in family C. Roman and Arabic numbers define the generation position and number of the individuals in a family pedigree.	**56**
Figure 3.17	Polyacrylamide electropherogram illustrating the pattern of alleles amplified with specific microsatellite markers flanking the *EXPH5* gene in family C. Roma and Arabic numbers define the generation position and number of the individuals in a family pedigree.	**56**
Figure 3.18	Polyacrylamide electropherogram illustrating the pattern of alleles amplified with specific microsatellite markers flanking the *COL7A1* gene in family C. Roman and Arabic numbers define the generation position and number of the individuals in a family pedigree.	**57**
Figure 3.19	Polyacrylamide electropherogram illustrating the pattern of alleles amplified with specific microsatellite markers flanking the *KRT5* gene in family C. Roman and Arabic numbers define the generation position and number of the individuals in a family pedigree.	**57**
Figure 3.20	Polyacrylamide electropherogram illustrating the pattern of alleles amplified with specific microsatellite markers flanking the *KRT14* gene in family C. Roman and Arabic numbers define the generation position and number of the individuals in a family pedigree.	**58**
Figure 3.21	Polyacrylamide electropherogram illustrating the pattern of alleles amplified with specific microsatellite markers flanking the *FERMT1* gene in family C. Roman and Arabic numbers define the generation position and number of the individuals in a family pedigree.	**58**

LIST OF TABLES

Table No	Title	Page No
Table 1.1	List of mutated genes causing EB	**9**
Table 1.2	List of mutated genes reported in ARCI	**13**
Table 2.1	Solutions with their composition and concentration used in the phenol-chloroform method	**27**
Table 2.2	Solutions and their composition used for 1% agarose gel electrophoresis	**28**
Table 2.3	PCR Conditions	**28**
Table 2.4	Microsatellite markers, flanking the selected genes, used in Genotyping families with EB	**29**
Table 2.5	Microsatellite markers, flanking the selected genes, used in Genotyping families with Ichthyosis	**30**
Table 2.6	List of primers used for *EXPH5* gene sequencing	**31**
Table 2.7	List of primers used for *CERS3* gene sequencing	**33**
Table 3.1	Analysis of the variant in family B	**52**

LIST OF ABBREVIATIONS

ABCA12	ATP binding cassette subfamily A member-12
ACMG	American College of Medical Genetics
AD	Autosomal Dominant
ALOX12B	Lipoxygenases 12B
ALOXE3	Lipoxygenases E3
APC	Antigen-presenting cells
APS	Ammonium persulphate
AR	Autosomal Recessive
BMP4	bone morphogenetic protein-4
CASP14	Cytochrome P450, Fami1y 4 Subfami1y F, Po1ypeptide 22
CD151	Tetraspanin
CDPX2	Chondrodysplasia Punctata 2 X-linked Dominant
CERS3	Ceramide Synthase 3
CHHS	Conradi–Hunermann–Happel Syndrome
CIE	Congenital Ichthyosiform Erythroderma
cM	centimorgan
COL17A1	Collagen XVII
CYP4F22	Cytochrome P450 Family 4 Subfamily F Member 2
DDEB	autosomal dominant Epidermolysis Bullosa
DEB	Dystrophic Epidermolysis Bullosa
DSP	Desmoplakin
DST	Dysonin
EB	Epidermolysis Bullosa
EBF	Early B-Cell factor
EBS	Epidermolysis Bullosa simplex
EDA	Ectodysplasin-A
EH	Epidermolytic Hyperkeratosis
EI	Epidermolytic Ichthyosis
Et. Br	Ethidium Bromide
EXPH5	Exophilin-5
FERMT1	Fermitin family homolog 1
FGF4	fibroblast growth factor-4
HGMD	Human Gene Mutation Database

HI	Harlequin Ichthyosis
HPMM	Highly Polymorphic Microsatellite Markers
IC	Inherited Ichthyoses
IRB	Institutional Review Board
ITGA3	Integrin α3A
ITGB4	Integrin β4
IV	Ichthyosis Vulgaris
JEB	Junctional Epidermolysis Bullosa
JUP	Plakoglobin
KEB	Kindler Epidermolysis Bullosa
KIND1	kindlin-1
KLHL24	Kelch-like protein 24
KPI	Keratinopathic Ichthyoses
KRT14	Keratin 14
KRT5	Keratins 5
LAMA3	Laminin-5 α3
LAMB3	Laminin-5 β3
LAMC2	Laminin-5 γ2
LC	Stratum Lucidum
LI	Lamellar Ichthyosis
LIPN	Lipase Family Member N
$MgCl_2$	magnesium chloride
NIPAL4	Non-Imprinted in Prader-Willi/Angelman Syndrome Region Protein 4
NS	Netherton syndrome
PHYH	Phytanoyl-CoA Hydroxylase
PKP1	Plakophilin-1
PLEC1	Plectin
PNPLA1	Patatin Like Phospholipase Domain Containing 1
RDEB	Autosomal Recessive Epidermolysis Bullosa
RS	Refsum Syndrome
RXLI	Recessive X-linked Ichthyosis
SB	Stratum Basale
SC	Stratum Corneum
SDR9C7	Short-Chain Dehydrogenase/Reductase Family 9C Member 7
SG	Stratum Granulosum

Shh	Sonic hedgehog
Slac2-b	Synaptotagmin-Like Protein Lacking C2 Domains B
SLS	Sjogren-Larsson Syndrome
SS	Stratum Spinosum
ST14	Suppression of tumorigenicity 14
STS	Steroid Sulfatase
SULT2B1	Sulfotransferase Family 2B Member 1
TBE	Tris-Borate-EDTA
TE	Tris-EDTA
TEMED	N, N, N'-Tetra methyl diamine
TFs	Transcription Factors
TGM1	Transglutaminase-1
TGM5	Transglutaminase-5
TMD	Transmembrane Domains
TTD	Trichothiodystrophy

ABSTRACT

The skin makes a barrier between the organism and its surrounding environment and plays a protective role along with the distinctive functions of lubrication and thermoregulation. The skin appendages, including nails, teeth, sweat glands, and hairs share common functions like immune surveillance, epidermal barrier, and role in pigmentation and defense mechanism in the skin. Genetic defects in the genes involved in the development of skin or associated appendages lead to inherited skin disorders collectively known as ectodermal dysplasia.

Epidermolysis Bullosa and Ichthyosis are two inherited skin disorders. Epidermolysis Bullosa is a hereditary skin disorder described by skin blister formations upon minor injury or mechanical traumas. On the other hand, ichthyosis is characterized by dry skin, scaling, and hyperkeratosis of the skin surface. It has syndromic as well as non-syndromic forms. The molecular basis of these manifestations being understood by the identification of variants in genes involved in skin barrier formation.

The current study in the thesis investigated the three consanguineous Pakistani families (A-C), segregating various types of autosomal recessive congenital skin disorders at clinical and genetic level. Family A presented Epidermolysis Bullosa, family B showed lamellar ichthyosis, and family C had the autosomal recessive type of ichthyosis phenotypes. Genotyping using microsatellite markers closely located to the previously ectodermal dysplasia-associated loci and haplotype analysis were used to establish linkage in these families. Sanger sequencing followed by in silico analysis were used to identify causative variants of the disease phenotypes.

Family A was found linked to the *EXPH5* gene located on chromosome 11q22.3 whereas, family B revealed linkage to the *CERS3* gene located on chromosome 15q26.3. Family C affected individuals were found heterozygous for various combinations of parental alleles thus, excluding family C from linkage to ten previously reported causative genes.

Sanger sequencing of coding regions and exon-intron borders of *EXPH5* and *CERS3* was performed by dideoxy chain termination method in families A, and B. In family A, all six

coding exons of the *EXPH5* gene were sequenced which revealed no pathogenic variant, suggesting that the variation might be exist in regulatory sequences of the *EXPH5* gene. In family B, Sanger sequencing data analysis depicted a novel splice site variant (c.466-1G>A) in exon eight of *CERS3* gene. The variant was found homozygous in the affected members, while heterozygous in phenotypically normal parents. Different prediction tools and ACMG classification predicted the variant likely pathogenic.

In conclusion, the present study identified a novel variant only in one of the three families. The finding will be helpful in prenatal screening, carrier testing, genetic counseling of the affected and other families in the Pakistani population. Moreover, the study will bring awareness among Pakistani population to prevent genetic disorders by minimizing cousin marriages where a positive family history for any inherited disorders is present. In searching for disease-causing variants in family A and C, it is recommended to use whole genome/exome sequencing to find the causative genes.

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CHAPTER 1

INTRODUCTION

INTRODUCTION

Skin

The skin is the human body's largest organ, which is the outermost flexible and soft tissue covering the whole body. The skin of an adult human weighs about five kilograms and has a surface area of about $2m^2$. Normal human skin thickness fluctuates depending on age, gender, and location, with a thickness range of 0.5 mm to 4.0 mm. The skin serves as the first line of defense against external threats and the environment. Skin acts as an epidermal barrier to aid the body in immunological surveillance, thermoregulation, lubrication, UV protection, insulation, sweating, sensation, pigmentation, vitamin D production, and protection of skin numerous stem cells niches (Ibrahim *et al*., 2022).

Skin Types

External features such as texture, oiliness, pH, hydration, pores, wrinkles, pigmentation, skin microrelief, stratum corneum water content, and loss of transepidermal water define skin types (Mercurio *et al*., 2013). Histologically, skin is divided into two categories: 1) Hairy skin and 2) Glabrous skin (Hairless). Hairy skin masks the entire body except for the palms and soles. It has a thin epidermis and contains hair follicles, sebaceous, and sweets glands. The palms and soles have glabrous skin, which is thick and hairless. Glabrous skin has a thick epidermis and sweat glands but no sebaceous glands and hair follicles (Ibrahim *et al*., 2022).

The skin and skin appendages originate from two embryonic layers: ectoderm and mesoderm (Hardy, 1992; Fuchs, 2007). Epidermis, dermis, hypodermis, and skin appendages are all components of the skin.

Human Skin Morphology

Histologically, skin has three layers: 1) Epidermis, 2) Dermis, and 3) Hypodermis.

Epidermis

The epidermis is the non-vascularized and stratified outermost epithelial layer of the skin originating from the ectoderm. The epidermis can self-renew, differentiate, and multiply

(Segre, 2006). The epidermis is thin, ranging from 1-2 mm in thickness. The epidermis receives nourishment via diffusion (Ibrahim *et al.,* 2022). Keratinocytes (95%) are the major cells in the epidermis. A cytoskeleton made up of keratin intermediate filaments gives keratinocytes their 'bricklike' shape. keratinocytes differentiation gives rise to five major strata: 1) Stratum corneum (SC), 2) Stratum lucidum (LC), 3) Stratum granulosum (SG), 4) Stratum spinosum (SS), and 5)Stratum basale (SB) respectively from top to bottom (Lai-Cheong and McGrath, 2013). The SC or corneocytes is the epidermis' topmost dead layer. The SL is a transparent layer, maybe a few layers thick with dead keratinized cells restricted to the palms and soles hairless skin. The SG contains intracellular granules of keratohyalin. The granular cell layer and spinous layer cytoplasm contain smaller lamellated granules. They release lipid constituents into the intercellular space, where they aid intercellular cohesiveness and barrier function within the SC. The SB is usually single-cell-layer thick continuous layer, however, maybe 2-3 cells layer thick in the hyperproliferative epidermis and glabrous skin. The basal cells are cuboidal, small in shape having dense cytoplasm that contains many ribosomes and large nuclei (McGrath and Uitto, 2016). The epidermis also contains Langerhans cells, Melanocytes, and Merkel cells. Langerhans cells have a crucial function in the skin acquired immunity (Holikova *et al*., 2001). Melanocytes are dendritic cells responsible for skin color (Agar and Young, 2005). Merkel cells have a key function in mechanosensory reception (Tobin, 2006).

Dermis

The dermis is present among the epidermis and the hypodermis and has a mesenchymal origin. The dermis is thicker than the epidermis and is made up of two layers of several cell types. The dermis is made up of a supporting matrix where polysaccharides and proteins are intertwined to form a network that gives the skin strength and a remarkable ability to retain water. Depending on the skin site, the dermis thickness ranges from 0.5-5 mm. The dermis contains a plentiful supply of blood. Collagen and elastic tissue are the two main forms of protein fiber. Collagen accounts for 80–85% of the dermis dry weight and gives tensile strength to the skin. Elastic fibers make up 2–4% of the dermis responsible for the skin's elasticity and durability. The non-collagenous glycoproteins like integrins, fibronectin, and fibulins in the dermis facilitate cell motility and cell adhesion.

Glycosaminoglycan/proteoglycan contribute 0.1–0.3% dermis dry weight and maintain hydration (McGrath and Uitto, 2016).

Hypodermis

The hypodermis is located beneath the dermis, also called subcutaneous or subcutis tissue. Hypodermis is made up of fatty connective tissue that is densely packed with blood vessels, adipocytes, sweat glands, nerves, lymphatic vessels, and hair follicle bases. It is significantly thicker than the dermis (McGrath and Uitto, 2016). It connects the dermis to the underlying skeletal component; muscles and bones (Freinkel and Woodley, 2001). It has an important role in storing energy, insulating, and protecting the body from harm.

Ectodermal Appendages

A class of organs (nails, teeth, glands, and hairs) that are developed from the outermost layer of the embryo (ectoderm) known as ectodermal appendages or skin appendages.

a. Nail

The nail is a translucent platelike keratinous structure. Nails protect the fingers and toes tips and are useful for pinching and prizing things. Anatomically, a nail is made up of four epithelial structures enlisted as the 1) Proximal nail fold, 2) Matrix, 3) Hyponychium,4) Nail bed, and nail plate (McGrath and Uitto, 2016). Signaling proteins including fibroblast growth factor-4 (FGF4), bone morphogenetic protein-4 (BMP4), Wnt7A, Sonic hedgehog (Shh) (Chuong *et al*., 1996), and transcription factors (TFs) *MSX1, LMXI,* and *Engrailed-1* involves in nail development (Chen *et al*., 1998; Jumlongras *et al*., 2001). Nail dysplasia might develop from variations in the genes involved in these pathways (Baran *et al*., 2012).

b. Teeth

Human teeth are the hardest calcium phosphate biomineral part of the craniofacial skeleton (Busch *et al*., 2001). The basic function of teeth is mastication. Anatomically a tooth is composed of three parts: 1) Crown, 2) Root, and 3) Pulp cavity. The mineral components are enamel, dentin, and cementum (Hu and Simmer, 2007). There are four major types of mammalian teeth: incisors, canines, premolars, and molars. The teeth development involves signaling pathways including BMP, Wnt, Shh, and FGF (Tucker and Sharpe,

2004; Hu and Simmer, 2007; Mikkola, 2007). Dental anomalies are caused by several hereditary, epigenetic, and environmental factors. Inherited dental disorders have been linked to several genes which play a role in tooth morphogenesis and development or contribute to tooth structure (Cabay, 2014).

c. Sweat Glands

Sweat glands are tubular coiled glands that can be found almost anywhere on the skin, except the nail bed, lips, nipples, and penis glans. They regulate body temperature and maintain homeostasis. Sweat glands are divided into two categories: 1) Eccrine and 2) Apocrine. Eccrine glands can be found on the entire body, with a larger density in the palms and soles compared to the trunk and extremities. Apocrine glands are present in the dermal or hypodermal layers of the skin, and their secretory ducts generally open into hair follicles. (Serri *et al*., 1963). Ectodysplasin-A (EDA) and Shh signaling are involved in sweat gland formation and subsequent ductal growth. *Foxi1* and *Foxa1* TFs from the *Fox* family regulate sweat gland secretions (Kunisada *et al*., 2009). Variations in these pathways' genes cause Hypohidrotic/anhidrotic ED with hypohidrosis/anhidrosis, hypotrichosis, and hypodontia (Keller *et al*., 2011).

d. Hairs

Hair is a flexible, keratinized dead epithelial cells thin tube on the outside, but it is a portion of live hair follicles on the interior of the skin. Humans have approximately five million hairs, found over most of the human skin surface (Lai-Cheong and McGrath, 2013) that function as the protective barrier in the form of the outer covering, reduce heat loss, and are a sign of human beauty (McKittrick *et al*., 2012). Hair is divided into three categories: 1) Lanugo hairs which are lost shortly after birth, 2) Vellus hairs are fine hairs that are usually found on the body, and 3) Terminal hairs are longer and thicker (Lai-Cheong and McGrath, 2013). Signaling pathways involved in hair follicle formation are Wnt/β-catenin, Notch, BMP, and Shh (Rishikaysh *et al*., 2014). Variations in genes involved in hair follicle growth, cycling, and morphogenesis have been linked to most hereditary hair loss disorders.

Genetic Skin Disorders

The word "genodermatoses" refers to hereditary skin diseases caused by defects in skin structure and function. Genodermatoses comprise a diverse category of rare diseases that make up approximately one-third of all hereditary skin diseases. Genodermatoses have a broad range of phenotypic manifestations including ectodermal dysplasia, Ichthyosis, Epidermolysis Bullosa, alopecia, abnormality of keratinization, and pigmentation. These abnormalities are infrequent, may be restricted to the skin and skin appendages, or may be part of a complex pathology with serious consequences. Several genodermatoses cause multisystem dysfunction, increasing mortality and morbidity (Hafsi *et al*., 2022).

Ectodermal Dysplasia

Ectodermal dysplasia (ED) is a large heterogeneous group of hereditary diseases described by genetic imperfections of ectodermal structures and its associated appendages i.e., nails, teeth, sweat glands, and hair (Itin, 2014). Frequently, the clinical manifestations could be seen in more than one ectodermal tissue (skin, nails, sweat glands, and hairs). There are two main groups of ED. In group one, due to the failure of signaling between ectoderm and mesoderm, hypoplasia of ectodermal tissues is seen. Group 2, besides the other epithelial structures, palmoplantar keratoderma is the most significant feature. To date almost 200 different types of ED have been identified (Garcia-Martin *et al*., 2013).

Epidermolysis Bullosa

Epidermolysis Bullosa (EB) is a heterogeneous group of skin fragility disorders described by variations in the epidermal and dermo-epidermal junction complex structural components, resulting in skin blister formation upon minor injury or mechanical traumas (Bruckner-Tuderman and Has, 2014; Fine *et al*., 2014; Uitto *et al*., 2017). In some subtypes, it also affects mucous membranes and other organs (Fine *et al*., 2008). Besides humans, it is also noticed in other mammals such as calves and dogs (Foster *et al.,* 2010). EB is organized into four subtypes based on blister formation such as 1) EB simplex, 2) Junctional EB, 3) Dystrophic EB, and 4) Kindler EB (Fine *et al*., 2014).

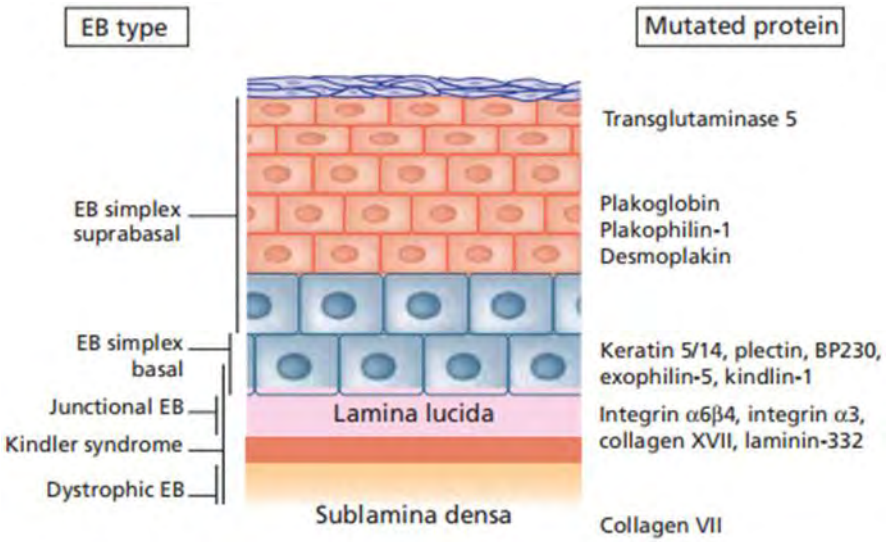


Figure 1.1 Epidermolysis Bullosa (EB) Types. The basement membrane and epidermis demonstrates the several levels where blisters arise in EB subtypes, as well as the location of the targeted proteins (McGrath, 2016).

a. EB Simplex

EB Simplex (EBS) is described by skin blister formation because of cleavage within the epidermis basal or suprabasal layer. EBS is transmitted both autosomal dominant (AD) and autosomal recessive (AR) pattern (Has *et al*., 2020). Based on the type of blister formation, EBS is classified into subtypes: basal and suprabasal EBS. Suprabasal EBS is defined by suprabasal keratinocyte acantholysis, which may be like palmoplantar keratosis or ichthyosis. Basal EBS is associated with a variety of disorders with varying severity (Laimer *et al*., 2015). Clinically, EBS is further subdivided into fourteen distinct clinical disorders namely acral peeling skin syndrome (MIM 609796), generalized intermediate EBS (MIM 131900), generalized severe EBS (MIM 619555), autosomal recessive EBS BP230 (MIM 615425), migratory circinate EBS (MIM 609352), muscular dystrophy with EBS (MIM 226670), mottled pigmentation with EBS (MIM 131960), pyloric atresia with EBS (MIM 612138), autosomal recessive EBS exophilin-5 (MIM 615028), EBS plakophilin-1 deficiency (MIM 604536), EBS desmoplakin deficiency (MIM 609638), EBS autosomal recessive keratin 14, EBS plakoglobin deficiency, EBS superficialis (Patrizi *et al*., 2022). The genetic cause of EBS has been associated with variations in eleven genes summarized in **(Table 1.1)**.

b. Junctional EB

Junctional EB (JEB) is an AR syndrome described by blister development within the dermo-epidermal basement membrane of lamina lucida. Exuberant granulation tissue development is a common characteristic feature in JEB. JEB clinical symptoms embrace skin and mucosal fragility, nail abnormalities, palmoplantar keratoderma and amelogenesis imperfecta (He *et al*., 2016). JEB is divided into JEB generalized and JEB localized. JEB generalized is further subdivided into generalized severe, generalized intermediate, generalized late-onset, generalized with pyloric atresia, and generalized with respiratory and renal dysfunction. Localized JEB is subdivided into localized inversa and localized Laryngo-onycho-cutaneous disorder (McGrath, 2016). The genetic cause of JEB has been linked with variations in the laminin (*LAMC2*, *LAMA3,* and *LAMB3,*), integrin (*ITGA3, ITGB4,* and *ITGA6*), and collagen (*COL17A1*) (Yenamandra *et al*., 2017) summarized in **(Table 1.1)**.

c. Dystrophic EB

Dystrophic EB (DEB) is a heterogeneous form of EB that is described by skin cleavage in the superficial part of the dermis underneath the lamina densa. DEB is inherited in both autosomal dominant EB (DDEB) and recessive EB (RDEB) patterns. Although RDEB is more severe than DDEB, the two forms share a lot of phenotypic similarities (Has *et al.,* 2020). DEB seems to be the most devastating group of EB due to severe abnormalities, higher vulnerability to squamous cell cancer, and severely low quality of life. The clinical feature involves the skin and mucosal blistering, dental anomalies, mitten deformities, nail dystrophy and loss, esophageal stenosis, and microstomia. DDEB is subdivided into DDEB acral, DDEB generalized, DDEB pruriginosa (MIM 131750), DDEB pretibial (MIM 131750), DDEB nails, and newborn bullous dermolysis. RDEB is subdivided into newborn bullous dermolysis, RDEB centripetalis, generalized intermediate RDEB, generalized severe RDEB, inversa RDEB (MIM 226600), localized RDEB, pretibial RDEB (MIM 226600), and pruriginosa RDEB (MIM 226600; Fine *et al.,* 2014). DEB is triggered by the mutated *COL7A1* (MIM 120120) gene, which codes for type VII collagen, a key factor of anchoring fibrils expressed in the basement membrane. Type VII collagen is a vital element of the anchoring fibril that provides stable dermal-epidermal adhesion beneath the upper dermis basement membrane (Shinkuma, 2015). To date, according to Human Gene Mutation Database (HGMD) professional 2021.1, 1031 sequence variants are reported for DEB in the *COL7A1* gene.

d. Kindler Epidermolysis Bullosa

Kindler Epidermolysis Bullosa (KEB; 173650) is a rare form of EB, with just about 250 cases reported globally since 1954 (kindler, 1954). KEB is an AR syndrome defined by variable degrees of skin cleavage beneath the basement membrane. KEB is commonly diagnosed in childhood, with photosensitivity and acral blisters at first, then atrophy and poikiloderma afterward (Laimer *et al*., 2015). KEB affects the skin and mucous membranes and has several extracutaneous signs such as gastrointestinal stenoses, urogenital, gingivitis, and periodontitis (Fine *et al*., 2014). The disruption of epithelial adhesion in KEB is caused by *KIND1* (syn. *FERMT1*) sequence variations that codes protein kindlin-1 (syn. Fermitin family homolog 1), which results in defective basal keratinocyte anchoring

to the extracellular matrix and cell signaling (Laimer *et al*., 2015). A total of 78 sequence variants are reported in the *KIND1* gene for KEB according to HGMD professional 2021.1.

Table 1.1. List of mutated genes causing EB (Has *et al*., 2020)

Targeted protein	Mutated gene	Inheritance	Cytogenetic Position	Gene MIM	References
EB Simplex					
Kelch-like protein 24	*KLHL24*	AD	3q27.1	611295	(He *et al*., 2016)
Plectin	*PLEC1*	AR, AD	8q24.3	601282	(Tu *et al.,* 2020)
Tetraspanin	*CD151*	AR	11p15.5	602243	(Vahidnezhad *et al*., 2018)
Exophilin-5	*EXPH5*	AR	11q22.3	612878	(Diociaiuti *et al*., 2020)
Dysonin	*DST*	AR	6p12.1	113810	(Ganani *et al*., 2021)
Keratins 5	*KRT5*	AR, AD	12q13.13	148040	(Lalor *et al*., 2019)
Keratin 14	*KRT14*	AR, AD	17q21.2	148066	(Vahidnezhad *et al*., 2016)
Transglutaminase-5	*TGM5*	AR	15q15.2	603805	(Pigors *et al*., 2012)
Plakophilin-1	*PKP1*	AR	1q32.1	601975	(Ersoy-Evans *et al*., 2006)
Plakoglobin	*JUP*	AR	17q21.2	173325	(Pigors *et al*., 2011)
Desmoplakin	*DSP*	AR	6p24.3	125647	(Al-Owain *et al*., 2011)
Junctional EB					
Laminin-5 γ2	*LAMC2*	AR	1q25.3	150292	(Pulkkinen *et al*., 1994)
Laminin-5 β3	*LAMB3*	AR	1q32.2	150310	(Floeth and Bruckner-Tuderman, 1999)
Laminin-5 α3	*LAMA3*	AR	18q11.2	600805	(Shaiq *et al*., 2012)
Collagen XVII	*COL17A1*	AR	10q25.1	113811	(Van den Akker *et al*., 2011)
Integrin α3A	*ITGA3*	AR	17q21.33	605025	(Has *et al*., 2012)
Integrin β4	*ITGB4*	AR	17q25.1	147557	(Inoue *et al*., 2000)
Dystrophic EB					
Collagen VII	*COL7A1*	AD, AR	3p21.31	120120	(Van den Akker *et al*., 2011)
Kindler EB					
Fermitin family homolog 1	*FERMT1*	AR	20p12.3	607900	(Shan and Zuo, 2022)

Ichthyosis

Ichthyosis is originated from the Greek word "ichthys," meaning fish was first time used by Robert Willan in 1808 to describe generalized cornification disorder. It is a clinically heterogeneous group of chronic superficial scaling and roughness of skin. Clinical features include cracking, blistering, dry scales, and shedding of skin. Ichthyosis can be X-linked, AD, AR, or autosomal semi-dominant, based on the inheritance pattern (Oji *et al*., 2016). The three components of SC such as cornified cell envelope, the breakdown products of keratin-filaggrin, and intercellular lipid layer in cornified cells cytoplasm are thought to be the etiology of ichthyosis (Williams *et al*., 2005). Most cases of ichthyosis are inherited, while acquired ichthyosis can arise from malignancy, viral disorders, autoimmune diseases, metabolic diseases, and drug induced (Souissi *et al*., 2022).

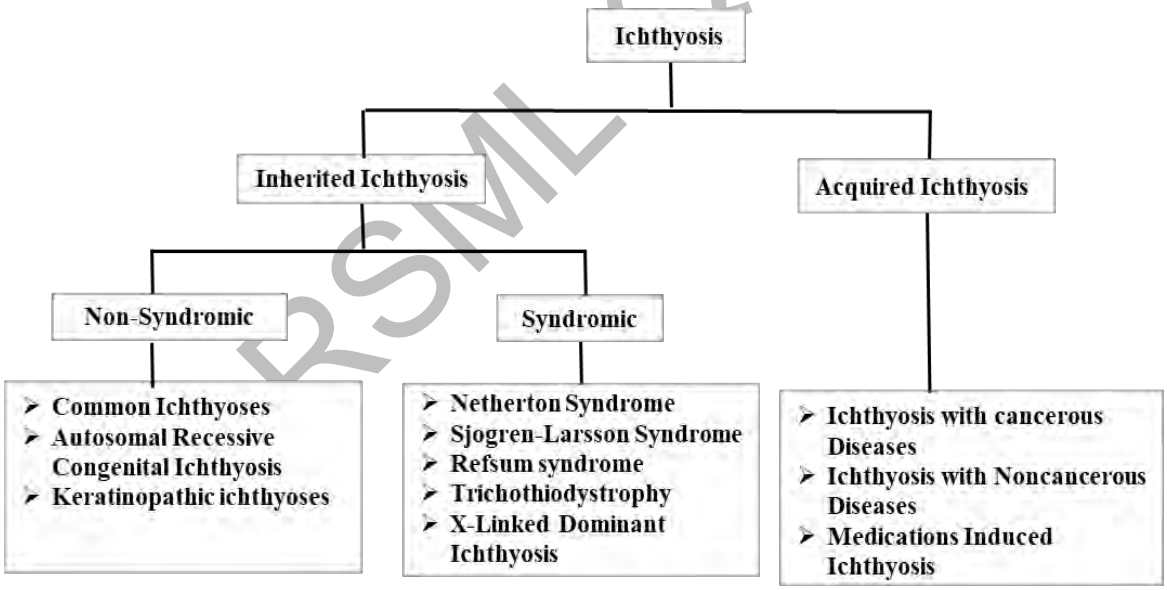


Figure 1.2. Ichthyosis classification

Classification of Ichthyoses

Inherited ichthyoses

Inherited ichthyoses (IC) are a cornification disorder marked by hyperkeratosis and scaly skin frequently linked with erythroderma disorders caused by abnormalities in skin barrier-forming genes. IC is divided into non-syndromic ichthyosis and syndromic ichthyosis (Oji *et al*., 2016).

Nonsyndromic Ichthyosis

Six subtypes are known in inherited nonsyndromic ichthyosis, starting with ichthyosis Vulgaris, recessive X-linked ichthyosis, harlequin ichthyosis, lamellar ichthyosis, congenital ichthyosiform erythroderma and epidermolytic ichthyosis.

Common Ichthyosis

a. Ichthyosis Vulgaris

Ichthyosis Vulgaris (IV; MIM 146700) is the mildest type of hereditary nonsyndromic ichthyosis, which is inherited in a semi-dominant (autosomal) pattern. Symptoms appear in early childhood and improve in summer usually. The hallmarks of IV are hyperkeratosis and erythema with fine, white, flaky scales (Richard and Ringpfeil, 2012). It has a significant link to atopic symptoms. The lower leg extensors and the back are the most affected. Palmoplantar hyperlinearity and keratosis pilaris can be seen. Its cause is a sequence variation in the gene filaggrin (*FLG*; Takeichi and Akiyama, 2016). *FLG* mutations cause poor epidermal barrier development and a significant decrease in natural moisturizing components, which are crucial for SC hydration.

b. Recessive X-linked Ichthyosis

Recessive X-linked Ichthyosis (RXLI; MIM 308100) is the mild scaling common type of congenital nonsyndromic ichthyosis. RXLI is inherited by asymptomatic female carriers which affect men. It is more severe than IV in terms of clinical manifestations, such as dryness and polygonal dark brown scales. Cutaneous symptoms frequently appear soon after birth and do not get improve with age. RXLI histology often shows mild acanthosis

and hyperkeratosis (Takeichi and Akiyama, 2016). It is characterized by steroid sulfatase (*STS*) deficiency caused by huge deletions in the *STS* gene present on chromosome Xp22.3 (Richard and Ringpfeil, 2012). Due to an enzyme deficiency cholesterol sulfate builds up in the epidermis. High levels of cholesterol sulfate block proteases which are essential for normal corneodesmosome breakdown. Serine protease activity was observed to be significantly lower in RXLI skin (Oji *et al*., 2016).

Autosomal Recessive Congenital Ichthyosis

Autosomal recessive congenital ichthyosis (ARCI) is a heterogeneous group of nonsyndromic ichthyosis seen at birth and characterized by hyperkeratosis and scaling**.** Harlequin ichthyosis, lamellar ichthyosis, and congenital ichthyosiform erythroderma are three subtypes of ARCI. To date, variants in thirteen different genes for ARCI have been reported **(Table1.2)**.

ARCI Subtypes

a. Harlequin Ichthyosis

Harlequin ichthyosis (HI; MIM 242500) is most dangerous critical type of ARCI. HI arises in 1 in every 300,000 births (Glick *et al*., 2017). Patients with HI frequently pass away in the early two weeks after birth, but once they have survived the neonatal stage, their phenotypes improve within a few weeks. A dense plate-like keratotic scale covering the whole body, ectropion, red fissures, eclabium, flattened ear and nose cartilage, generalized erythema, prematurity and palmoplantar keratoderma are all symptoms. Due to skin barrier deterioration, dehydration, unbalanced thermoregulation, elevated metabolic needs, pulmonary edema, and sepsis is observed. Sequence variations in the *ABCA12* gene lead to HI. *ABCA12* transports lipids like glucosylceramides into lamellar bodies required for epidermal barrier development. Lamellar bodies release lipid glucosylceramides and hydrolytic enzymes such as proteases, lipases, and proteins necessary for desquamation (Oji *et al*., 2016).

Table 1.2. List of mutated genes reported in ARCI

Targeted Protein	Gene	Location	Gene MIM	References
Transglutaminase-1	*TGM1*	14q12	190195	(Ullah *et al*., 2016)
Lipoxygenases 12B	*ALOX12B*	17p13.1	603741	(Vahlquist *et al*., 2010)
Lipoxygenases E3	*ALOXE3*	17p13.1	607206	(Vahlquist *et al*., 2010)
ATP-binding cassette subfamily A member-12	*ABCA12*	2q35	607800	(Akiyama, 2010)
Cytochrome P450 Family 4 Subfamily F Member 2	*CYP4F22*	19p13.12	611495	(Lefevre *et al*., 2006)
Non-Imprinted in Prader-Willi/Angelman Syndrome Region Protein 4	*NIPAL4*	5q33.3	609383	(Lefevre *et al*., 2004)
Lipase Family Member N	*LIPN*	10q23.31	613924	(Israeli *et al*., 2011)
Ceramide Synthase 3	*CERS3*	15q26.3	615276	(Radner *et al*., 2013)
Patatin Like Phospholipase Domain Containing 1	*PNPLA1*	6p21.31	612121	(Ahmad *et al*., 2016a)
Suppression of tumorigenicity 14	*ST14*	11q24.3	606797	(Ahmad *et al*., 2018c)
Cytochrome P450, fami1y 4, subfami1y F, po1ypeptide 22	*CASP14*	19p13.12	605848	(Kirchmeier *et al*., 2017)
Short-chain dehydrogenase/reductase family 9C member 7	*SDR9C7*	12q13.3	609769	(Shigehara *et al*., 2016)
Sulfotransferase Family 2B Member 1	*SULT2B1*	19q13.33	604125	(Heinz *et al*., 2017)

b. Lamellar Ichthyosis

Lamellar Ichthyosis (LI; MIM 242300) is illustrated by enormous platelike dark brown hyperkeratosis spanning the entire body with moderate palmoplantar involvement. LI have milder phenotypes than HI with a prevalence of 1/200,000 to 1/300,000 patients Associated symptoms can include nail dystrophy, eclabium, scalp alopecia, and ectropion (Bolognia *et al*., 2014). Neonate is often born as a collodion baby. To date, eight genes; *TGM1*, *CERS3*, *ABCA12*, *ALOX12B*, *ALOXE3, CYP4F2*, *NIPAL4*, *PNPLA1,* and *LIPN* have been reported in LI. Sequence variations in *ABCA12* and *TGM1* gene is most frequently reported in Asian populations (Takeichi and Akiyama, 2016).

c. Congenital Ichthyosiform Erythroderma

Congenital Ichthyosiform Erythroderma (CIE; MIM 242100) or Epidermolytic hyperkeratosis (EH) is congenital ichthyosis characterized by extensive scaling and erythroderma without blister development. CIE is inherited in an AR pattern (Richard and Ringpfeil, 2012). Children are usually born with CIE as collodion babies. Erythroderma and scaling are frequently accompanied by ectropion, keratoderma, eclabium, and nail dystrophy. In severe CIE, the erythroderma is widespread and long-lasting. In mild CIE it improves during childhood. Sequence variations in several genes have been reported for CIE including *TGM1, ABCA12, CERS3, ALOXE3, ALOX12B, LIPN, CYP4F22, NIPAL4,* and *PNPLA1* (Takeichi and Akiyama, 2016). All these genes contribute to the development of SC and the cornified cell envelope. Sequence variation in any one of these genes leads to disruption in skin barrier function (Oji *et al*., 2016). Sequence variations in *ALOX12B* and *ALOXE3* genes in patients with CIE were identified from the Mediterranean zone (Jobard *et al*., 2002).

Keratinopathic Ichthyoses

Keratinopathic ichthyoses (KPI) are an extremely rare group of cornification disorders. Erythroderma, erosions, and scales are common at birth in KPI patients. The word 'keratinopathic' refers to all types of ichthyoses characterized by genetic variations in keratin family genes (Oji *et al*., 2010). KPI comprises epidermolytic ichthyosis (EI), superficial EI, autosomal recessive EI, annular EI, congenital reticular ichthyosiform

erythroderma, and epidermolytic nevi. All KPI types are associated with sequence variations in the keratin family genes keratin 1 (*KRT1*; MIM 139350), keratin 2 (*KRT2*; MIM 600194), and keratin 10 (*KRT10*; MIM 148080) (Takeichi and Akiyama, 2016). Variations disrupt keratin oligomerization and epidermal cellular integrity, resulting in blistering and cytolysis (Richard and Ringpfeil, 2012).

Syndromic Ichthyosis

In syndromic ichthyosis affected individuals possess defects in other organs of the body other than the skin (Oji *et al*., 2010). The syndromic forms of ichthyosis include Netherton Syndrome, Sjogren-Larsson Syndrome, Refsum syndrome, Trichothiodystrophy, and X-Linked Dominant Ichthyosis (syn. CDPX2; 302960).

a. Netherton Syndrome

Netherton syndrome (NS: MIM 256500) is an AR congenital multisystem syndrome described by the SC premature desquamation and skin barrier dysfunction (Saleem *et al*., 2018). Clinicall featured include ichthyosiform dermatosis, atopic diathesis, and hair shaft abnormalities. Scaling like CIE, enteropathy, chronic skin cracking, intellectual disability, aminoaciduria, growth retardation, hypoalbuminemia, and immunological disorders are some of the other symptoms (Saleem *et al*., 2018). NS is caused by *SPINK5* (MIM 605010) gene sequence variations which code for a serine protease inhibitor (Boskabadi *et al*., 2013). The *SPINK5* is expressed in SS, trachea, tonsils, thymus, hair follicles parathyroid glands. It has 15 domains, two of which are cysteine-rich and aid in disulfide bonding (Furio and Hovnanian, 2011).

b. Sjogren-Larsson Syndrome

Sjogren-Larsson syndrome (SLS; MIM 270200) is an AR neurocutaneous condition characterized as a lipid metabolism inborn defect. The diagnostic triad of SLS includes ichthyosis, spastic paraparesis, and mental retardation (Bindu, 2020). Generalized ichthyosis in particularly neck, and lower abdomen (Dutra *et al*., 2012). Clinical features include premature birth, delayed speech, macular dystrophy, pruritus, seizure, thin scalp hair, brownish-yellow skin, and leukoencephalopathy (Bindu, 2020). Genetic variations in the aldehyde dehydrogenase 3 (*ALDH3A2*; MIM 609523) caused SLS. *ALDH3A2* codes

the enzyme fatty aldehyde dehydrogenase (FALDH). FALDH forms fatty acids from long-chain aliphatic aldehydes. The deficiency of FALDH causes abnormal fatty aldehyde oxidation to fatty acids, resulting in the accumulation of fatty aldehyde precursors. As a result, skin and neurological problems appear (Laurenzi *et al*., 1996; Rizzo and Carney, 2005).

c. Refsum Syndrome

Refsum syndrome (RS: MIM 266500) is a rare AR neurocutaneous lipid storage syndromic ichthyosis described by impaired hearing and vision, ataxia, mild ichthyosis, and neuropathy. Genetic variation in the PHYH gene is responsible for RS, which encodes a human phytanoyl-CoA hydroxylase that is required for phytanic acid oxidation (Jansen *et al*., 2000). Adult RS is triggered by genetic variation in the *PEX7* gene, which encodes peroxin 7. This gene serves as a receptor for the PHYH protein, allowing it to enter peroxisomes (Van Den Brink *et al*., 2003; Horn *et al*., 2007). The aberrant functioning of phytanoyl-CoA hydroxylase triggers the buildup of phytanic acid in tissues and plasma (Oji *et al*., 2016).

d. Trichothiodystrophy (Tay Syndrome):

Trichothiodystrophy (TTD; MIM 601675) is a heterogeneous group of a neurocutaneous syndrome described by Sulphur deficient brittle hairs because of a reduction in matrix cysteine-rich protein level. Small stature, unusual facial features, mental retardation, ichthyotic skin, and photosensitivity are some of the other clinical features (Faghri *et al*., 2008). There are two sub-types of TTD: photosensitive and non-photosensitive (Stefanini *et al*., 2010). The DNA repair genes *GTF2H* (p8/TTDA), *ERCC2* (*XPD*) or *ERCC3* (*XPB*) are mutated in most photosensitive patients. Variations in *MPLKIP* (*TTDN1*) have been linked to a few cases of non-photosensitive TTD (Hashimoto and Egly, 2009; Stefanini *et al*., 2010; Moslehi *et al*., 2010). TFIIH core proteins XPB and XPD have ATP-dependent helicase activity required during transcription for nucleotide excision repair. Any defect in these genes can cause TTD (Coin *et al*., 1999).

e. X-Linked Dominant Ichthyosis

X-linked dominant syndrome (MIM 302960) synonyms Conradi–Hunermann–Happel Syndrome (CHHS) or Chondrodysplasia Punctata 2 X-linked Dominant (CDPX2) mostly affects women and is severe in men. Asymmetrical limb shortening, mild ichthyosis, cataracts, generalized erythroderma, scarring alopecia, and follicular keratosis are some of the symptoms (Feldmeyer *et al*., 2006). Sequence variations in the TF Early B-Cell factor (*EBF*) gene cause CHHS. EBF is involved in the late steps of cholesterol biosynthesis, and neuronal differentiation in the hindbrain and spinal cord, enabling the precise placement of neurons across the neurogenic pathway (Garcia Dominguez *et al*., 2003).

Acquired Ichthyosis

Acquired ichthyosis develops in adults because of underlying illnesses or drugs. It seems like IV. Clinical features include pruritus, ichthyosis, and hair loss. A humid and warm temperature can improve such problems (Ugonabo *et al*., 2019).

a. Ichthyosis with cancerous Diseases

Some cancerous diseases such as Hodgkin lymphoma, Kahler's disease (multiple myeloma), lymphoproliferative diseases, cutaneous lymphoma, Kaposi sarcoma, cancer of the lung, breast, liver, and bladder have been reported with ichthyosis (Ugonabo *et al*., 2019).

b. Ichthyosis with Noncancerous Diseases

Noncancerous Diseases like 1) Infectious disorders such as acquired immune deficiency syndrome, pulmonary phthisis, and Hansen's disease. 2) Inflammatory disorders such as lupus erythematosus, glomerulonephritis, and hematopoietic stem cell transplantation. 3) Metabolic disorders include dietary deficiency, nephropathy, adenosis, and diabetes can be associated with Ichthyosis.

c. Medications Induced Ichthyosis

Drugs such as hydroxyurea, maprotiline, butyrophenone, clofazimine, acitretin, isoniazid, dixyrazine, cimetidine, and allopurinol have been reported to induce ichthyosis (Ugonabo *et al*., 2019).

The objective of the study, presented here, was to search for the genetic variants causing hereditary skin disorders in three consanguineous Pakistani families (A-C). Family A, identified with EB, was sampled from the province Sindh, Pakistan. Family B belonged to the province of Punjab, Pakistan and characterized by AR ichthyosis. Family C, belonged to Sindh, was characterized by dryness, pigmentation, and scales on the skin. To achieve the goals of the study, highly polymorphic microsatellite markers (HPMM), linked to candidate genes, were typed in each family. Family A was found linked to the *EXPH5* gene located on chromosome 11q22.3 whereas, family B revealed linkage to the *CERS3* gene located on chromosome 15q26.3. In family C linkage was tested by typing HPMM associated with candidate genes including *ABCA12* (2q35), *CERS3* (15q26.3), *LIPN* (10q23.31), *ST14* (11q24.3), *PNPLA1* (6p21.31), *EXPH5* (11q22.3), *KRT5* (12q13.13), *COL7A1*(3p21.31), *KRT14* (17q21.2), and *FERMT1* (20p12.3) revealed no linkage to the assessed loci. It suggests involvement of novel genes in causing diseased phenotypes. Sanger sequencing of all six coding exons of the *EXPH5* gene revealed no sequence variant in family A. In family B, Sanger sequencing data analysis revealed a novel variant (c.466-1G>A) locates at the splice acceptor site of exon eight of *CERS3* gene.

CHAPTER 2

MATERIALS AND METHODS

MATERIALS AND METHODS

Ethical Consent

Approval of the research work was taken from the Institutional Review Board of Quaid-i-Azam University Islamabad, Pakistan. Written consents were taken from the legal guardians of the affected members who participated in this study.

Recruitment of Families

Families that participated in the current study belonged to Sindh and Punjab, provinces of Pakistan. Blood samples and photographs of the affected individuals were collected by visiting the hometowns of these families. Guardians of the affected members were interviewed to get information for detailed family history. All the affected members were examined at local hospitals. Pedigree was drawn by following the Bennett *et al*. (1995) protocol. In pedigree, females and males were represented by the symbols of circles and squares, respectively. Normal family members were shown unfilled while affected members were shown by filled symbols. Deceased members of the family were shown through the slashed line on the symbols. Generation number and the members within the generation were represented with Roman and Arabic numerals, respectively. The marriage line was shown by a horizontal line between male and female, while the generation line was shown by a vertical line. Consanguineous marriage was shown with double lines. Blood (3 to 5 ml) was collected from the affected and the normal family members in EDTA tubes (BD Vacutainer® K3 EDTA, Franklin Lakes NJ, USA).

Genomic DNA Extraction

The genomic DNA of family members was extracted by phenol-chloroform method (Sambrook *et al*., 1989) and Thermo Scientific DNA Extraction Kit.

a. Phenol-Chloroform Method

Day 1 Protocol

- 750µl of both blood and solution A was mixed by gentle inversion in an Eppendorf tube of 1.5 ml volume. An incubation time of 20-30 min at room

temperature was given to it. Solutions with their compositions used in Phenol-Chloroform method are given in **(Table 2.1)**.

- The Eppendorf was centrifuged for sixty seconds at 13,000 rpm and after discarding the supernatant, solution A (500 µl) was used to dissolve the pellet.
- Then again the mixture was centrifuged for sixty seconds at 13,000 rpm.
- Pellet was dissolved in 500 µl solution B, 12µl of 20% SDS and 8µl of proteinase K. After this, the sample was kept for incubation at 40°C overnight.

Day 2 Protocol

- A freshly prepared mixture of solutions C and D (500µl) was added and for ten min centrifuged it at 13,000 rpm.
- Three prominent layers were visible; the upper layer consists of DNA was picked in a new Eppendorf tube.
- Solution D (500µl) was added to the DNA and for ten min centrifuged it at 13,000 rpm. This resulted in the formation of three layers and DNA was collected from the upper layer.
- DNA was precipitated by adding 500µl Isopropanol (chilled) and 55µl of 3M Sodium acetate to the Eppendorf. DNA thread was seen by inverting the Eppendorf several times. Centrifuged it for ten min at 13,000 rpm and discarded the supernatant.
- This time 200 µl chilled 70% ethanol was added to dissolve the pellet of the DNA by centrifugating it at 13,000 rpm for seven min.
- After centrifugation, a DNA pellet was formed in the Eppendorf. The solution was discarded carefully to dry the pellet in the vacuum concentrator for ten min at 45°C.
- Dissolved the DNA pellet in 100µl-120 of TE buffer and then incubated overnight at 37°C in order to completely suspend the DNA pellet and protect from DNase or RNase.

b. Thermo Scientific DNA Extraction Kit

A commercially available Genomic DNA Extraction kit of Thermo Scientific, Lithuania was used for the extraction of DNA.

- 200µl of whole blood was added in an Eppendorf and then added 400µl of lysis buffer. The mixture was thoroughly mixed and then add 20µl of Proteinase K.
- Then the Eppendorf tube was incubated in a water bath for 10 min at 56°C with shaking or vertexing after 5 min. After incubation 200µl of 100% ethanol was added to Eppendorf. Then Eppendorf was vortexed for thirty seconds followed by short spin.
- After this, the mixture was poured into collection tube containing column and centrifuged at 6,000 x g for sixty seconds.
- Solution collected in the collection tube was discorded and then wash buffer I (500 µl) was used to wash the column by centrifuging for sixty seconds. Solution collected in the collection tube after centrifugation was thrown away.
- Added 500µl wash buffer II to the column to wash it again to remove any impurity. For washing, the solution for three min was centrifuged at 12,000 x g and discarded the solution collected in the collection tube.
- Then column was run at 13,000 x g for sixty seconds to give an empty spin to remove any residual solution from the column. Discard the collection tube and then transfer the purification column to an Eppendorf.
- For the elution of the DNA, 200µl elusion buffer was added to it and centrifuged at the speed of 8,000 x g for sixty seconds, after incubation for two min.
- Purification column was discarded and extracted DNA was used for further analysis.

Agarose Gel Electrophoresis (1%)

For genomic DNA qualitative analysis, 1% agarose gel was used to resolve the DNA. Chemicals used in an agarose gel are given in (**Table 2.2)**. Gel was prepared by adding 5ml 10X TBE buffer and 45 ml distilled water in a beaker. Then 0.5 g agarose was added to the beaker. The agarose was dissolved in the solution by heating it for two min in a microwave oven. After heating Et. Br (5 µl) was added to the beaker mixed it thoroughly and transferred into the gel tank. To create wells a comb was placed for loading DNA later. Solidification of gel was attained at room temperature by leaving for ninety min. Loading dye and extracted DNA in an equal quantity of 3µl were mixed to load into the wells of the

gel. Thereafter, electrophoresis was performed for one hour at 90 volts in a 1X TBE buffer. Et. Br-coated DNA bands were then visualized by a modern gel doc system (Biometra, Gottingen, Germany).

DNA Quantification and Dilution

Colibri micro-volume spectrophotometer (Titertek Berthold, Germany), an instrument that was used to calculate the quantity of DNA in samples under 260 nm optical density wavelength. 1µl of Tris-EDTA (TE) buffer was used as a blank and then a DNA sample of 1µL was loaded in the spectrophotometer and DNA concentration was measured in ng/µl units. DNA was diluted to 20-30 ng/µl by adding PCR water.

Genotyping and Linkage Analysis

The families with EB and Ichthyosis were subjected to genotyping using HPMM specific to the genes responsible for these phenotypes. UCSC genome browser (human GRCh37/hg19 assembly) was used to get details of the genes tested in the linkage analysis. The list of microsatellite markers used for the linkage analysis of EB, and ichthyosis are listed in **(Tables 2.4 and 2.5)**, respectively. The markers with greater than 80% average heterozygosity were selected for the genotyping.

Polymerase Chain Reaction

First 1.5µl of template DNA was added into a PCR tube and then added 0.3µl of each forward and reverse marker. To make a total of 25µl of PCR reaction mixture, 23µl of the master mix was used. To prepare the master mix, PCR buffer (2.5µl), magnesium chloride ($MgCl_2$:1.5µl), dNTPs (0.5µl), Taq polymerase (0.5µl), and 18µl double filtered PCR water was added into an Eppendorf tube. Then the mixture was given a short spin for 10 seconds for proper mixing.

Polyacrylamide Gel Electrophoresis

After amplification, polyacrylamide gel (8%) was used to resolve the PCR amplified product. To prepare a single plate of polyacrylamide gel, 13.5 ml acrylamide solution (30%; prepared by acrylamide; MERCK Darmstadt, Germany) and N, N' Methylene-bis-acrylamide (BDH, Poole, England) added in 29:1 ratio, 5 ml 10 X Tris-Borate-EDTA (TBE; prepared by 0.89 M Tris, 0.89 M Borate and 0.02 M EDTA), 350µL of 10%

ammonium persulphate (APS; 5 g APS in 50 ml distilled water), 25µL N, N, N'-Tetra methyl diamine (TEMED; Sigma-Aldrich, USA), and distilled water to made the total volume of 50 ml in a beaker. Two glass plates were fixed together with the help of clips. The space between the two plates were 1.5 mm. Then gel solution was poured between the plate and a comb was used to make wells for loading of the sample and kept it for an hour for polymerization. Before loading the sample into the well, firstly, mixed the amplified product and bromophenol blue dye [bromophenol blue (0.25%) with sucrose (40%) solution]. Then electrophoresis occurred at 147 volts and took two to three hours to complete. For electrophoresis 1-X TBE was used as a running buffer. 10 µg/ml Et. Br solution was used for staing the gel which then help in the visualization of the bands on UV transilluminator (Biometra, Gottingen, Germany). Electrophoresis analysis system DC 290 (Kodak, Digital Sciences, New York, USA) was utilized for photograph of the gel.

Primer Designing

Primers for the exons of the linked genes were designed. The online available Primer3 software (https://primer3.ut.ee/) tool was used to design primers of the genes. Primer specificity was checked through online available tool primer stats (https://www.bioinformatics.org/sms2/pcr_primer_stats.html). Primers subjected to PCR amplification are mentioned in **(Table 2.6)** and **(Table 2.7)**.

Pre sequencing PCR

To prepare the reaction mixture for amplification PCR tube (200 µl) was used. The total reaction mixture prepared was 25 µl. The reaction mixture was made up of DNA (1.5 µl), forward primer (0.5 µl), reverse primer (0.5 µl), PCR buffer (2.5 µl), $MgCl_2$ (1.5µl), dNTPs (0.5µl), Taq polymerase (0.5µl), and PCR water (17.5 µl). PCR tube was then put into the thermocycler. PCR amplification was carried out in a thermocycler by following the conditions mentioned in **(Table 2.3)**. After the completion of the PCR reaction, the product was then checked on 2% agarose gel.

Agarose gel electrophoresis (2%)

2% horizontal agarose gel was used for the amplified product analysis. Gel was made by adding 2 g agarose into a solution of 10 ml 10X TBE buffer and 90 ml distilled water in a beaker. This mixture was heated for two min in a microwave oven. After heating Et. Br (7

µl) was added to the beaker mixed it thoroughly and transferred into the gel tank. To create wells a comb was placed for loading DNA later. Solidification of gel was attained at 37°C by leaving for 30-40 min. Put the gel into the gel tank containing 1X TBE buffer as a running buffer after solidification. Equal amounts (3µl) of both DNA and bromophenol blue were mixed before being loaded into the gel. Then the electrophoresis was performed for sixty min at 95 volts and the bands were then visualized in the gel doc.

Purification of the amplified product

After the confirmation of the amplification, the amplified product was purified by a commercially prepared GeneJET PCR Purification kit of Thermo Scientific, Lithuania following the instruction given by the company.

- A binding buffer to the amplified product present in the PCR tube was added in 3:1 volume. Transfer this mixture into a purification column having a collection tube.
- The mixture in the column was centrifuged for sixty seconds at 13,000 rpm. Filtrate from the collection tubes was discarded.
- 450 µl wash buffer was added to the column and centrifuged for sixty seconds at 13,000 rpm. Filtrate was removed from the collection tubes.
- 300 µl wash buffer was added this time and centrifugated it for sixty seconds at 13,000 rpm. Discard the collection tube and use a new collection tube.
- To avoid any contamination, the purification column and the collection tube were subjected for two min empty spin at 13,000 rpm.
- After an empty spin, the purification column was shifted to an Eppendorf tube and kept for incubation of two min at room temperatures after adding 20-25 µl of elution buffer into the column.
- Then the Eppendorf tube containing the purification column underwent ten min centrifugation at the resolution speed of 13,000 rpm. Then bands of purified DNA were checked on 2% agarose gel electrophoresis. and store the product at 4°C.

Sanger Sequencing

The purified products were subjected to commercially available Sanger sequencing. For sequencing**,** DTCS-Quick-Start Sequencing Kit (Beckman Coulter, Fullerton, CA, USA) was used. BioEdit sequence alignment editor (http://www.mbio.ncsu.edu/bioedit.html/) version 7.1.3.0 was used to analyzed sequence variation among the affected individuals and normal gene sequence.

Table 2.1. Solutions with their composition and concentration used in the phenol-chloroform method

Solution Used	Composition	Concentration
Solution A	Sucrose	0.32M
	Tris (pH 7.5)	10Mm
	$MgCl_2$	5mM
	Triton X-100	1%v/v
Solution B	Tris (pH 7.5)	10mM
	NaCl	400mM
	EDTA	2mM
Solution C	Saturated Phenol	-
Solution D	Chloroform	24:1 (by volume)
	Isoamyl Alcohol	
SDS 20%	SDS+ distilled water	10g in 50ml

Table 2.2. Solutions and their composition used for 1% agarose gel Electrophoresis

Chemicals	Composition
Agarose	0.5 g
Ethidium bromide	5 µg/mL
Gel loading dye	0.25%bromophenol blue 40% Sucrose
10X (TBE buffer)	0.032M EDTA (PH 8.3) 0.025M Boric acid 0.89M Tris
1X (TBE buffer)	0.032M EDTA (PH 8.3) 0.025M Boric acid 0.89M Tris

Table 2.3. PCR Condition

Steps	Sub Cycles	Temperature	Time
Initial Denaturation		96°C	2 min
40 PCR cycles	1. Denaturation	96°C	15 sec
	2. Primer annealing	52 – 63°C	20 sec
	3. Primer Extension	72°C	40 sec
Final Extension		72°C	2-4

Table 2.4. Microsatellite markers, flanking the selected genes, used in Genotyping families with EB

Serial No.	Gene	Cytogenetic position	Marker	CM
1	*FERMT1*	20p12.3	D20S835	16.16
			D20S905	18.96
			D20S194	19.79
			D20S846	20.8
			D20S602	23.25
2	*EXPH5*	11q22.3	D11S900	108.77
			D11S898	109.28
			D11S2017	112.89
			D11S1178	113.13
			D11S1391	115.14
3	*PLEC1*	8q24.3	D8S1520	160.14
			D8S1741	164.27
			D8S1729	165.8
			D8S373	171.32
			D8S1926	171.62
4	*COL7A1*	3p21.31	D3S3649	68.92
			D3S1568	71.04
			D3S3629	71.04
			D3S3661	71.31
			D3S3688	71.1
5	*KRT14*	17q21.2	D17S966	62.37
			D17S838	65.53
			D17S649	66.44
			D17S800	67.26
			D17S967	67.94
6	*KRT5*	12q13.13	D12S361	65.25
			D12S1677	66.17
			D12S297	67.04
			D12S1604	69.22
7	*DST*	6p12.1	D6S951	77.05
			D6S2410	77.63
			D6S1344	78.02
			D6S1960	80.98
			D6S1053	84.58

Table 2.5. Microsatellite markers, flanking the selected genes, used in Genotyping families with Ichthyosis

Serial no.	Gene	Cytogenetic position	Marker	Cm
1	*FLG*	1q21.3	D1S2343	157.06
			D1S2347	156.56
			D1S2346	158.66
			D1S2858	159.93
			D1S305	159.2
2	*TGM1*	14q12	D14S261	0.98
			D14S122	5.03
			D14S50	9.33
			D14S990	10.93
			D14S64	15.36
			D14S972	15
			D14S1041	16.11
			D14S729	17.07
			D14S80	20.53
			D14S608	21.3
3	*ALOX12B*	17p13.1	D17S1149	16.67
			D17S960	19.66
			D17S1353	20
			D17S1844	21.35
			D17S786	22.46
			D17S1791	23.59
4	*ABCA12*	2q35	D2S143	212.85
			D2S1345	213.02
			D2S2361	214.40
			D2S137	215.32
			D2S2382	216.19
5	*LIPN*	10q23.31	D10S523	105.18
			D10S215	107.31
			D10S1411	108.01
			D10S1735	108.23
			D10S2470	110.42
6	*ST14*	11q24.3	D11S4091	144.06
			D11S4123	145.13
			D11S4463	150.29
			D11S4131	152.89

			D11S1304	155.18
7	*PNPLA1*	6p21.31	D6S1618	56.26
			D6S291	57.66
			D6S943	60.29
8	*CERS3*	15q26.3	D15S985	121.77
			D15S966	122.56
			D15S87	126.53
			D15S642	131.77
			D1S305	159.2

Table 2.6. List of primers used for *EXPH5* gene sequencing

Exon	Primer	5' to 3' Sequences	Product size (bp)	Tm (°C)
1	EXPH5-1F	GGGGCGGACTTATTATTGGC	387	62.8
	EXPH5-1R	TTTTCTCTTCCCGACCCGC		62.4
2	EXPH5-2F	GTTGCAGTGAGACGAGGTTG	557	60.6
	EXPH5-2R	ACCCACTATGCTATCTTGCCT		62,4
3	EXPH5-3F	GCCCTGTTTCTAGTCTGCATAA	494	58.5
	EXPH5-3R	GCAGCTCGTTAGAAGTTGGATT		59.9
4	EXPH5-4F	AACACGCACAAAGATCAGGC	654	61.2
	EXPH5-4R	GCAACACGTGGCAAAATATCA		61.8
5	EXPH5-5F	GAGAAGTTGCAGGTCAGTTACA	610	57.1
	EXPH5-5R	GTCTCAAACTCCTGACCCCT		58.2
6-1	EXPH5(6)-1F	ACGGCGTTAATGGAAGGTTT	827	60.6
	EXPH5(6)-1R	ACATACTTGTCAGCGGGATCA		58.4
6-2	EXPH5(6)-2F	ACCAGCAGAGTCCAAAGAGA	806	60.4
	EXPH5(6)-2R	GAATGAGGATGCTAGAGTCTGA		60.8
6-3	EXPH5(6)-3F	CCACATCCTTGGCAGTTTGA	727	60.4
	EXPH5(6)-3R	TGGATTTATCTGTGTGCGGT		58.4
6-4	EXPH5(6)-4F	ATGGACCAGACAAACAAGGC	705	60.4
	EXPH5(6)-4R	AGGCACAGGACTATCATTTCTCT		58.7
	EXPH5(6)-5F	CCTTGATGCTCCTGTGGTTC		62.6

6-5	EXPH5(6)-5R	GGACATGACATAGCCCTGTTG	696	62.4
6-6	EXPH5(6)-6F	AGCCACAGAGAGAATGACAAATG	645	64.5
	EXPH5(6)-6R	GGTGTTCCTGACTGCTCTCG		61
6-7	EXPH5(6)-7F	ACCTCCTTCAACAGTATACACAA	558	59.2
	EXPH5(6)-7R	TACCACTCCCTGTACACTCC		62.4
6-8	EXPH5(6)-8F	CCTCTAGTCTTCCAGCTCTTTCA	645	62.8
	EXPH5(6)-8R	ACTCCACTGTGCCAAGAGAT		62.4
6-9	EXPH5(6)-9F	CTAAAGCCAGCAGAAAATTCCC	544	60.8
	EXPH5(6)-9R	GCACTCCTTTGTTGCTGCA		60.2
6-10	EXPH5(6)-10F	CTGAGCCCTCCTTTTCCACT	841	62.4
	EXPH5(6)-10R	GGGACATTTCAGAGCAGACAC		62.6

Table 2.7. List of primers used for *CERS3* gene sequencing

Exon	Primer	5' to 3' Sequences	Product size (bp)	Tm (°C)
1	CERS3-1F	CTGGCATTTTGAGCCAAGGT	318	60.4
	CERS3-1R	GCCCATTAGTGGCAACCTCT		62.4
3	CERS3-3F	ATCCCCAGTACCGAAGCTCT	353	60.8
	CERS3-3R	ACACCACATCAGTGGGTGAC		61.2
4&5	CERS3-4&5F	CATCTAATAAGTGGGGCAGAGC	888	62.4
	CERS3-4&5R	GCCAGGGATGACACAGTAGT		62.4
7	CERS3-7F	GCTCCCTCACAAAGATTGTGTTTA	536	60.4
	CERS3-7R	ATGCAACCTTCAGGAGTGGAAT		62.7
9	CERS3-9F	AATCCTCTAGCCCCATCACC	566	62.4
	CERS3-9R	GAACATTCAACAGTCCGGGC		62.4

CHAPTER 3

RESULTS

RESULTS

Family A

Family A, diagnosed with EB, was sampled from the rural area of the province Sindh, Pakistan. A four-generation pedigree was constructed after getting information from elders of the family **(Figure 3.1)**. The pedigree depicted a consanguineous pattern of marriages and AR mode of inheritance. Out of eight individuals in the fourth generation, one male (IV-4), and one female (IV-5), were found with EB. Both affected individuals have skin problems. The sole and palm sides were affected and had white patches. Swelling of joints and knees was found in both the patients. They had thick, dome-shaped nails **(Figure 3.2)**. Teeth were missing however hairs were not affected. For genetic analysis, blood of two affected members (IV-4, IV-5) and three normal members (III-1, III-2, IV-2) were collected.

Family B

Family B was sampled from province of Punjab, Pakistan. Pedigree of a four-generation was constructed after collecting information from elders of the family **(Figure 3.6)**. The pedigree consists of forty-five individuals, including eight affected members (IV-2, IV-3, IV-4, IV-5, IV-13, IV-16, IV-17, IV-18). The Pedigree drawing showed an AR pattern of inheritance. The affected individuals displayed clinical features of congenital ichthyosis with black scaling on the skin. Scales appeared and disappeared from time to time. The skin of the hands was stiff and hard while wrinkle and hard at the joints of affected individuals **(Figure 3.7)**. Blood samples from fifteen members including ten normal (III-1, III-4, III-5, III-6, III-8, III-9, IV-1, IV-2, IV-7, IV-14) and five diseased (IV-2, IV-4, IV-5, IV-16, IV-17) participants were collected.

Family C

Family C was sampled from the rural area of the province of Sindh, Pakistan. A four-generation pedigree was constructed for the family **(Figure 3.11)**. The pedigree depicted a consanguineous pattern of marriages and AR mode of inheritance. Affected individuals

were produced from two normal carrier parents in the fourth generation. Out of seven individuals in the fourth generation, one male (IV-5) and two female (IV-6, IV-7) were found with skin disorders. The clinical features of affected individuals include dryness, pigmentation, and scales on the skin that become severe in summer. No other deformity was identified in the affected members. Blood samples from six participants, involving three diseased (IV-5, IV-6, IV-7) and three healthy (III-2, IV-2, V-4) individuals were collected. Their DNA was subjected to molecular analysis for identification of the pathogenic sequence variants.

Genotyping and Linkage Analysis

All the three families (A-C) in the current study were analyzed for linkage to previously known loci for EB and ichthyosis through HPMM tested within respective candidate regions. HPMM for genotyping were chosen from the regions of respective candidate genes. The candidate genes included *FERMT1* (20p12.3), *EXPH5* (11q22.3), *PLEC1* (8q24.3), *COL7A1* (3p21.31), *KRT14* (17q21.2), *KRT5* (12q13.13), *DST* (6p12.1), *FLG* (1q21.3), *TGM1* (14q12), *ALOX12B* (17p13.1), *ABCA12* (2q35), *LIPN* (10q23.31), *ST14* (11q24.3), *PNPLA1* (6p21.31), *CERS3* (15q26.3) were analyzed for linkage. Analysis of HPMM was performed using PCR protocol and the products were subsequently resolved on polyacrylamide gel (8% non-denaturing). PCR amplified products were stained with Et. Br and visualized on UV Transilluminator (Biometra, Gottingen, Germany). The gels were photographed using a camera EDAS 290 (Kodak, NY, USA). Analysis of the allele pattern was performed to establish or exclude linkage in the family. The markers were considered linked to the disease gene, if the normal individuals exhibited heterozygous banding patterns and affected individuals were homozygous for the same microsatellite marker.

In family A, DNA extraction of two affected members (IV-4, IV-5) and three unaffected participants (III-1, III-2, IV-2) were carried and tested to genotyping study. The results obtained from typing HPMM linked to *FERMT1* (20p12.3), *EXPH5* (11q22.3), *PLEC1* (8q24.3), *COL7A1* (3p21.31), *KRT14* (17q21.2), *KRT5* (12q13.13), *DST* (6p12.1) are listed in **(Table 2.4)**. Genotyping analysis revealed a linkage of the family to *EXPH5* gene with cytogenetic location 11q22.3 **(Figure 3.3)**. Markers in linkage interval were found in the

homozygous state in both the affected members while heterozygous in unaffected members of the family. Markers D11S898 (109.28 cM) and D11S2017 (112.89 cM) flanked the linkage interval. Analysis of haplotypes showed a 3.61 Mb region on chromosome 11q22.3 homozygous in both affected individuals **(Figure 3.4)**.

In family B, DNA samples of six members were extracted from blood, involving two affected members (IV-4, IV-5) and three healthy (III-4, III-5, IV-7) members were selected for homozygosity mapping with HPMM for *FLG* (1q21.3), *TGM1* (14q12), *ALOX12B* (17p13.1), *ABCA12* (2q35), *LIPN* (10q23.31), *ST14* (11q24.3), *CERS3* (15q26.3). Genotyping results revealed linkage to the ceramide synthase (*CERS3*) gene located on chromosome 15q26.3 of the family. The HPMM D15S87 (126.53 cM) was found linked **(Figure 3.8)**. Haplotypes of family B are presented in (**Figure 3.9)**.

In family C, autozygosity was carried out in five DNA samples. Of five individuals two healthy (III-2, IV-1) and three affected (IV-5, IV-6, IV-7) members were screened for autozygosity mapping using HPMM for ten genes *ABCA12* (2q35), *CERS3* (15q26.3), *LIPN* (10q23.31), *ST14* (11q24.3), *PNPLA1* (6p21.31), *EXPH5* (11q22.3), *KRT5* (12q13.13), *COL7A1*(3p21.31), *KRT14* (17q21.2), *FERMT1* (20p12.3). Genotyping results of HPMM failed to establish linkage in the family to the tested genes.

Sequencing *EXPH5* Gene

Genotyping results illustrate family A linkage to the *EXPH5* gene located on chromosome 11q22.3. To identify pathogenic variant in coding and/or intron and exon borders, primers for all six coding exons were designed using Primer3 software (https://primer3.ut.ee/). Designed primers sequences are listed in **(Table 2.6)**. Sanger sequencing data of all six coding exons analysis failed to reveal a pathogenic variant **(Figure 3.5)**. Hence, it is possible that the variant could be present in regulatory region of the gene.

Sequencing *CERS3* Gene

Homozygosity mapping of family B displayed linkage to ceramide synthase gene (15q26.3). Primers of selected exons (1,3, 4, 5, 7, and 9) were designed with help of primer3 software. A list of primers is given in **(Table 2.7)**. Sanger sequencing of selected

coding exons of the affected individual (IV-4) was performed. Sanger sequencing data analysis revealed a novel splice site variant (c.466-1G>A) in exon eight of the *CERS3* gene. Sequencing of the variant in rest of the family members (III-4, III5, IV-5, IV-6) validated its segregation with the disorder within the family **(Figure 3.10)**. The variant was predicted to be pathogenic by bioinformatic tools, including varSEAK, Varsome, DANN, BayesDel_addAF, FATHMM-MKL, EIGEN, CADD, and Mutation Taster. According to American College of Medical Genetics (ACMG) classification, the identified variant was interpreted as "likely pathogenic" with PVS1, PM2, PP3 values **(Table 3.1).**

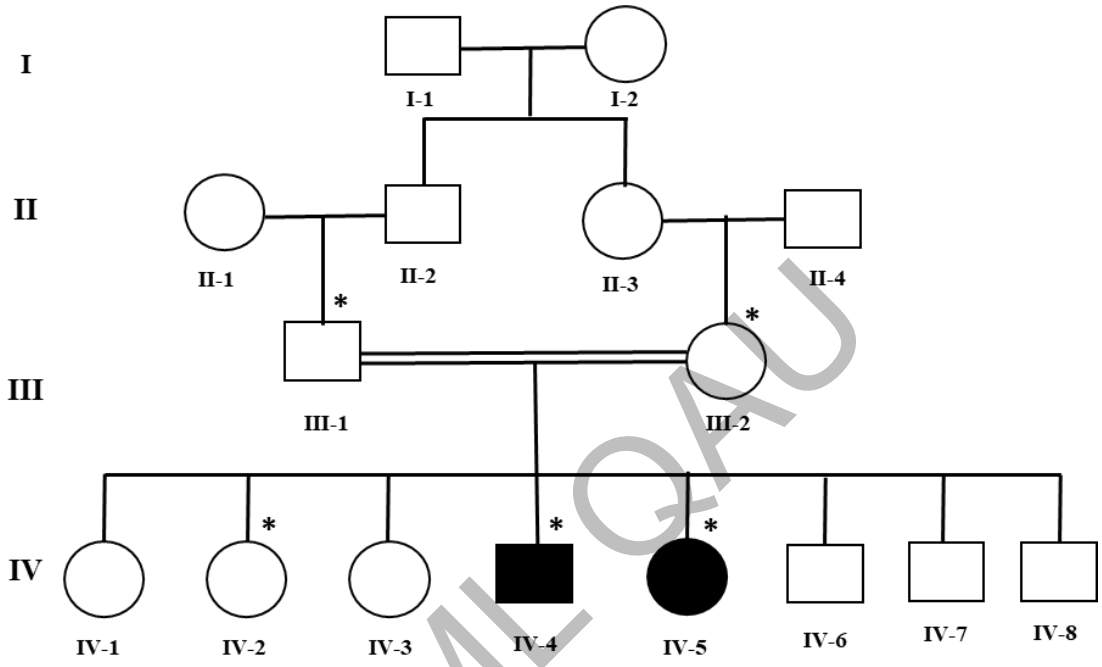


Figure 3.1. Pedigree sketch of family A, segregating in the AR pattern of EB simplex. The circles symbolize females, and the squares males. A shaded circle or square represents an affected while an unshaded symbol represents normal individuals. Double lines specify consanguineous marriages. Roman and Arabic numbers define the generation position and number of the members in a family pedigree. Those members whose blood were collected are represented with asterisks (*) in the pedigree.

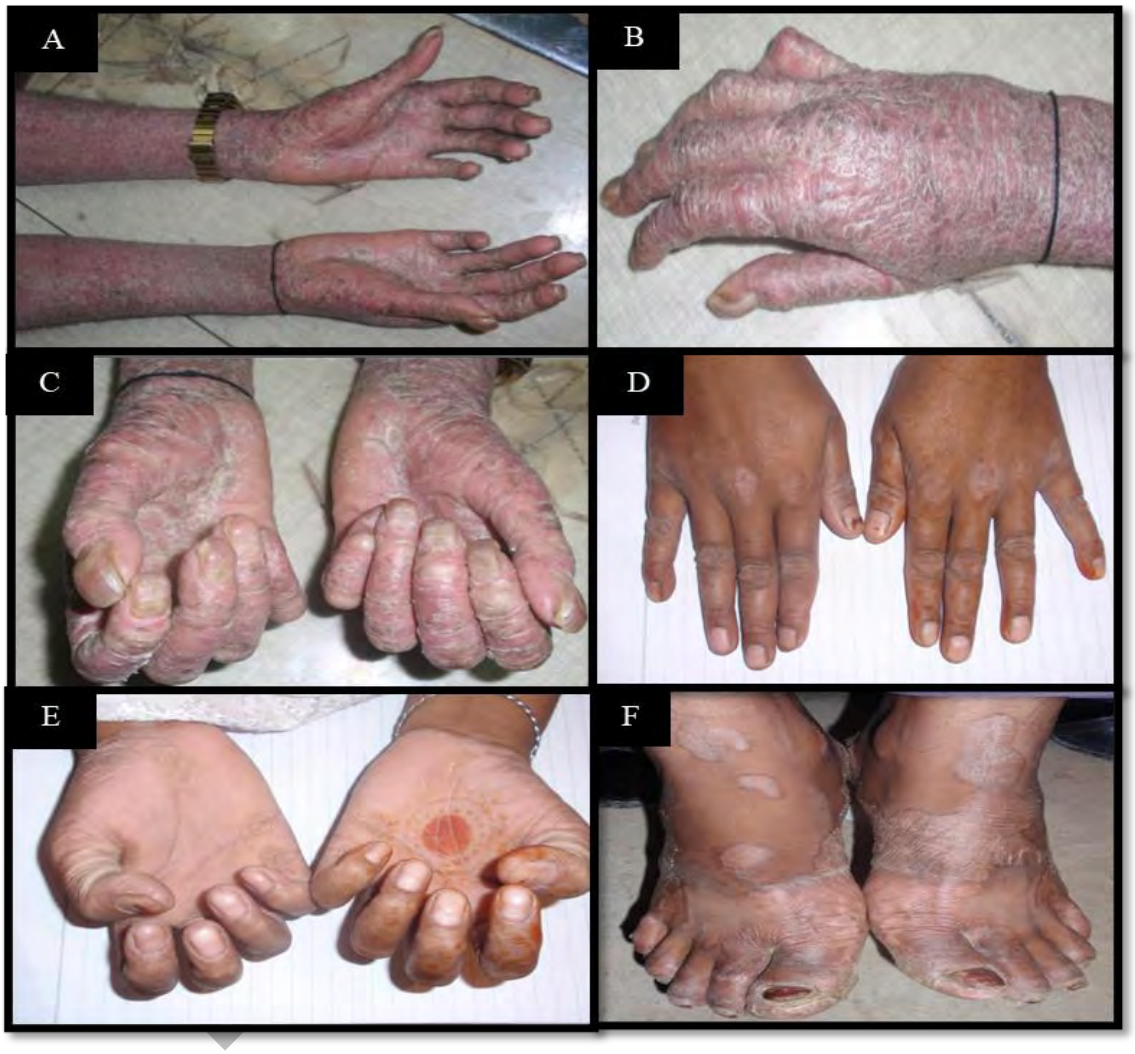


Figure 3.2: Clinical representation of affected individuals (IV-4, IV-5) in family A. **(A-C)** Extreme dryness and white scales on hands with nail dystrophy in IV-4. **(D-F)** Swelling and white patches on fingers joints, abnormal dome-shaped nails. Sole was more affected and had cuts on the heel side in IV-5.

Family A: *EXPH5* gene

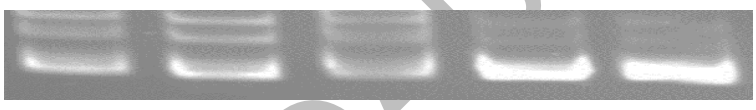
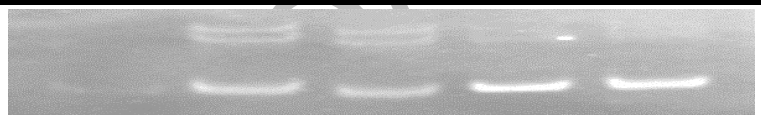
Marker ID	Map Unit (cM)	1-N	2-N	3-N	4-A	5-A
D11S898	109.28					
D11S2017	112.89					
		1-III-2 (Normal)	2-III-2 (Normal)	3-IV-2 (Normal)	4-IV-4 (Affected)	5-IV-5 (Affected)

Figure 3.3: Polyacrylamide electropherogram illustrating homozygosity among the affected members (IV-4 and IV-5) for microsatellite markers D11S898 and D11S2017 flanking the *EXPH5* gene on chromosome 11q22.3. Roman and Arabic numbers define the generation position and number of the members in a family pedigree.

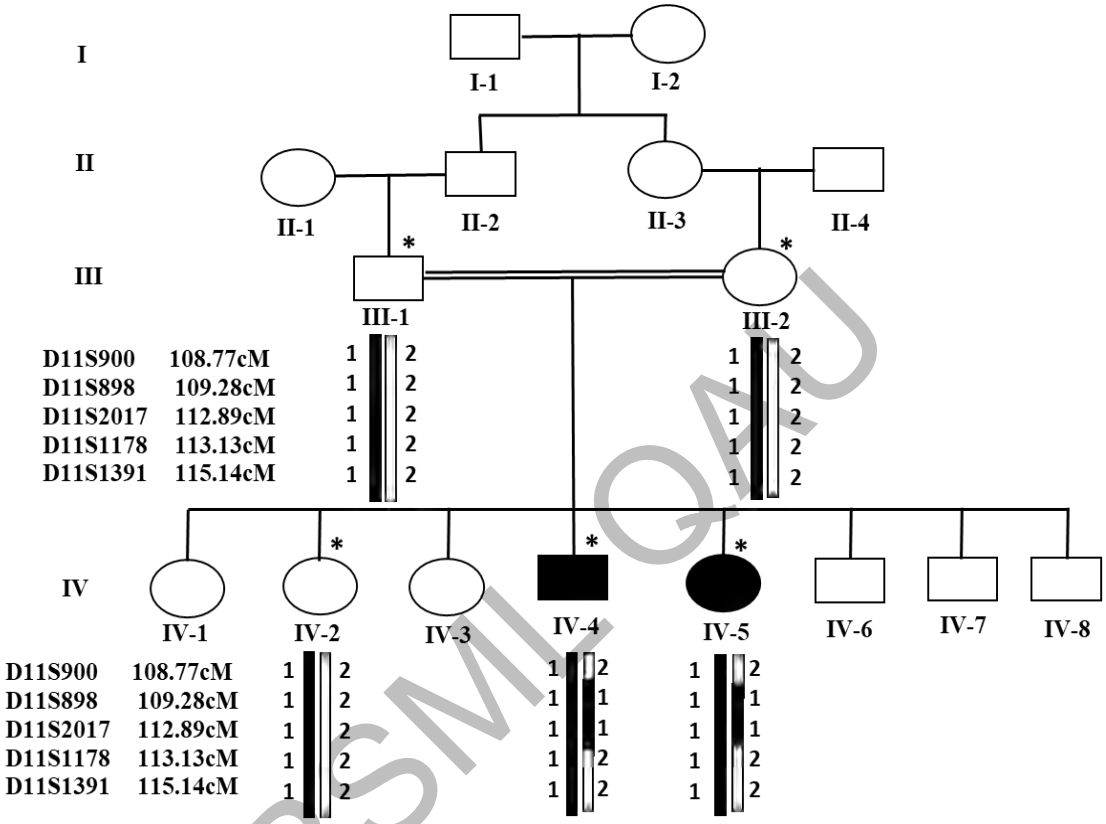


Figure 3.4: Pedigree illustrates of family A, segregating AR type of EB simplex with haplotypes of the closely linked locus-specific microsatellite markers under each genotyped member. Cytogenetic position presented in centimorgan (cM). Black symbols depict affected members whereas blank symbols represent normal members of the family. Genotypes with 1 1 represents homozygous while 1 2 heterozygous states of the marker.

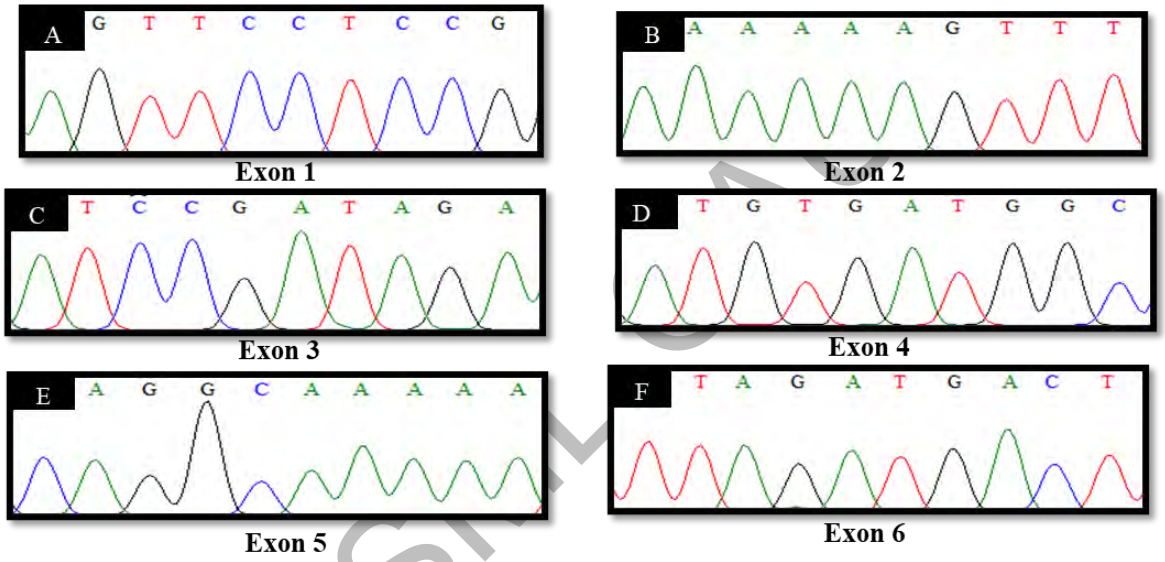


Figure 3.5: Partial sequencing chromatogram of all six coding exons of *EXPH5* in an affected member (IV-4) of family A.

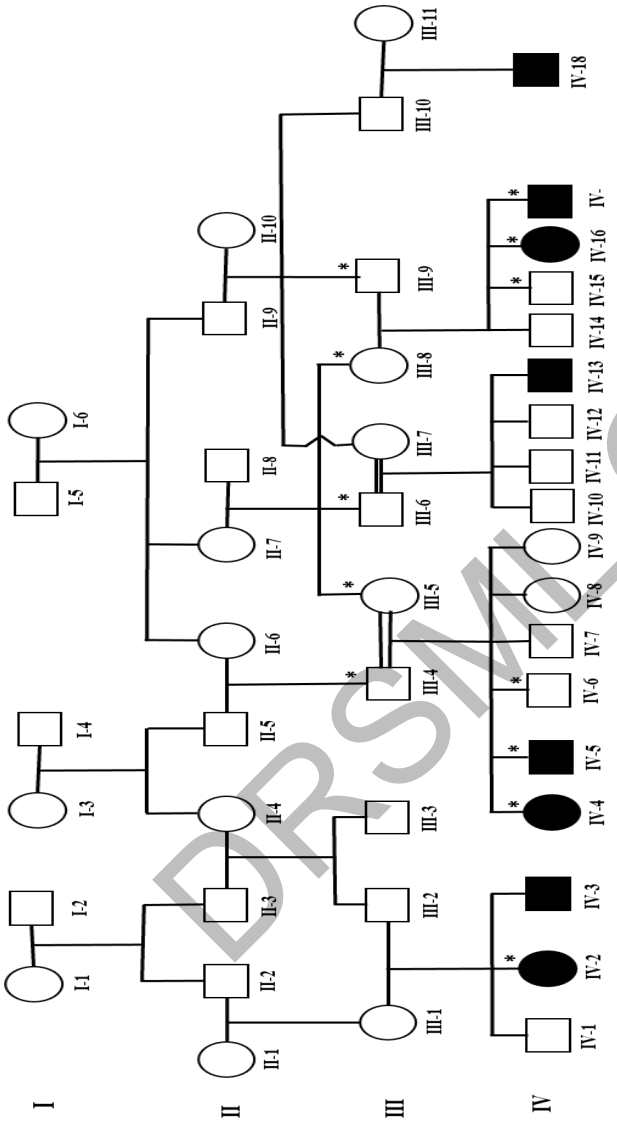


Figure 3.6: Pedigree of family B, segregating hereditary AR congenital ichthyosis (ARCI). The circles symbolize females and the squares males individuals of the family. A shaded circle or square represents an affected while an unshaded symbol represents normal individuals of family. Double lines specify consanguineous marriages. Roman and Arabic numbers define the generation position and number of the members within a generation in pedigree. Those members whose blood were collected are represented with asterisks (*) in the pedigree.

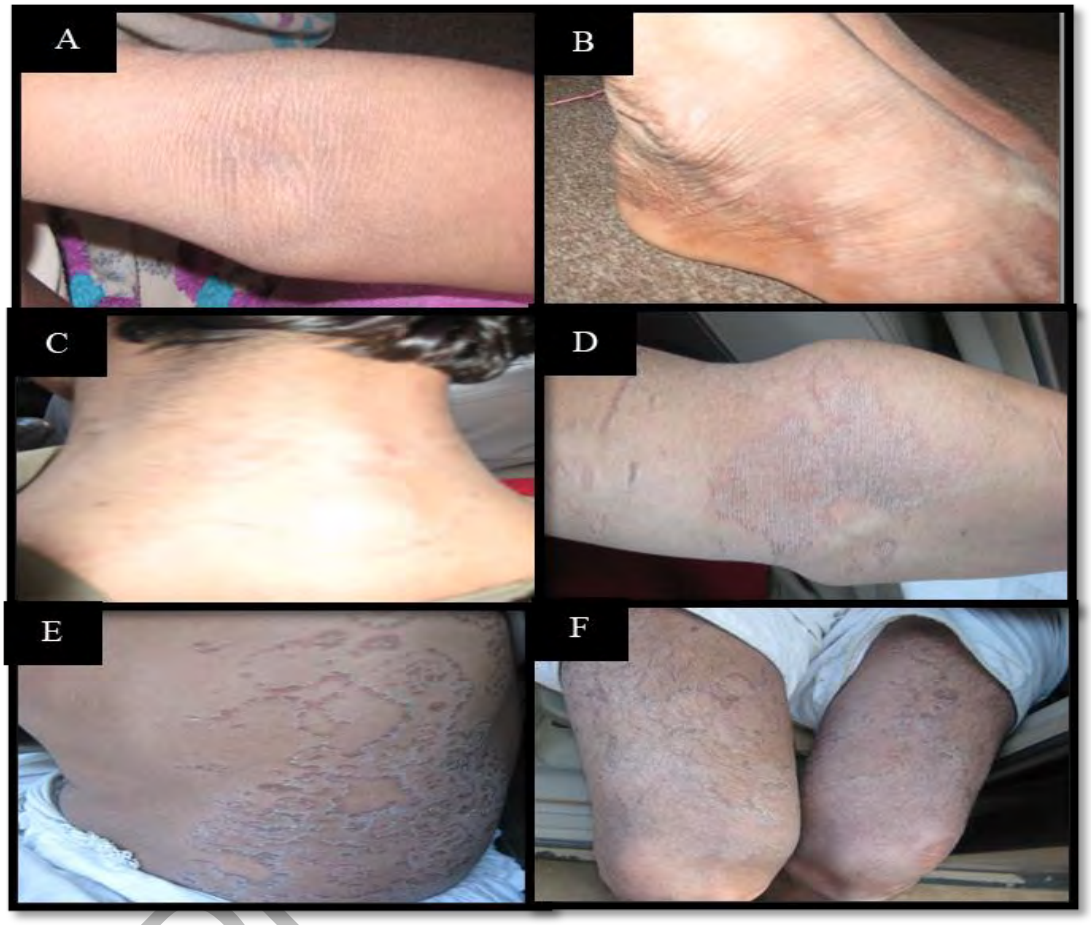


Figure 3.7: Clinical features of affected individuals (IV-4, IV-5) segregating autosomal recessive congenital ichthyosis (ARCI) in family B. **(A-B)** Stiff and hard skin at hands and hyperkeratosis over feet in an affected individual IV-4. **(C-F)** Affected individual IV-5 had skin dryness and black scales on the back of the neck, elbow, back, and legs.

Family B: *CERS3* gene

Marker ID	Map Unit (cM)	1-N	2-N	3-N	4-A	5-A
D15S87	126.53					
		1-III-2 (Normal)	2-III-2 (Normal)	3-IV-2 (Normal)	4-IV-4 (Affected)	5-IV-5 (Affected)

Figure 3.8: Polyacrylamide electropherogram illustrating homozygosity among the affected members (IV-4 and IV-5) for microsatellite marker D15S87 flanking the *CERS3* gene on chromosome 15q26.3. Roman and Arabic numbers define the generation position and number of the members in a family pedigree.

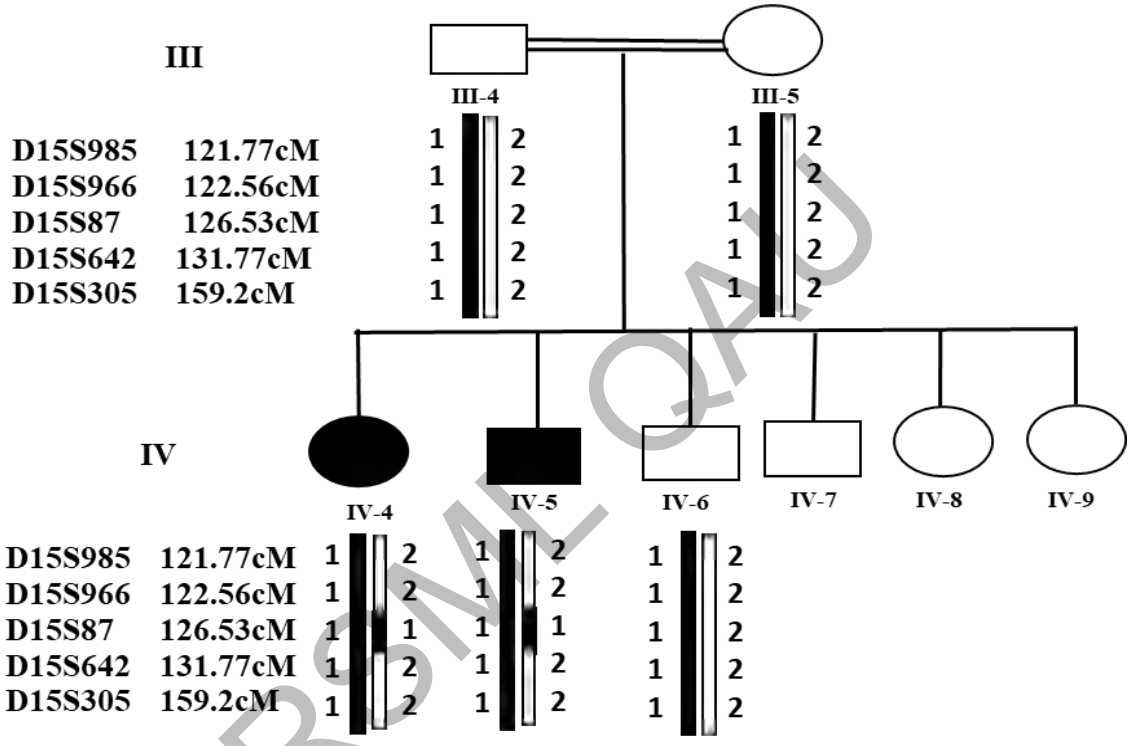


Figure 3.9: Pedigree illustrates family B; segregating AR congenital ichthyosis haplotypes of the closely linked locus-specific microsatellite markers under each genotyped member. Cytogenetic position is represented in centimorgan (cM). Black symbols depict affected members whereas blank symbols represent normal members of the family.

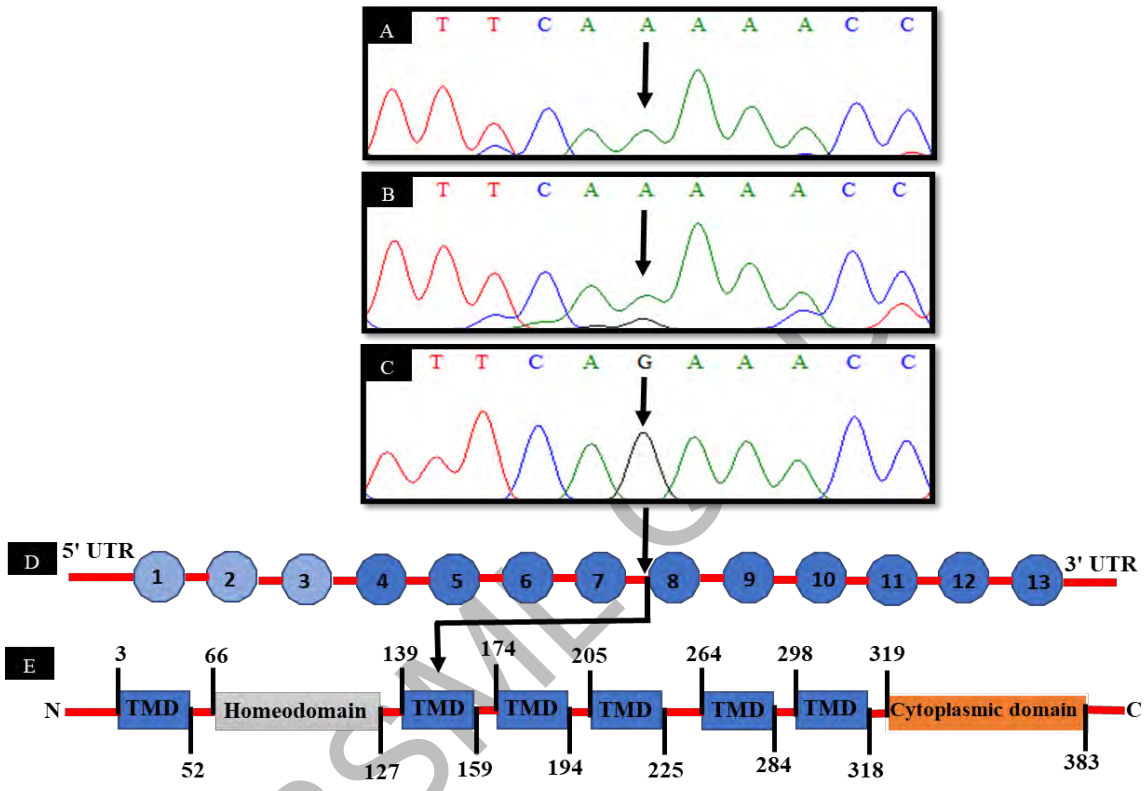


Figure 3.10: Sequencing chromatogram illustrating sequencing of the coding exon of *CERS3* indicating a G to A transition at splice acceptor site (c.466-1G>A). **Panel A** (upper) shows the DNA sequence in the affected member (IV-4), **panel B** (middle) shows the DNA sequence in the heterozygous carrier (III-4) and **panel C** (bottom) shows the DNA partial sequence *CERS3* in the normal member (IV-6) of family B. The black arrow indicates the nucleotide change in the sequence. **Panel C, D** manifesting *CERS3* gene and protein structure. The gene consists of thirteen exons. *CERS3* has a homeobox domain (gray), six transmembrane domains (TMD; dark blue), and a cytoplasmic domain (orange).

Table 3.1: Analysis of the variant in family B

***CERS3* Variant Description**		
Chromosome	**15**	
Genomic position	**101019684**	
Mutation Type	**Splice-site**	
Zygosity	**Homozygous**	
GnomAD allele count	**Absent**	
Bioinformatics Prediction Tools Variant Prediction		
Prediction Tool	**Prediction**	**Score**
SpliceAI		**0.99**
Varsome	**-**	**7**
BayesDel_addAF	**Damaging**	**0.618**
DANN	**-**	**0.992**
EIGEN	**Pathogenic**	**0.9698**
FATHMM-MKL	**Damaging**	**0.9849**
CADD	**-**	**33**
Mutation Taster	**Disease-causing**	**1**
ACMG Classification	**Likely Pathogenic**	**-**
PVS1	**Very Strong**	**-**
PM2	**Moderate**	**-**
PP3	**Strong**	**-**

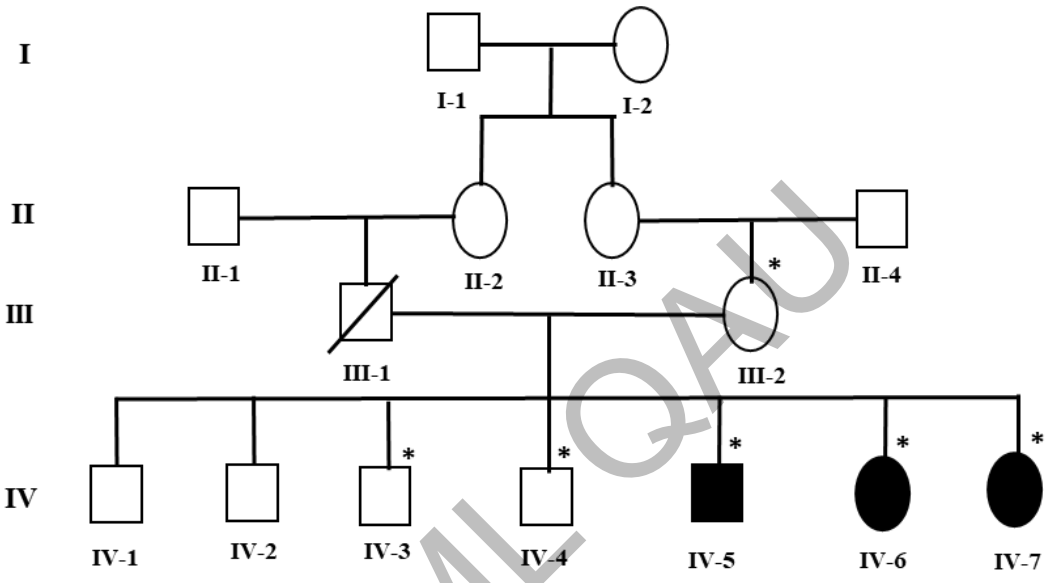


Figure 3.11: Pedigree of family C, segregating hereditary AR congenital ichthyosis. The circles symbolize females and the squares males individuals of the family. A shaded circle or square represents an affected while an unshaded symbol represents normal individuals of a family. Double lines specify consanguineous union. Cross lines over the symbols represent deceased individuals. Roman and Arabic numbers define the generation position and number of the members in a family pedigree. Those members whose blood were collected are represented with asterisks (*) in the pedigree.

Family C: *ABCA12* gene

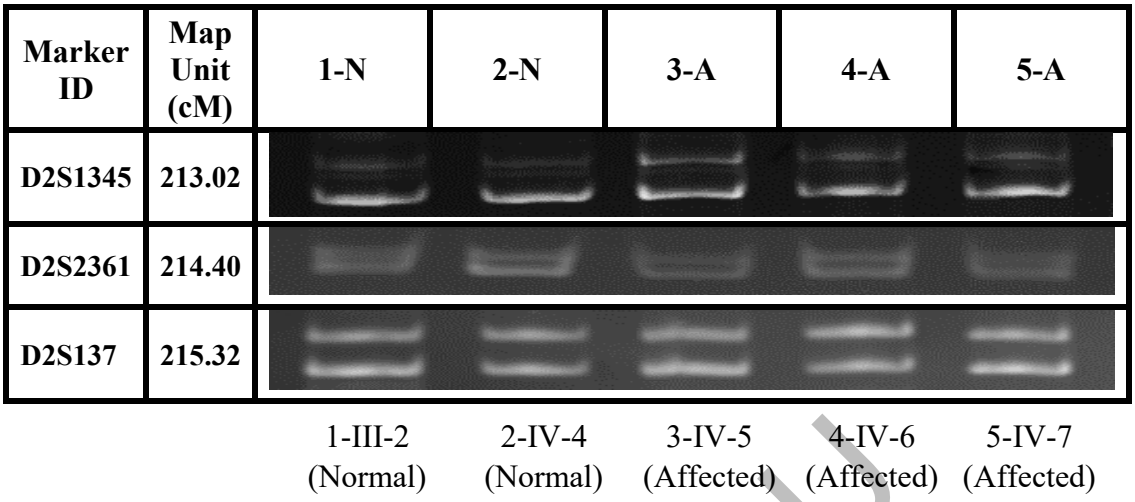


Figure 3.12: Polyacrylamide electropherogram illustrating the pattern of alleles amplified with specific microsatellite markers flanking *ABCA12* gene in family C. Roman and Arabic numbers define the generation position and number of individuals in a family pedigree.

Family C: *CERS3* gene

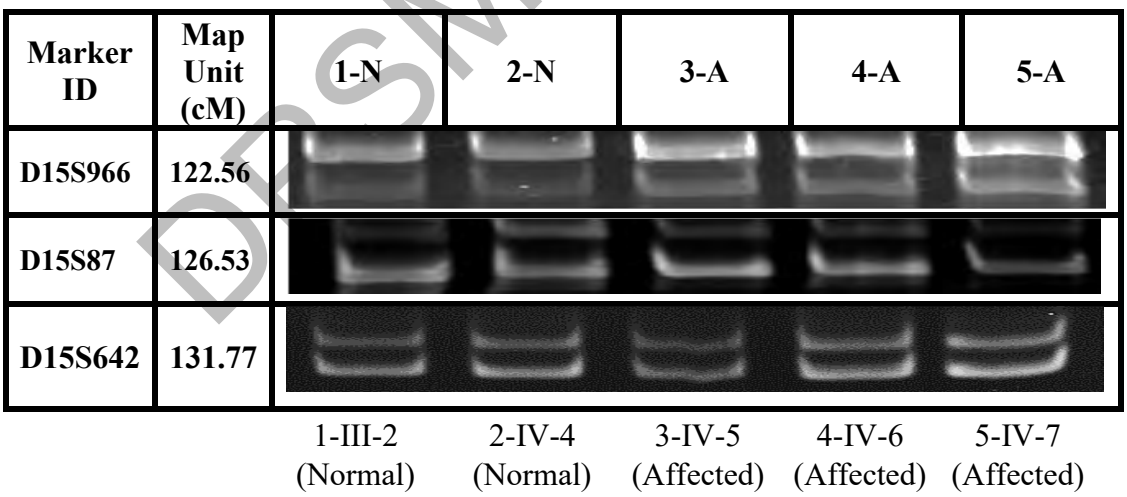


Figure 3.13: Polyacrylamide electropherogram illustrating the pattern of alleles amplified with specific microsatellite markers flanking *CERS3* gene in family C. Roman and Arabic numbers define the generation position and number of the individuals in a family pedigree.

Family C: *LIPN* gene

Marker ID	Map Unit (cM)	1-N	2-N	3-A	4-A	5-A
D10S215	107.31					
D10S1411	108.01					
D10S2470	110.42					

1-III-2 (Normal) 2-IV-4 (Normal) 3-IV-5 (Affected 4-IV-6 (Affected) 5-IV-7 (Affected)

Figure 3.14: Polyacrylamide electropherogram illustrating the pattern of alleles amplified with specific microsatellite markers flanking *LIPN* gene in family C. Roman and Arabic numbers define the generation position and number of the individuals in a family pedigree.

Family C: *ST14* gene

Marker ID	Map Unit (cM)	1-N	2-N	3-A	4-A	5-A
D11S4123	145.13					
D11S4463	150.29					
D11S4131	152.89					

1-III-2 (Normal) 2-IV-4 (Normal) 3-IV-5 (Affected 4-IV-6 (Affected) 5-IV-7 (Affected)

Figure 3.15: Polyacrylamide electropherogram illustrating the pattern of alleles amplified with specific microsatellite markers flanking *ST14* gene in family C. Roman and Arabic numbers define the generation position and number of the individuals in a family pedigree.

Family C: *PNPLA* gene

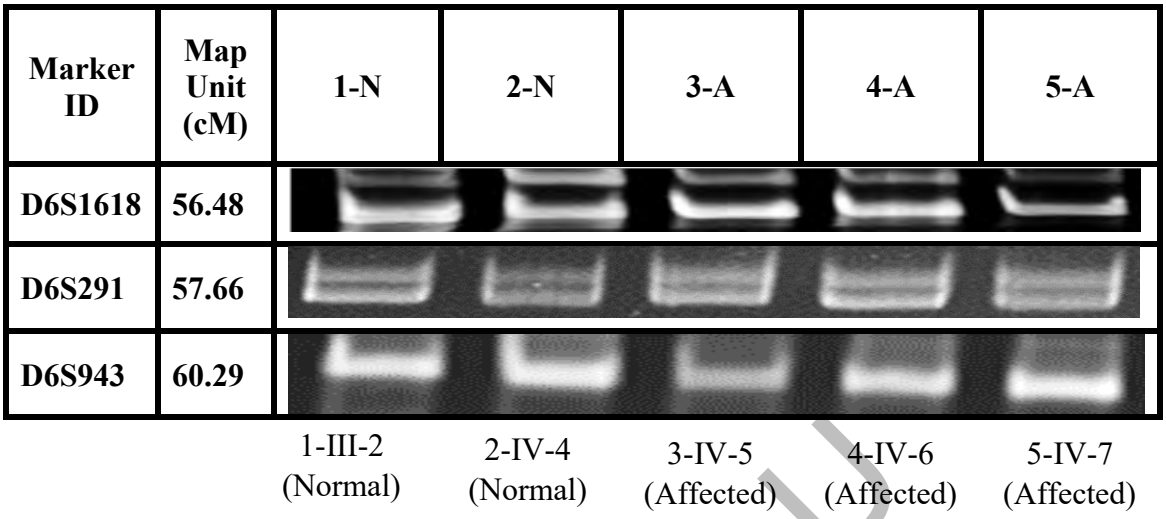


Figure 3.16: Polyacrylamide electropherogram illustrating the pattern of alleles amplified with specific microsatellite markers flanking the *PNPLA* gene in family C. Roman and Arabic numbers define the generation position and number of the individuals in a family pedigree.

Family C: *EXPH5* gene

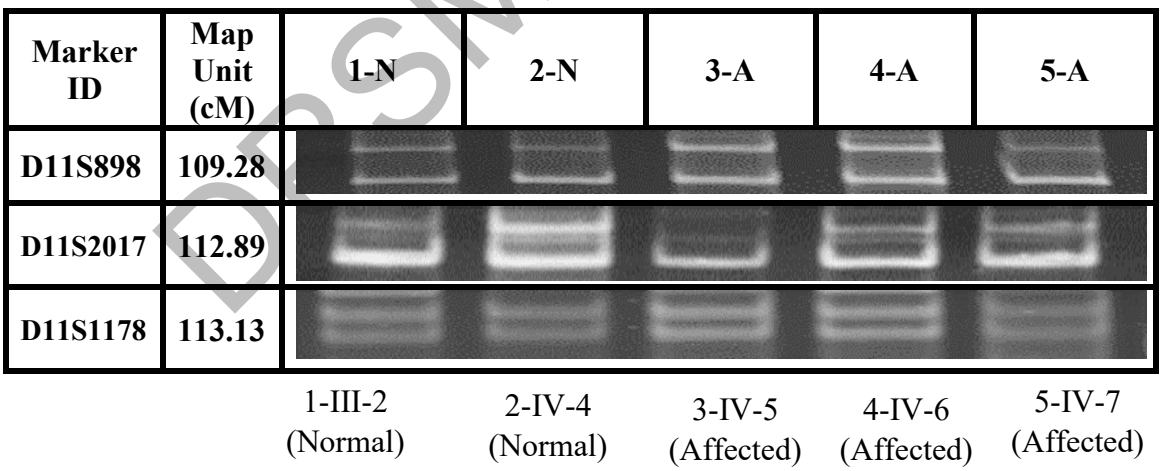


Figure 3.17: Polyacrylamide electropherogram illustrating the pattern of alleles amplified with specific microsatellite markers flanking the *EXPH5* gene in family C. Roma and Arabic numbers define the generation position and number of the individuals in a family pedigree.

Family C: *COL7A1* gene

Marker ID	Map Unit (cM)	1-N	2-N	3-A	4-A	5-A
D3S3649	68.92					
D3S3629	71.04					
D3S3661	71.31					
		1-III-2 (Normal)	2-IV-4 (Normal)	3-IV-5 (Affected)	4-IV-6 (Affected)	5-IV-7 (Affected)

Figure 3.18: Polyacrylamide electropherogram illustrating the pattern of alleles amplified with specific microsatellite markers flanking the *COL7A1* gene in family C. Roman and Arabic numbers define the generation position and number of the individuals in a family pedigree.

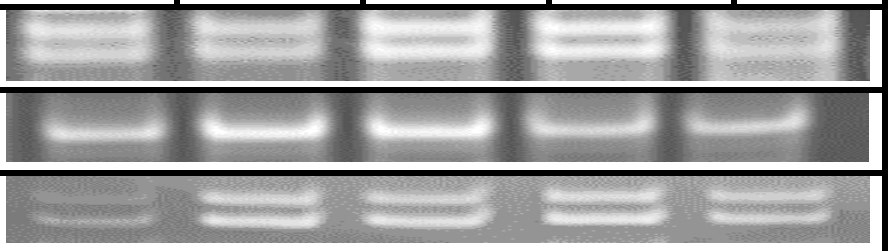
Family C: *KRT5* gene

Marker ID	Map Unit (cM)	1-N	2-N	3-A	4-A	5-A
D12S1677	66.17					
D12S297	67.04					
D12S1604	69.22					
		1-III-2 (Normal)	2-IV-4 (Normal)	3-IV-5 (Affected)	4-IV-6 (Affected)	5-IV-7 (Affected)

Figure 3.19: Polyacrylamide electropherogram illustrating the pattern of alleles amplified with specific microsatellite markers flanking the *KRT5* gene in family C. Roman and Arabic numbers define the generation position and number of the individuals in a family pedigree.

Family C: *KRT14* gene

Marker ID	Map Unit (cM)	1-N	2-N	3-A	4-A	5-A
D17S838	65.53					
D17S649	66.44					
D17S800	67.26					

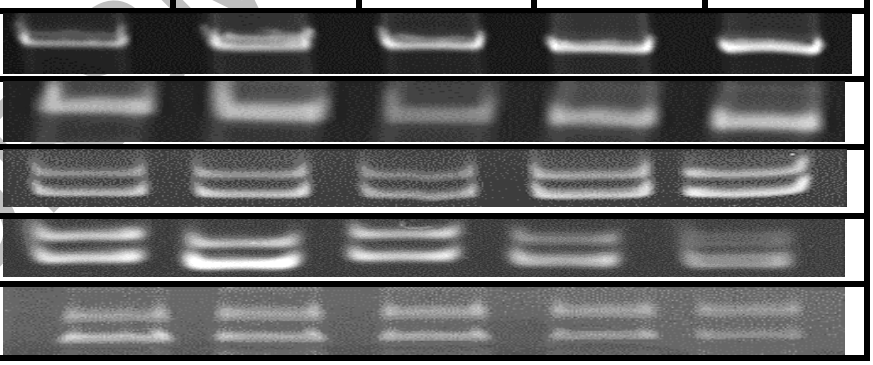


1-III-2 (Normal) 2-IV-4 (Normal) 3-IV-5 (Affected) 4-IV-6 (Affected) 5-IV-7 (Affected)

Figure 3.20: Polyacrylamide electropherogram illustrating the pattern of alleles amplified with specific microsatellite markers flanking the *KRT14* gene in family C. Roman and Arabic numbers define the generation position and number of the individuals in a family pedigree.

Family C: *FERMT1* gene

Marker ID	Map Unit (cM)	1-N	2-N	3-A	4-A	5-A
D20S835	16.16					
D20S905	18.96					
D20S194	19.79					
D20S846	20.8					
D20S602	23.25					



1-III-2 (Normal) 2-IV-4 (Normal) 3-IV-5 (Affected) 4-IV-6 (Affected) 5-IV-7 (Affected)

Figure 3.21: Polyacrylamide electropherogram illustrating the pattern of alleles amplified with specific microsatellite markers flanking the *FERMT1* gene in family C. Roman and Arabic numbers define the generation position and number of the individuals in a family pedigree.

CHAPTER 4

DISCUSSION

DISCUSSION

In the present study, three consanguineous Pakistani families (A-C) with different types of inherited AR skin disorders sampled from Sindh and Punjab provinces of Pakistan were investigated at clinical and genetic level. Their blood was collected for this reason, and DNA was extracted from those samples. To find out the autozygous region in these families, HPMM specific for previously reported genes were used to amplified DNA. If linkage was established, the candidate gene in that region was subjected to sequencing to find out any pathogenic sequence variation. So, the research is conducted in two parts: 1) Homozygosity mapping with HPMM and 2) Sanger sequencing of the putative gene (s) located in the linkage interval.

Family A, presented the clinical features of EB, was sampled from the rural area of the province of Sindh, Pakistan. The clinical features of affected individuals include affected sole and palm sides and white patches. Swelling of joints and knees was found in both the patients. They have thick, dome-shaped nails. Teeth were missing in both affected individuals. For autozygosity mapping, a minimum of 5-6 HPMM were used in all available individuals for genotyping the candidate region of different genes. Seven known genes for EBS were genotyped. These include *FERMT1* (20p12.3), *EXPH5* (11q22.3), *PLEC1* (8q24.3), *COL7A1*(3p21.31), *KRT14* (17q21.2), *KRT5* (12q13.13), and *DST* (6p12.1). Analysis validated family linkage to the *EXPH5* gene located on chromosome 11q22.3. Its sequencing was performed by designing primers for all the coding exons. All six coding exons were sequenced in one of the affected members of family. Sanger sequencing results were unable to reveal any pathogenic variation, suggesting that the variation responsible for diseased phenotypes may be exist in the regulatory region of the *EXPH5* gene.

EXPH5 has six exons encoding for exophilin-5 protein synonym synaptotagmin-like protein lacking c2 domains b (*Slac2-b*) contains 1989 amino acids. It is expressed in keratinocytes (Liu *et al*., 2014). The *slac2b* is a member of the Ras superfamily of guanosine triphosphatases (GTPases). It is a ras-related protein Rab-27B effector protein

and has an N-terminal synaptotagmin-like homology domain (Bare *et al*., 2021). Rab GTPases plays a critical function in controlling secretory pathways, endocytic transport, and intracellular traffic by recruiting proteins to membrane surfaces. This in turn can 1) direct vesicle docking, 2) control organelle motility, and 3) drive cargo collection at membrane surfaces (Liu *et al*., 2014). Variation affecting *EXPH5* protein has been linked to genodermatoses characterized by aberrant skin pigmentation or cornification (Malchin *et al*., 2016). Loss of exophilin-5 causes intraepidermal skin fragility, and disruption of keratin filaments especially in the lower epidermis (McGrath, 2016). To date, in HGMD professional 2021.1, thirteen variants are reported for in the *EXPH5* gene underlying EB.

Family B was identified with hereditary AR congenital ichthyosis (ARCI). The clinical features of affected individuals include ichthyosis with black scaling on the skin. Scales appeared and disappeared from time to time. The skin of the hands was stiff and hard while wrinkled and hard at the joints of affected individuals. DNA samples of six individuals, two affected individuals(IV-4, IV-5) and three healthy (III-4, III-5, IV-7) members were tested to homozygosity mapping by typing HPMM flanking the genes causing ARCI. Minimum 5 or 6 HPMM were selected for genotyping in the main region of known genes/loci. The genes/loci assessed for linkage analysis include *FLG* (1q21.3), *TGM1* (14q12), *ALOX12B* (17p13.1), *ABCA12* (2q35), *LIPN* (10q23.31), *ST14* (11q24.3), *CERS3* (15q26.3). Family B was linked to the HPMM located on chromosome 15q16 harboring *CERS3* gene. Primers for selected six coding exons (1,3, 4, 5, 7, and 9) as most of the sequence variants are reported in these exons were designed. Sanger sequencing of the selected coding exons of affected individual (IV-4) was performed using gene-specific primers. Data analysis of the sequenced ceramide synthase gene revealed a homozygous splice acceptor site variant (c.466-1G>A) in exon 8. The variant was segregated with disease phenotypes among the rest of the family members (III-4, III5, IV-5, IV-6).

The novel homozygous sequence variant (c.466-1G>A) found in the DNA of the affected individuals locates at the splice acceptor site of exon eight of *CERS3* in the present family. Different variant effect prediction tools including varSEAK, Varsome, BayesDel_addAF, DANN, EIGEN, FATHMM-MKL, CADD, and Mutation Taster predict the identified

variant " disease-causing." According to ACMG classification, the identified variant was interpreted as "likely pathogenic" with PP3 (strong), PVS1 (very strong), and PM2 (Moderate). The identified variant is likely to disrupt the normal splicing of its mRNA and hence protein functions. The mis-splicing is predicted to cause skipping of exon eight in the CERS3 mature mRNA thus, leading to abnormal or absence of normal *CERS3* protein function, which significantly impairs the epidermal ceramide synthesis and leads to the patient's ichthyosis skin phenotypes.

The *CERS3* gene is located on chromosome 15q26.3. Its longest transcript is composed of thirteen exons which encode a 46.2kDa protein known as ceramide synthase 3 of 383 amino acids. It has a homeodomain, six transmembrane domains, and a cytoplasmic domain (Mullen *et al*., 2012). It is an integral membrane protein of the ER that produces ceramide from sphinganine and acyl-CoA substrates with a significant preference for ceramides with very long and ultra-long chains (chain length greater than C22) in the epidermis (Radner *et al*., 2013). Ceramides are the SC lipids most prevalent constituents in mammalian epidermis, acting as a barrier against the penetration of harmful microbes and chemicals as well as the uncontrolled loss of water and an absolute requirement for the development of an intact SC (Feingold, 2007; Uchida and Holleran, 2008). *CERS3* gene sequence variations are particularly human rare AR `knockout' alleles cases with predicted loss of function (Saleheen *et al*., 2017). To date, in HGMD professional 2021.1, thirteen sequence variants are reported for ARCI in the *CERS3* gene. All reported patients including our family in this dissertation had consanguineous parents and autosomal recessive genetics.

Affected individuals of family C presented phenotypes of AR ichthyosis. It included dryness, pigmentation, and scales on the skin that become severe in summer. Homozygosity mapping of two healthy members (III-2, IV-2) and three affected individuals(IV-5, IV-6, IV-7) was checked by at least three HPMM for each candidate gene. These genes include *ABCA12* (2q35), *CRS3* (15q26.3), *LIPN* (10q23.31), *ST14* (11q24.3), *PNPLA1* (6p21.31), *EXPH5* (11q22.3), *KRT5* (12q13.13), *COL7A1* (3p21.31), *KRT14* (17q21.2), and *FERMT1* (20p12.3). Results from HPMM for the mentioned

genes/loci showed no linkage to the assessed loci. It suggested exclusion of family from the pre-reported ten genes. Further, it is highly likely that a novel gene participates in the pathogenesis of the family.

In conclusion, the study, presented in the thesis, attempt was made to investigate the skin disorders pathogenesis at clinical and genetic levels in three families. Following characterization at clinical levels, genotyping and Sanger sequencing were used for the characterization of the families at the genetic level. A novel splice site variant was identified in the *CERS3* gene only in family B. For families A and C, it is recommended to use exome sequencing to search for the responsible gene. Overall, the study will aid to provide further insight into the genetic characterization of inherited ED. Clinical and genetic research main goal is to establish phenotype-genotype correlation. Once the genes linked to these rare diseases are identified, gene therapy would be a convenient approach for the diseased treatment. Studies involving searching for the causative genes and variants facilitate genetic counseling of not only the families involved but also other families carrying similar features in the local population.

CHAPTER

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