DNA Sequencing of *TGM1* Gene in Families Affected with Autosomal Recessive Congenital Ichthyosis (ARCI)

Riaz Ahmad

Reg. No: 02271513009

A thesis submitted in partial fulfillment of the requirements of Quaid-i-Azam University for the degree of Master of Philosophy in Biotechnology

Department of Biotechnology
Faculty of Biological Sciences
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Certificate

This thesis submitted by Riaz Ahmad is accepted in its present form by the Department of Biotechnology, Faculty of Biological Sciences, Quaid-i-Azam University Islamabad, as satisfying the thesis requirement for the degree of Master of Philosophy in Biotechnology.

Supervisor: _____

Muhammad Naeem, Ph.D.
Associate Professor
Department of Biotechnology
QAU, Islamabad

External Examiner: _____

Prof. Mahmood Akhtar Kayani
Department of Biosciences
COMSATS Institute of
Information Technology,
Islamabad

Chairman: _____

Muhammad Naeem, Ph.D.
Associate Professor
Department of Biotechnology
QAU, Islamabad

Date:

Dedication

To my parents

Declaration of Originality

I hereby declare that the work accomplished in this thesis is my own research effort carried out in the Medical Genetics Research Laboratory, Department of Biotechnology, Quaid-i-Azam University Islamabad and is written and composed by me.

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LIST OF ABBREVIATIONS

%	Percentage
µl	Microliter
µg	Microgram
µM	MicroMolar
° C	Degree Celsius
ABCA12	ATP-binding cassette subfamily A member 12
Acral SHCB	Acral self-healing collodion baby
ALOX12B	Arachidonate 12-lipoxygenase
ALOXE3	Arachidonate lipoxygenase 3
ARCI	Autosomal recessive congenital ichthyosis
ATP	Adenosine triphosphate
Bp	Base pair
BSI	Bathing suit ichthyosis
C	Cytosine
CCE	Cornified cell envelope
CERS3	Ceramide synthase 3
CIE	Congenital ichthyosiform erythroderma
CYP4F22	Cytochrome p450 family 4 subfamily F
DNA	Deoxy ribonucleic acid
EDTA	Ethylene diamine tetra acetic acid

FLG	Filaggrin
G	Guanine
HI	Harlequin ichthyosis
His	Histidine
IV	Ichthyosis valgaris
LI	Lamellar ichthyosis
LIPN	Lipase family member N
mA	Milliampere
MEDOC	Mendelian disorder of cornification
Mg	Milligram
mM	Millimolar
NaCl	Sodium chloride
Ng	Nano gram
nM	Nano mole
OMIM	Online Mendelian inheritance in man
P	Short arm of chromosome
PCR	Polymerase chain reaction
PNPLA1	Patatin –like phospholipase domain 1
Pro	Proline
Q	Long arm of chromosome
RPM	Revolution per minute
RXLI	Recessive X-linked ichthyosis
SC	Stratum corneum

SDR9C7	Short chain dehydrogenase/reductase family 9C,member 7
Sec	Second
SHCB	Self-healing collodion baby
SNP	Single nucleotide polymorphism
STS	Steroid sulfatase
T	Thymine
Taq	Thermus aquaticus
TBE	Tris borate EDTA
TE	Tris EDTA
TGase	Transglutaminase
TGM1	Transglutaminase 1
TTD	Trichothiodystrophy

ABSTRACT

Autosomal recessive congenital ichthyosis (ARCI) is a genetically and clinically heterogeneous class of non-syndromic ichthyosis that consists of three major types, harlequin ichthyosis, lamellar ichthyosis and congenital ichthyosiform erythroderma. Its minor sub-classes are bathing suit ichthyosis, self-healing collodion baby and acral self-healing collodion baby. ARCI is generally characterized by epidermal scales accompanying generalized erythema in some cases, hyperkeratosis, defective trans-epidermal water loss, collodion membrane, ectropion, eclabium and sometimes alopecia. The prevalence rate of ARCI is almost 1:200000 individuals and 30-50% cases are due to mutations in *TGM1*.

In the present study, three families, DER48, DER49 and DER58 affected with ARCI were studied. All these families were recruited from Khyber Pakhtunkhwa Province of Pakistan. Patients of DER48 and DER49 family showed the clinical features of congenital ichthyosiform erythroderma (CIE), while patients of the family DER58 present features of bathing suit ichthyosis. Keeping in view the high prevalence rate of *TGM1* mutations in ARCI patients, the families were screened for mutations in *TGM1* through Sanger sequencing method. Two heterozygous mutations (c.550C>T) and (IVS5-1G>A) were detected in the affected individuals of DER48 which are the likely cause of ichthyosis in this family based on the segregation analysis. Whereas, no pathogenic mutations in *TGM1* gene were found in DER49 and DER58.

Chapter 1

INTRODUCTION

Skin is the largest organ of the body constituting an almost 15% of total body weight of an adult. Epidermis, dermis and subcutaneous are three major layers of the skin as shown in the figure 1.1. Its main function is to protect the body from chemical, physical and biological agents and to avoid the loss of excess water and thermoregulation of the body (Kanitakis, 2002).

1.1 Epidermis

Epidermis contains group of cells (keratinocytes) that are essential for the protection of human skin while dermis, is basically composed of structural protein known as collagen. Panniculus or subcutaneous tissue is the third layer of skin, which contains fat cells or lipocytes. All these layers are different in thickness, in various parts of the body. For example, the epidermis layer of the eyelid is thinner (0.1 mm) than soles and palms of hands (1.5 mm) (James et al., 2006). Other than melanocytes, epidermis also consists of merkel and Langerhans cells. The following are the four layers of the epidermis: stratum basale (basal cell), stratum spinosum (squamous cell), stratum granulosum (granular cell), and stratum corneum (horny or cornified cell) (James et al., 2006). Langerhans, melanocytes, merkel and keratinocytes are four types of epidermis cells and these are responsible for the following functions. Langerhans cells are mostly involved in defense system or immunity while for touch sensation, merkel cells play a vital role. Melanocytes are types of cells that produce melanin. Keratinocytes produce keratin proteins having a protective role in the skin. Keratinocytes and melanocytes make 90% and 8% of the epidermis, respectively. Keratinocytes cells continuously differentiate after moving from lower layer i.e. stratum basale to stratum corneum. In the epidermis, no blood vessels are present for its own function and depend on blood vessels of dermis for metabolic processes, removal of wastes, oxygenation and nutrition (Gaboriau and Murakami, 2001).

1.1.1 Stratum basale

This layer consists of keratinocytes in a row form and makes a junction between epidermis and dermis. In this layer, the active epidermal stem cells are present that undergo division actively and

maintain the high nucleo-cytoplasmic ratio. Two genes highly expressed in keratinocytes, K5 and K14 encoding for keratin, a filamentous protein that plays an essential role in the protection against microorganism, chemicals and heat (McLafferty et al., 2012).

1.1.2 Stratum spinosum

It is a layer of the epidermis that consists of different cells based on structure, size and cellular properties and these cells exist in various locations within the layer. In upper layer (stratum spinosum) lamellar granules containing large cells while in lower layer polyhedral cells containing oval nuclei are present. These cells become flattened when pushed toward the surface of the human skin. The layer of these cells is responsible for the synthesis of keratin (protein) and intracellular adhesion of the stratum corneum (Haake et al., 2001; Kolarsick et al., 2011).

1.1.3 Stratum granulosum

This layer is composed of flattened cells having a large number of keratohyalin granules. These molecules or granules contain different proteins like keratin10, keratin1, loricrin, profilaggrin and cysteine-rich proteins. All these proteins are essential for keratinization process to provide barrier function of the skin. Formation of the cornified envelope is stimulated by this layer with the help of secretion of lamellar bodies and condensation of keratin filaments (McLafferty et al., 2012).

1.1.4 Stratum corneum

The outermost layer of the epidermis is composed of corneocytes that are terminally differentiated, large polyhedral and flattened cells filled with keratin. Corneocytes lack nucleus and organelles but at the last stage of differentiation, cornified cell envelope is formed that works as a barrier of the skin (Madison, 2003). This layer is essential to provide protection against different foreign substances and maintains the balance of water level in the skin (Harding, 2004). Nearly, 90% of skin barrier function is due to stratum corneum which was confirmed with biochemical and morphological studies. Two components are essential to provide barrier function both are keratinocytes derived structures: the corneocytes and the inter-corneocytes lipids (in form of bilayer); the first one provides structural support to stratum corneum (SC) while bilayer is for transport of substances to the external environment (Lee et al., 2006; Darlenski et al., 2011). The major elements of SC involved in skin barrier function are intercellular lipid layers, cornified cell

envelop and degradation product like filaggrin/keratin as shown in figure 1.2 (Akiyama, 2011). Defective intercellular lipid layer may form due to abnormal keratinocyte lipid metabolism, secretion and transport. Lipoxygenase-3, 12 R- lipoxygenase, ATP-binding cassette (ABC) transporters, cytochrome P4, steroid sulphatase and ichthyin are the molecules that are essential for the synthesis of this lipid layer of stratum corneum. Defect in these molecules leads to the abnormal formation of intercellular lipid layer and as a result the skin becomes scaly and dry (Madison et al., 1987). In an interior surface of cell membrane, cornified cell envelop is formed. Transglutaminases are the enzymes that express in the epidermis and mediate cross-linked among different proteins like loricrin, involucrin and small proline-rich proteins. Specially, transglutaminase 1 plays a vital role in the formation of cornified envelop. Scaling and dryness of the skin may be caused due to deficiency of this enzyme (Kalinin et al., 2002). Keratin 2 (*KRT2*), filaggrin (profilaggrin), keratin 1, and keratin 10 are the molecules that are required for the formation of keratohyalin granules. For the integrity of corneocytes these molecules are important. However, deficiencies of these molecules may also cause dry and scaly skin (Porter and Lane, 2003).

1.2 Ichthyosis

The *ichthys* word is derived from Greek which means "fish" and refers to the scaling of the human skin (DiGiovanna, 2004). Ichthyosis is a group of disorders that is genetically and clinically heterogeneous. A single gene is involved in these types of cornification diseases, also known as Mendelian disorders of cornification (MEDOC). Mostly, skin and its derivative structures are involved in this group of disorders (Williams and Elias, 1986). Epidermis provides barrier function to the mammalian skin in which stratum corneum layer has a unique role. When a change occurs at the molecular level in corneocytes, it leads to defects in the synthesis and functioning of SC. The resulting defect is known as cornification disorder (Williams and Elias, 2000). For epidermal barrier homeostasis, 50 different genes have been reported (Williams and Elias, 2000). The epidermis is a self-renewing and dynamic tissue in which the old cells of epidermis undergo desquamation, a process in which shedding of the outer layer of the skin occurs, and new corneocytes replace the old cells by cell division. For the smooth appearance of human skin, the equilibrium between cell division and desquamation is essential. Scaling and drying of the skin may occur because of the disturbance in these two (desquamation, cell division) normal processes

of the life (Menon et al., 2012). Rapid active cell division or delay in the desquamation are the main causes of various degrees of dryness and scales of the human skin. These also constitute the medical symptoms of ichthyosis (Oji and Traupe, 2006).

1.3 Classification of ichthyosis

According to the recent classification for ichthyosis, there are two types of inherited ichthyosis i.e. syndromic and non syndromic. In syndromic form, skin and other organs are involved while in non syndromic ichthyosis the phenotypic manifestation is only limited to the skin (Oji et al., 2010). The prevalence rate of syndromic ichthyosis is lower than non-syndromic ichthyosis (Dreyfus et al., 2014).

1.3.1 Syndromic ichthyosis

This subtype is the rare form of the ichthyosis in the skin as well as other organ or even systems, for instance, deafness-related ichthyosis, hair abnormalities and ichthyosis-related neurological signs. The second most common system that is involved after skin is nervous system in the syndromic form of ichthyosis, therefore, neurological signs are also significant evidence for diagnostic purposes (Saral et al., 2016). Major syndromes in which hair, nervous system and other system are affected are discussed below.

1.3.1.1 Trichothiodystrophy (OMIM: 601675)

The following organs are affected in this heterogeneous disorder; hair, skin and central nervous system. Its pattern of inherence is autosomal recessive and the phenotype of this disorder lacks sulfur proteins of hair shaft leading to brittle hair (Price et al., 1980). Based on clinical features, there are two types of trichothiodystrophy (TTD); congenital and non-congenital ichthyosis TTD. Congenital one is also known as Tay's or (P) IBIDS syndrome, which stands for photosensitivity, ichthyosis, brittle hair, impaired intelligence, decreased fertility and short stature and its causative gene is *ERCC2*/*XPD* which encodes for subunits of transcription factor TFIIH. This complex (DNA repair complex) composed of ten proteins and due to instability, may cause photosensitivity in half patients. Collodion membrane or congenital ichthyosiform erythroderma (CIE) is present at the time of birth frequently, and later these manifestations improve with age and become fine and generalized ichthyosis (Faghri et al., 2008; Morice-Picard et al., 2009). Non-congenital

trichothiodystrophy is without the involvement of mutation in nuclear proteins and its phenotype does not show photosensitivity but fine whitish scales and mild erythema (Faghri et al., 2008).

1.3.1.2 Netherton syndrome (OMIM: 256500)

The most common syndromic ichthyosis is caused due to mutation in *SPINK5* gene which is located on chromosome 5q32. Its prevalence rate is 1/100000 and follows the autosomal recessive mode of inheritance. Usually, the phenotype is born with CIE manifestation while later the hair became brittle and short. In a very small number of cases the phenotypes show severity. The additional frequent features are atopy, pruritus, increased IgE, and hyper level of eosinophils (Bitoun et al., 2002).

1.3.1.3 Sjogren-Larsson syndrome (OMIM: 270200)

This syndrome is less severe and prominently nervous system is involved in it. It also follows autosomal recessive pattern of inheritance. *ALDH3A2* mutations are the reason of this disorder which encodes for dehydrogenase (fatty aldehyde dehydrogenase) enzyme responsible for oxidation of aldehydes that results from metabolism of the lipid. Accumulation of alcohol in the tissues occurs due to defective dehydrogenase because for lipid metabolism this enzyme is important (De Laurenzi et al., 1996). Spastic paraparesis, a neurological and a mild form of epilepsy are also the conditions that were experienced in Sjogren–Larsson syndrome (Rizzo, 2007).

1.3.1.4 Keratitis-ichthyosis-deafness syndrome (OMIM: 148210)

Keratitis ichthyosis deafness is a rare neonatal syndrome that follows autosomal dominant mode of inheritance. The *GJB2* mutations is a cause of this disorder which encodes for connexin 26. Erythrokeratoderma and follicular occlusion are the most frequent symptoms while keratitis improves after infancy and exists in maximum number of the patients. In these individuals, vision is also affected severely (Mazereeuw-Hautier et al., 2007).

1.3.2 Non-syndromic ichthyosis

When abnormal skin is the only phenotypic organ and all other systems/organ are not affected, such type of ichthyosis will be non-syndromic ichthyosis e.g. common ichthyosis (ichthyosis

vulgaris, recessive X-linked ichthyosis), autosomal recessive congenital ichthyosis, keratinopathic and some other forms (Oji et al., 2010).

1.3.2.1 Common ichthyosis

Ichthyosis vulgaris (IV) and recessive X-linked ichthyosis (RXLI) are classified as common ichthyosis by Oji et al in 2010 because its prevalence rate were higher than other types of ichthyosis. Frequently, their symptoms appear later in life than other types of inherited ichthyosis (Oji et al.,2010).

1.3.2.1.1 Ichthyosis vulgaris (OMIM: 146700)

Ichthyosis vulgaris is the most common type of non syndromic ichthyosis. Its prevalence rate is 1:100-250 and follows an autosomal semi-dominant mode of inheritance. After 2 months the symptoms start while in term of severity it is a mild form of inherited ichthyosis and improves in summer. Mild scaling, inflammation and pruritus, dryness and atopic manifestations of the skin are the mildest conditions in ichthyosis vulgaris (Wells, 1966; Oji et al., 2010). *FLG* is located at the long arm of autosomal chromosome 1q21.3 and consists of three exons. The first two exons are very small in size, 15 bps and 159 bps, respectively, while the third exon is 12.7 kb in size (Oji et al., 2010). For this disease, mutations in *FLG* (OMIM 135940) encoding for abnormal filaggrin, a protein that is essential for the synthesis of barrier function of the skin to enable terminal differentiation of epidermis. In last the stage of differentiation, this protein cross-links with cornified cell layer to form a complex insoluble barrier structure to defend against different agents of the external environment and also retains water level in the epidermis (Candi et al., 2005; Naz and Samdani, 2011). Hence, it is an important epidermal protein for the barrier function and lower expression of this gene leads to mildly dry and scaly skin which is the main clinical features of ichthyosis vulgaris (Gruber et al., 2011).

1.3.2.1.2 Recessive X-linked ichthyosis (OMIM: 308100)

It is also a common type after ichthyosis vulgaris its prevalence rate is 1: 2000-6000, and mode of inheritance is recessive. Based on its pattern of inheritance chances of males affection are common than females. Polygonal, brownish dark scaling and drying of the skin are the common phenotypic manifestations of X-linked recessive ichthyosis. At the time of birth or after 2 months, mild collodion along with thick scaling or erythrodermic symptoms may appear. In most cases, the

clinical symptoms improve in summer but not recover throughout the life (Oji et al., 2010). The color of scales in XLRI is brown dark or light grey and in size may be fine or mostly large. Scales cover many parts of the skin but particularly neck is highly affected, however, the soles and palms remain unaffected (Elias et al., 2014). Molecular analysis has shown that *STS* gene is involved in the phenotypic manifestation of XLRI. This gene is present at X chromosome and its position is 22.31, which encodes for steroid sulfates enzyme. *STS* gene is composed of 10 exons and these encode for 583 amino acids and the size of this gene is 6521bp long. Almost in 90% cases of RXLI, *STS* deleterious mutation is the main reason to form the defective enzyme. Normal desquamation process is disturbed due to mutation in *STS* leading to formation of abnormal proteins which accrues in the SC and as a result scaling and drying of the skin occurs (Elias et al., 2014).

1.3.2.2 Autosomal recessive congenital ichthyosis (OMIM: 190195)

It is a form of ichthyosis which includes lamellar ichthyosis (LI), congenital ichthyosiform erythroderma (CIE) and harlequin ichthyosis (HI). All these are non-blister forming and non-syndromic forms of ichthyosis. Harlequin ichthyosis is more fatal and severe form than CIE and LI (both are mild forms of ARCI). Overlapping clinical symptoms common in all types of ARCI are palmar plantar hyperkeratosis, collodion membrane at birth, ectropion, alopecia, hypohidrosis, eclabium, erythema and epidermal scaling of the skin (Herman et al., 2009). Only skin is involved and scales appear on entire body but at the birth time most patients are born as collodion babies. Later, collodion membrane of collodion babies gradually changes into scaling and erythema of the skin (Oji et al., 2010; Rodríguez-Pazos et al., 2013). The prevalence rate of ARCI is less than 1:100000 (Traupe et al., 2014) and it is almost 1:200000 (Bale and Doyle, 1994). The occurrence rate of ARCI in the Europe is 1: 200000 whereas in the USA its rate is 1:200000-300000 subjects (Bale and Doyle, 1994). Mutations in eleven genes (*TGM1, ALOXE3, ABCA12, ALOX12B, NIPAL4, CYP4F22, LIPN, ST14, PNPLA1, CERS3 and SDR9C7*) are related with ARCIs (Shigehara et al., 2016). *TGM1* is the most common mutated gene as compared to other genes (*ABCA12, ALOX12B, ALOXE3, CERS3* and *CYP4F22*) of ARCI (Fischer, 2009).

Other than three main phenotypes of ARCI minor subtypes are bathing suit ichthyosis (BSI), self-healing collodion baby (SHCB) and acral self-healing collodion baby (ASHCB) (Takeichi and Akiyama, 2016). LI and CIE are clearly defined phenotypes but the exact diagnosis of each type of ARCI is challenging, due to overlapping of clinical symptoms (Williams and Elias, 1985). LI

is commonly congenital and after few days of birth, the collodion membrane of this phenotype changes into a dark, lamellar scale ((plate (amore) like)). The frequent cause of LI is mutations in *TGM1* which encodes transglutaminase 1. CIE patient can also be born as a collodion baby. Fine white scales with generalized redness is common in this type of ARCI. *TGM1* mutations are also reported in some cases of CIE (Laiho et al., 1997). Clinical features of both LI and CIE overlap with each other because of common genes (Bale et al., 1996). In 2001, Akiyama et al reported two mutations (frame shift and missense mutation) in *TGM1* for the patient that was showing all clinical features of congenital ichthyosiform erythroderma. Erythrodermic as well as scaly skin (fine, light brown or grey scales) were the main features in the patient.

In addition, Choate et al in 1998, showed reduce the activity of transglutaminase 1 in CIE phenotypes. Out of 100 cases of ARCI, 30-50 cases may be due to mutation in *TGM1* (transglutaminase 1) (Richard and Bale, 2014; Liu et al., 2015), while 17 % cases of ARCI are due to *ALOXE3* and *ALOX12B*. Moreover, 12%, 8% and 5% cases of the ARCI are due to *NIPAL4*, *CYP4F22* and *ABCA12* genes, respectively. Other genes like *CERS3*, *LIPN* and *PNPLA1* are the rarely mutated genes in the case of ARCI (Richard and Bale, 2014).

1.3.2.2.1 Major types of autosomal recessive congenital ichthyosis

1.3.2.2.1.1 Harlequin ichthyosis (HI)

Harlequin ichthyosis (harlequin fetus) (OMIM: 242500) is an infrequent and severe type of autosomal recessive congenital ichthyosis. The survival rate of neonates having HI is frequently lower after birth, but several cases have been reported that show long term survivals. The pattern of inheritance is assumed to be autosomal recessive. *ABCA12* is a gene in which homozygous mutations lead to insufficiency of the transporter (Epidermal lipid transporter) and cause hyperkeratosis and abnormal barrier functions in the most affected individuals (Habib et al., 2011). Its prevalence rate is 1:300000 (Belengeanu et al., 2009). Eclabium, ectropion, flattened ears and scales (thick and large plate-like) over the whole body are severe features at birth causing a solidified skin surface (Akiyama et al., 2005). The main gene linked with HI is *ABCA12* (Thomas et al., 2006). However, its mutated forms have also been reported in lamellar ichthyosis (Lefèvre et al., 2003) and congenital ichthyosiform erythroderma (Sakai et al., 2009). The *ABCA12* (adenosine triphosphate-binding cassette A12) gene was first time identified in 2005 and its mutations were linked to HI (Akiyama et al., 2005). It main function is to bind adenosine

triphosphate and helps in transport of many molecules through the cell membrane because it is from the ABC transporter family (Annilo et al., 2002). The position of this gene is 2q35 that consists of 53 exons and 206 kbps long. It encodes for ATP binding cassette that is a transporter molecule and works as a keratinocyte trans-membrane lipid transporter. In HI phenotype, stratum corneum becomes thicker due to defective lamellar granule-mediated lipid transport in the upper epidermal (keratinocyte) layers because of sever deficiency of *ABCA12*. For this layer synthesis, the transport of lipid into lamellar granules and its metabolism is necessary. *ABCA12* protein plays vital role in lipid transport metabolism. Loss of function of the *ABCA12* molecule in lamellar granules causes the collection of lipids molecules and results in abnormal intercellular lipid layer.

Deleterious mutations in *ABCA12* are frequent as well as the main reason of abnormal formation of this transporter molecules. Intercellular lipid layer synthesis is essential for the barrier function of the epidermis. When any type of disrupting occurs, it results in the loss of barrier activity and severe hyperkeratosis (Akiyama et al., 2005).

1.3.2.2.1.2 Lamellar ichthyosis (LI)

The phenotypic manifestation of lamellar ichthyosis (OMIM: 146750) includes, brown or dark grey colored large scales on the whole skin surface. In addition, collodion membrane at birth, mild to moderate everting of eyelid and lips, and palmoplantar keratoderma (PPK) are the most common clinical features of LI. Nail abnormality and alopecia are also seen in some cases while hair and teeth are mostly observed normally. All the above features rarely improve with age (Williams and Elias, 1985; Akiyama et al., 2003). The prevalence rate of LI is 1 out of 200,000 individuals and it is the most frequent in those families having consanguineous marriages. It may be fatal for newborn babies as compared to adults according to the higher risk of death rate because the pathogenicity of this disease ranges from mild to severe (Deshmukh, 2014). In the mild form of LI, large thick and dark scales only appear on certain body parts (foreheads, trunk, upper arms, and lower legs). While sometimes, face and the extremities seem normal and no hyperkeratosis on soles or palms is obvious. In some milder forms of LI, tiny, white to grey scales are occasionally seen on the extremities, neck and other remaining parts of the body (Laiho et al., 1999). Clinical heterogeneity was observed in phenotypes of LI and CIE (Williams and Elias, 1985). There are two main dissimilarities between both phenotypes that are the size and color of the scales and strength of erythroderma. These are the significant manifestations which point out differences in

the two phenotypes (LI and CIE) (Akiyama, 1998). Usually, in LI the color of scales are dark-brown and in size, these scales are thick on the whole body while in the less severe form of LI, these scales limited to few body area containing forehead, upper arms, lower legs and trunk. In case of CIE, the scales are thin, white to grey and in powder form on the entire body surface along with mild to severe erythroderma. Sometimes, the scales present on the lower legs are observed like LI scales. Therefore, the clinical features are sometimes confusing but these manifestations can be differentiated to recognize the exact class of ARCI (Akiyama, 1998; Laiho et al., 1999; Akiyama et al., 2001). Cornified cell envelop is the most important for the barrier function of epidermis so defect in this layer is the main cause of LI phenotype. This layer is formed at the inner site of the plasma membrane. Different protein molecules (involucrin, loricrin) are present in this CCE layer and their cross-linking is catalyzed by transglutaminases to make CCE the most insoluble component of the epidermis. So CCE plays a vital role as water barrier of the skin (Yoneda et al., 1992). The causative genes of LI include *ALOX12B, ALOXE3, NIPAL4, ABCA12, CERS3, CYP4F22, LIPN, PNPLA1* and *TGM1*. Among these genes, *TGM1* is the most commonly mutated gene for LI (Radner et al., 2013; Rodríguez-Pazos et al., 2013). *TGM1* mutation spectrum was expanded by Terrinoni et al in 2012 by screening coding sequence of the gene in 16 patients of lamellar ichthyosis. Two putative splice sites, two nonsense mutations and two transition/ transversion were found in this case study. Most of them were present in the catalytic core domain of *TGM1* (Terrinoni et al., 2012).

1.3.2.2.1.3 Congenital ichthyosiform erythroderma (OMIM: 242100)

In this type of ARCI, typically baby born as a collodion baby and later gradually this mild membrane changes into the erythrodermic and scaly skin with lack of blister formation (Williams and Elias, 1985). The scales are whitish or light grey covering the whole skin surface along with mild eclabium and ectropion. Hearing, teeth and hair are approximately normal in the most cases while palms and soles are severely affected due to severe hyperkeratosis. Sometimes, scaling and erythroderma improve with age while in some studies these features became severe in the first two to three years. Mode of inheritance of this disorder is autosomal recessive and affecting 1-200 000 individuals (Akiyama et al., 2003). CIE phenotype also includes the features like contractures of the digits and painful fissures while in severe form loss of eyebrows, ectropion and eclabium have been reported (Richard and Bale, 2014). Eight genes (*TGM1, PNPLA1, NIPAL4, CYP4F22,*

CERS3, ALOXE3, ALOX12B and *ABCA12*) have been reported for CIE (Grall et al., 2012; Oji et al., 2010; Radner et al., 2013). The most mutated gene for CIE phenotype was *ABCA12* in Japanese population while for Malaysian population, *TGM1* is the most frequent (Sakai et al., 2009). Defects either in the cornified cell envelop or keratinocytes lipid metabolism leads to the CIE phenotype. Hence, two type of genes, one coding for the synthesis of enzyme-linked with cornified cell envelop and another coding for molecules associated with keratinocytes lipid metabolism (Kawashima et al., 2005). The possible method for diagnosis of CIE might be succeeded via Sanger sequencing (conventional method) of genomic DNA or cDNAs fragments, but this one is laborious to find the mutations in the candidate genes. Instead of it, whole exome (Takeichi et al., 2015) as well as target-captured DNA analysis (Scott et al., 2013) of genes are methods that can efficiently detect mutations involved in ichthyosis. For example, Inoue et al in 2015 reported a case of CIE through next generation sequencing (NGS) and the mutation was found in *ABCA12*. Therefore, NGS is important for exact diagnosis in a short time.

Genetically, ARCI is heterogeneous but the *TGM1* is the most frequently mutated gene for it. So this gene should be the key for the screening of mutations in ARCI disorder (Farasat et al., 2009). The same study also revealed that *TGM1* mutations in ARCI patients having features such as alopecia, collodion membrane at the time of birth and eye abnormalities are observed four times more than other genes of ARCI.

1.3.2.2.2 Minor types of autosomal recessive congenital ichthyosis

These are the less common subtypes of ARCI which include; Bathing suit ichthyosis (BSI), self-healing collodion baby (SHCB) and acral SHCB. For these phenotypic variants, mutations in *TGM1* have been reported (Oji et al., 2006; Mazereeuw-Hautier et al., 2009).

1.3.2.2.2.1 Bathing suit ichthyosis (BSI)

Bathing suit ichthyosis word was first time used in South Africa for the phenotype of LI having strange phenotypic manifestation. A minor type of ARCI, in which neck, the skin of the head, trunk and upper limbs are affected by lesions while extremities and face are not affected with any type of scales or lesions (Schultz, 1994). *TGM1* is responsible for this minor variant of ARCI. Genetic, biochemical and ultrastructural studies have confirmed that *TGM1* is a causative gene for BSI patients (Oji et al., 2006). Twenty different missense mutations in *TGM1* have been reported

for BSI phenotypes; 9 out of 20 mutations are just associated with BSI patients while the 11 missense mutations were linked to ARCI and BSI, although, no clear genotypic-phenotypic correlation is present. Thus in *TGM1*, mutations can cause both ARCI and BSI phenotypes. A similar mutation can express in a different way in two different environments (Kit et al., 2009; Benmously-Mlika et al., 2014). The BSI phenotype is closely linked with temperature. It is also proposed that bathing suit ichthyosis is a temperature sensitive phenotype (Oji et al., 2006). The operative activity of transglutaminase 1, is sensitive to temperature at more than 33°C (Kit et al., 2009). In 2010, Hackett et al expanded the spectrum of mutation in *TGM1* because he reported pathogenic mutations for three BSI phenotypes. These phenotypes present collodion membrane at birth, thick dark scales or lamellar scales on the scalp and trunk with sparing of the limbs and central face. Among these phenotypes, one patient showed prominent scaling in the axillae and also in auditory canals because of too much desquamation. These both parts of the body have a higher temperature so the functional activity of transglutaminase 1 is reduced.

1.3.2.2.2 Self-healing collodion baby

Also known as self-improving collodion baby, characterized by collodion membrane at the time of birth and then after three months clear visible scales appear on the body of an infant. Out of 100 cases of ARCI, almost 10 cases are of SHCB. This one is also a minor variant of ARCI (Akiyama et al., 2003; Oji et al., 2010). *TGM1, ALOX12B* and *ALOXE3* are responsible for SHCB because these have been reported with this clinical phenotype (Raghunath et al., 2003; Vahlquist et al., 2010).

1.3.2.2.2.3 Acral self-healing collodion baby

In a case study, the proband was born as a collodion membrane and this membrane was limited to the extremities. After 3 weeks the skin become normal while a sister of the proband was also born as collodion baby but with age, her skin condition changed into lamellar ichthyosis. This subtype of ichthyosis was only reported in 2009 first time in which *TGM1* mutations was the reason of these phenotypes. Compound heterozygous (p.Arg396His and p.Val359Met) mutations were reported in the proband and elder sister of proband was also compound heterozygous for c.1922_1926+2delGGCCTG and p.Arg396His. *TGM1* encodes for transglutaminase 1 and is essential for epidermis barrier function so later structure modeling for these three mutations was also performed. Structure modeling of p.Val359Met mutation showed minor damage of protein as

compared to p.Arg396His, thus the expression level of protein reduced due to both mutations. The first mutation showed residual activity while the p.Arg396His mutation displayed loss of activity. Therefore, the proband became normal later in life because the activity of enzyme is moderate while in the neonatal stage the activity of transglutaminase was low (Mazereeuw-Hautier et al., 2009).

1.3.2.3 Genes involved in ARCI

Mutations in eleven genes (*TGM1, ALOXE3, ALOX12B, NIPAL4, ABCA12, CYP4F22, LIPN, ST14, PNPLA1, CERS3 and SDR9C7)* are related with the phenotypic manifestation of ARCI (Shigehara et al., 2016).

1.3.2.3.1 *TGM1*

TGM1 (OMIM: 190195) is located on chromosome 14, at position 14q11.2 and comprises of 15 exons responsible for encoding 817 amino acid of transglutaminase 1, an enzyme that is required for formation of cornified cell envelope (Kim et al., 1992). Transglutaminases are present in the body in different tissues (liver cells, red blood cells, keratinocyte, chondrocytes and epidermal transglutaminase in the skin) and body fluids, for example, coagulation factor XIII in the blood (Aeschlimann, 1994). Transglutaminase 1 catalyzes the cross linking between precursor proteins in cornified envelope (Fischer, 2009). It assembles these proteins by forming N-(glutamyl) lysine isopeptide bonds (Zambrano et al., 2014). This enzyme forms cross-links or strong bond among the proteins molecules and leads to form cornified cell envelope while these cross linking is important for its maintenance and stability (Aeschlimann, 1994). Among the precursor proteins of the cornified cell envelope, transglutaminase 1 catalyzes the formation of e-(g-glutamyl) lysine cross-links. In the end stage of epidermal differentiation, this cornified cell envelope is formed which is insoluble and thick (15-20 nm) (Rice and Green, 1977). Loricrin, involucrin and proline rich proteins are precursor of the cornified cell envelope (Rice and Green, 1979; Mehrel et al., 1990; Hohl et al., 1995).

To date, 180 mutations in *TGM1* have been reported however all these are closely linked with ichthyosis including splicing, nonsense/missense, deletions (small and gross), regulatory, and small insertions. The majority of these mutations are single-base change (HGMD;.http:// www.hgmd.cf.ac.uk). Epidermal TGase-1 comprises of three domains, N-terminal, a catalytic core

and C-terminal domain while the last domain is separated into beta-barrel 1 and beta-barrel 2, that are also known as C-terminal end domain (Terrinoni et al., 2012). Maximum number of mutations in *TGM1* for ARCI, are present in the second or catalytic domain (Russell et al., 1995). Basically, for the formation of the cornified cell envelope, crosslinking of different proteins is essential. Skin cells are covered by the cornified cell envelope and protect the body from different type of infections as well as dehydration (Avrahami et al., 2008). These mutations cause defect in intercellular lipid layers of the stratum corneum (outer most layer of the epidermis) as a result the abnormal function of stratum corneum leads to ichthyotic or scaly skin phenotypes (Elias et al., 2002). Most of the mutations almost 90% in *TGM1* were reported for LI but also reported in patients with clinical features of CIE (Liu et al., 2015). Almost 55% phenotypes of ARCI have partial or complete loss of transglutaminase 1 because of *TGM1* mutations (Farasat et al., 2009). So the cornified cell envelope will be absent in patients having *TGM1* mutations while, the activity of the enzyme (transglutaminase-1) could be lower or completely lost (Huber et al., 1997). Up to 2009, the mutation, c.877-2A>G, has been found more frequently (34% mutated allele) than p.Arg142His, the second most common mutation in *TGM1*. Thus, these mutations look like hotspot mutations (Laiho et al., 1997; Shevchenko et al., 2000; Herman et al., 2009).

1.3.2.3.2 *ALOX12B and ALOXE3*

ALOX12B (OMIM: 603741) and *ALOXE3* (OMIM: 607206) are present on short arm of chromosome 17p13.1 (Krieg et al., 2001). Both consist of 15 exons and encode for epidermal lipoxygenases, 12-lipoxygenase enzyme (12R-LOX) and lipoxygenase 3 (eLOX-3) (Brash et al., 2007). Lipoxygenases (LOXs) are iron containing dioxygenases for deoxygenation of fatty acids. Conversion of arachidonic acid to hydroperoxide of fatty acid and formation of epoxy alcohol metabolites occur with the help of catalyzing activity of 12-lipoxygenase. *ALOXE3* encodes for enzyme that acts as hydroperoxide isomerase for production of epoxy alcohols (Traupe et al., 2014). For the formation of lipid barrier in the epidermis, epoxy alcohol is required which are produced by both lipoxygenases. Both these enzymes (12-lipoxygenase enzyme and lipoxygenase 3) play an essential role in hepoxilin pathway, which forms an end product that is involved in the formation of intercellular lipid of the epidermis. 12-lipoxygenase forms bonds among trioxilin B37 and hepoxilin B3 in order to produce 20-carboxy-trioxilin A3, a product essential for regulation of epidermis. Therefore, mutations in these both genes (*ALOX12B* and *ALOXE3*) disturb the lipid

processing in epidermis and lead to abnormalities in skin surface (Bland et al., 2015; Rosenberger et al., 2015). LI and CIE are the main types of ARCI, in which *ALOX12B* and *ALOXE3* mutations have been reported (Rosenberger et al., 2015).

1.3.2.3.3 *NIPAL4 (ichthyin)*

NIPAL4 (OMIM: 609383) is located on chromosome 5 at position 5q33 and contains 6 exons. This is also known as *ichthyin* and encodes for the transmembrane enzyme (ichthyin). This gene was first time found in 2004 that it may be associated with ARCI (Lefèvre et al., 2004). The expression level of this enzyme is higher in skin, brain, stomach, lungs and leukocytes while the lower level of expression in other tissues. In skin layers, the expression of ichthyin was found higher in stratum granulosum. Among the *in vitro* culturing of keratinocytes and fibroblast, expression of ichthyin was found higher in keratinocytes while lower in cultured fibroblast (Dahlqvist et al., 2012). Ichthyin protein acts as transporter as well as receptor, involved in the transport of Mg^{+2} through transmembrane while it is receptor for trioxilin A3 and B3. These are the components of hepoxilin pathway which is essential for developing an operative skin barrier (Muñoz-Garcia et al., 2014).

For the lamellar bodies, synthesis and transportation from keratinocytes into the extracellular environment are vital processes in stratum corneum because the release components are necessary for normal desquamation. Ichthyin is involved in both formation and secretion of lamellar bodies. Therefore, an impermeable lipid containing membrane forms that work as a water barrier (Descargues et al., 2005). Hepoxilin pathway and formation and secretion of lamellar bodies are the essential mechanisms that provide barrier function to the skin, so mutations in *NIPAL 4* might disturb these processes to lead impaired function of the epidermis and cause LI and CIE phenotypes.

1.3.2.3.4 *CYP4F22 and CERS3*

CYP4F22 (OMIM: 611495) is present on chromosome 19 at position 19p13.12. It consists of 12 exons that encode 531 amino acids. It is a member of cytochrome P450 protein family 4, subfamily F. 20-carboxy-trioxilin A3 is the end product of hepoxilin pathway catalyzed by *CYP4F22*. Mutations in this gene are linked with CIE and LI phenotypes because of reduced level of lipid barrier formation (Rodríguez-Pazos et al., 2013). *CERS3* (OMIM: 615276) is located on long arm of chromosome 15 at position 15q26.3. It encodes 394 amino acid to form ceramide synthase 3.

This member of ceramide synthases is involved in the formation of dihydroceramide from sphinganine by N-acylation of the sphingoid base. These enzymes are expressed in both, stratum granulosum and stratum corneum to metabolize long acyl chain that is crucial for lipid barrier formation (Traupe et al., 2014).

1.3.2.3.5 *LIPN*

LIPN (OMIM: 613924) is present on the long arm of chromosome 10 at position 10q23.31 and comprises of 9 exons responsible for encoding 398 amino acid or lipase-N. Lipase-N from AB hydrolase superfamily. Lipase-N is highly expressed in the granular layer of the epidermis and essential in the epidermal differentiation (Richard and Bale, 2014). For triglyceride metabolism in the epidermis, it plays a vital role to stimulate the process (Krieg and Fürstenberger, 2014).

1.3.2.3.6 *PNPLA1*

PNPLA 1 (OMIM: 612121) is present on chromosome 6 at position 6p21.31. It consists of 8 exons that is a member of the family of human patatin-like phospholipases and spans 2.63 Kb. It consists of three isoforms that expressed in stratum granulosum. The protein is localized to the keratin intermediate filament bundles (Demmer et al., 2014). The operative activity of this protein is undecided but biochemically it may show function in lipid organization in the lipid envelop. Mutation in this gene has shown loss of lipid barrier function of the skin. For clinical phenotype of CIE, two homozygous (one nonsense and one missense) mutations were reported in six members of two consanguineous families. These mutations were in the conserved catalytic domain of *PNPLA* 1 in six homozygous individuals (Demmer et al., 2014; Li and Wang, 2014).

1.3.2.3.7 *ABCA12*

ABCA12 (OMIM: 607800) consists of 53 exons and is a member of ABC transporters (family). This transporter protein is attached to adenosine triphosphate and helping in the transportation of various molecules through the plasma membrane (Dean and Allikmets, 2001). Lipid molecule like glucosylceramides is transported with the help of *ABCA12* via lamellar granules to the top surface of granular keratinocytes for the formation of lipid layer in the cornified cell envelope. Mutations in this gene cause loss or deficiency of intercellular lipid level that is essential for barrier function in the stratum corneum. This works as a keratinocyte transmembrane lipid transporter. In HI phenotype, stratum corneum becomes thicker due to defective lamellar granule-mediated lipid

transport in the upper epidermal (keratinocyte) layers because of severe deficiency of *ABCA12*. For the layer synthesis, the transport of lipid into lamellar granules and its metabolism is necessary. *ABCA12* protein is playing a vital role in lipid transport metabolism. Loss of function of the *ABCA12* molecule in lamellar granules and the collection of lipids molecules occurs resulting in abnormal intercellular lipid layer (Akiyama et al., 2005). Intercellular lipid layer synthesis is essential for the barrier function of the epidermis. When any type of disruption occurs, it results in the loss of barrier activity and severe hyperkeratosis (Akiyama et al., 2005).

1.3.2.3.8 *ST14*

ST14 encodes for matriptase and might be associated with ARCI, characterized by congenital ichthyosis, and hypotrichosis with curled, thin hair with follicular atrophoderma (Lestringant et al., 1998). Matriptase is a product of *ST14*, which plays a vital role in growth and differentiation of keratinocytes. Four mutations of *ST14* in homozygous form have been reported in which one splice site, one small deletion and two missense mutations are included (Basel-Vanagaite et al., 2007; Avrahami et al., 2008; Alef et al., 2009). In 2015, the mutation spectrum of *ST14* was further expanded by Takeichi et al through whole exome sequencing to detect a novel splice site mutation, IVS5+1G>A, in homozygous form in four-year-old girl. At the time of birth, she was found erythrodermic having scaly skin with mild palmoplantar keratoderma and diffuse hypotrichosis.

1.3.2.3.9 *SDR9C7*

SDR9C7 is located on long arm of the chromosome at position 12q13.3. It encodes 313 amino-acid or short-chain dehydrogenase/reductase family 9C, member 7. The gene is abundantly expressed in cornified and granular layers of the normal mammalian skin (Shigehara et al., 2016). *SDR9C7* belongs to SDR family of oxidoreductases and metabolizes vitamin A, converting trans retinal to trans retinol in the existence of NADH (Kowalik et al., 2009). Vitamin A downregulates corneodesmosomal proteins and consequently elevates desquamation of stratum corneum (DiGiovanna et al., 2013). The abnormal vitamin A metabolic pathway starts in the absence of vitamin A-metabolizing enzyme might slow down the process of keratinocyte differentiation, growth and desquamation, consequently leading to retention of hyperkeratosis. Hence, for different cutaneous disorders vitamin A derivatives are used traditionally for treatment (DiGiovanna et al., 2013). In 2016, Shigehara et al first time reported this new causative gene

(*SDR9C7)* for ARCI. In which two missense (p.Ile200Thr and p.Arg72Trp) homozygous mutations were found in three consanguineous Lebananies families. The affected phenotypes have been noticed with dry and scaly skin and scales were large on the entire body. In addition, palmoplantar keratoderma and severe hyperkeratosis over knees and elbows were also noticed.

In the most recent study, a novel frameshift mutation in *SDR9C7* with affected individuals of ARCI has been reported. The affected individuals were born as collodion babies and later their skin becomes dry with fine whitish scales on the trunk and upper limbs while large brown scales on the lower limbs. Palms, soles and dorsal area of feet and hands were severely affected with hyperkeratosis. Hypothyroidism and hypotrichosis, and gluten sensitivity were not seen in these patients. In this Pakistani family, the phenotypes were more severe than Lebanese patients that may be due to more pathogenic frameshift mutation in this study (Karim et al., 2017).

1.4 Diagnostic approaches

Genodermatosis is a group of inherited integument disorders having more than 350 genetic conditions with different phenotypic manifestations (Spitz, 2005). In 1987, the first mutation in ichthyosis was reported in the X-linked ichthyosis. (Bonifas et al., 1987). In 2003, the project of the human genome was completed that was a goal for the establishment of databases for mutations like single nucleotide polymorphisms (Consortium, 2005; Consortium, 2010). Further advancement was the next generation sequencing, through which whole exome and genome can be screened and this was a great achievement for detection of various types of mutated genes. (Metzker, 2010; Bamshad et al., 2011; Lai-Cheong and McGrath, 2011). About, 1000 genetic conditions and 3500 genes are associated with the skin (Feramisco et al., 2009; Lander, 2011).

Inherited skin disorders are mostly very rare but their diagnosis plays a vital role for therapies as well as for investigation of linked abnormalities. Because many skin diseases are also associated with other conditions, thus with the help of diagnosis, their therapies could be possible in future (Akiyama, 2011). For the diagnosis of ichthyosis, only one factor should not be kept in mind because it is based on clinical manifestations displayed by patients, their complete family and medical history, skin biopsies and many other types of biochemical examinations (Long, 2014).

In addition, screening of mutations is used to confirm the diagnostic process for the proper genetic counseling. Furthermore, initial clinical manifestations and onset time of disorder are the main

factors that may categorize common ichthyosis from congenital ichthyosis. Common ichthyosis are ichthyosis vulgaris and recessive X-linked ichthyosis can be diagnosed through skin biopsy or screening of filaggrin mutation and steroid sulfatase testing, respectively (Oji and Traupe, 2006). Electron microscopic studies can provide clues like a defect in hepoxilin pathway or other defects to differentiate ARCI major types (HI, LI and CIE) (Richard et al., 2013).

Research in molecular genetics now offers a platform to find out the disease and to understand about it pathogenic mechanism. Human genome information and databases are expanding which plays important role in exact diagnosis, planning enhanced therapies, better genetic counseling and DNA based prenatal diagnosis. As a result, a number of molecules and genes have been identified for ichthyosis through advances in molecular genetic research (Akiyama, 2011).

1.5 Treatment and management

The removal or reduction of scales and dryness of the skin is the main goal for the treatment of ichthyosis. Different aspects like gender, age, and lesion area of the affected individual should be taken into consideration before treatment (Vahlquist et al., 2008). Every day bathing for the ARCI phenotypes is suggested for the removal of the scales. Ten to fifteen minute's immersions of patient in water makes it easier to eliminate scales. Several studies also reveal that the addition of sodium bicarbonate (to denaturalize the keratins) to the water makes water alkaline and helps in removal of the scales (Traupe and Burgdorf, 2007).

1.5.1 Topical treatment

Keratolytic agents such as alpha-hydroxy acid or urea-based creams, lubricants and petroleum-based creams are used along with daily bathing to keep the skin supple and soft (Lodén, 2012). Retinoids (tazarotene, adapalene and tretinion) are also used topically and frequently give rise to painful fissures and irritation. Therefore, proper dressing is preferred for treatment of specific area to improve the activity of various moisturizers or keratolytic agents (Lodén, 2012). These agents are important in helping desquamation process and make better epidermis barrier functions (Van Scott and Ruey, 1980). For common ichthyosis (IV and XLRI), glycerin-based or urea-based ointments are most commonly used (McLean and Smith, 2012). While for autosomal recessive ichthyosis patients are counseled to take bath at least once a day and rub the body surface with

microfiber cloth or sponge to enhance the desquamation process. CIE phenotype shows inflammation so the patients are advised to use glycerin and macrogol (Richard et al., 2013).

1.5.2 Systemic treatment

Oral retinoids and drugs are used systematically for the treatment because it has a keratolytic activity to remove severe hyperkeratosis and various level of scales. The Proper dosage of oral retinoids should be used because it may cause side effects such as ligamentous calcification, bone toxicity and teratogenicity. Low doses for CIE patients show complete response because it is due to its less severity as compared to other form of the ichthyosis (DiGiovanna et al., 2013; Lacour et al., 1996). For the systemic treatment of ARCI, etretinate and acitretin are effective because of their keratolytic effects to eliminate the scales and also stop hyperkeratosis of the skin (Vahlquist et al., 2008). Liarozole (drug) is more effective as compared to acitretin and granted by different agencies for the treatment of CIE, LI and HI (Verfaille et al., 2007).

1.6 Aims of the present study

The present study was aimed to find out the possible reason of non-syndromic ichthyosis in three unrelated Pakistani families DER48, DER49 and DER58 affected with ARCI. Direct DNA sequencing technique was used to find out possible mutations in *TGM1*, associated with ARCI.

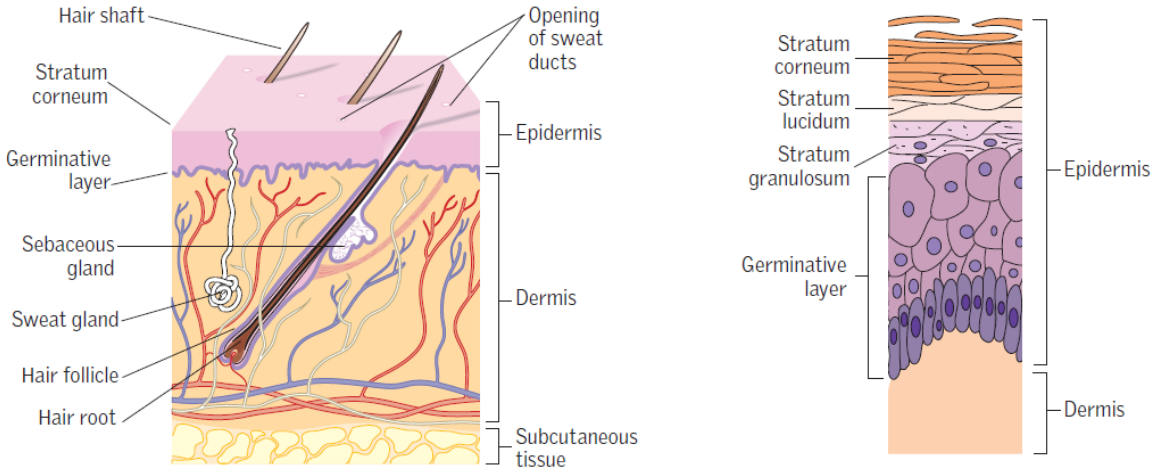


Figure 1.1: Structure of the human skin consisting of three layers i.e. subcutaneous, dermis and epidermis layer (McLafferty et al., 2012).

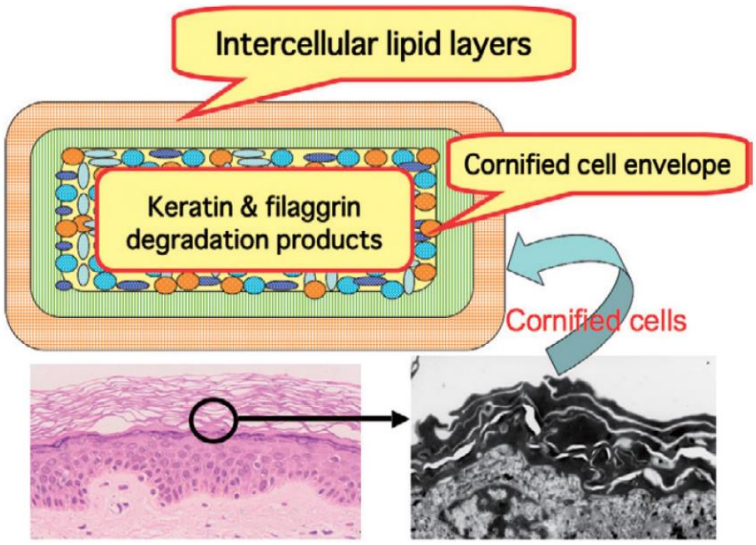


Figure 1.2: Stratum corneum layer of the epidermis. It consists of major components of skin barrier functions such as cornified cell envelope, intercellular lipid layers and keratin/filaggrin degradation products (Akiyama, 2011).

Table 1.1 Summary of common ichthyosis and major types of ARCI

Phenotype	OMIM	Mode of inheritance	Onset	Prevalence	Causative Gene
Common occurring ichthyosis					
X-link recessive ichthyosis	308100	XR	At birth or late onset	1-2000/6000	*STS*
Ichthyosis vulgaris	146700	AD/AR	After 2-6 months of birth	1-100/250	*FLG*
Autosomal recessive congenital ichthyosis					
Congenital ichthyosiform erythroderma	242100	AR	At birth	1-200000	*TGM1*, *ABCA12*, *NIPAL4*, *ALOXE3*, *ALOX12B*, *LIPN*, *CYP4F22*, *CERS3*, *PNPLA1*
Lamellar ichthyosis	146750	AR	At birth	1-200000	*TGM1*, *ABCA12*, *NIPAL4*, *ALOXE3*, *ALOX12B*, *LIPN*, *CYP4F22*, *CERS3*, *PNPLA1*
Harlequin ichthyosis	242500	AR	At birth	1-300000	*ABCA12*

Chapter 2

MATERIAL AND METHODS

2.1 Human subjects

Three families (DER48, DER49 and DER58) showed congenital ichthyosiform erythroderma (CIE) and bathing suit ichthyosis (BSI), respectively, were recruited from Khyber Pakhtunkhwa (KPK) province of Pakistan. Blood samples, history and pictures were collected from these families. Pedigrees were drawn according to the collecting history from their guardian or elders.

2.2 Pedigree construction

HaploPainter was used to construct three pedigrees. In the pedigree, the square is used to represent males and circles are used to represent females while black filled symbols were used for affected and unfilled for normal individuals. Consanguinity was also shown between partners with double horizontal lines. From pedigree, mode of inheritance was then inferred.

2.3 Blood collection

From these three families, blood was drained in both 5 ml and 10 ml syringes from all affected and available normal individuals. Vacutainers were used for collecting blood samples that contained ethylene diamine tetra acetate (EDTA). These collected blood samples were taken to Medical Genetics Research Laboratory, at Department of Biotechnology, Quaid-i-Azam University Islamabad and were refrigerated at 4^{o}C.

2.4 Genomic DNA extraction

For genomic DNA extraction, organic (phenol-chloroform extraction) method was used to isolate total genomic DNA from blood samples.

2.4.1 Organic method

Before starting this protocol, fresh and old blood samples were placed for 30 minutes and one hour, respectively, in racks at room temperature.

- Blood and solution A of same volume (750µl) were mixed in a 1.5mL microcentrifuge tube and then kept for 10-15 minutes at room temperature and centrifuged at 13000 rpm for 1 min.
- The supernatant was discarded and the pellet obtained was again centrifuged at same condition but well dissolved in 400µL of solution A before centrifugation.
- The supernatant was again discarded and the pellet was re-suspended in 400µl of solution B, 12µl of 20% SDS (25µl of 10% SDS) and 5-8µl of proteinase K (20µl/mL stored at -20^{o}C).
- The pellet (via tapping) was well dissolved and then incubated overnight at 37^{o}C.
- Equal volumes (500µl) of solution C and D were added to incubated Eppendorf and centrifuge at 13000 rpm for 10 mins. After centrifugation, the upper layer was collected into a new tube.
- Same volume (500µl) of solution D was added into the same new tube and centrifuged at 13000rpm for 10 minutes and the upper layer was again transferred to a new Eppendorf.
- The DNA was precipitated by adding 55µl of sodium acetate and 500µl of chilled isopropanol (-20^{o}C) while the tube was inverted several times to precipitate the DNA. After precipitation, at the same condition, the tube was centrifuged (13000rpm for 10 mins).
- The supernatant was discarded carefully without disturbing the DNA pellet. For washing, 70% ethanol about 200µl was added and then centrifuged at 13000 rpm for 7 minutes.
- Ethanol was discarded from tube carefully because it makes disturbance in downstream processing. The tube was kept in the concentrator at 45^{o}C for 10 minutes to evaporate the residual ethanol.
- In the last step, the pellet DNA was dissolved in 80-100µl of TE/deionized water and incubated overnight at 37^{o}C.

2.5 Molecular analysis of extracted DNA

Agarose gel electrophoresis method was used to analyze extracted DNA. Agarose gel 1% was used which consists of 0.5g agarose, 45 ml of distilled water, 5ml of 10X Tris-Borate EDTA (TBE) buffer and 5µl of ethidium bromide.

First of all, 45 ml of distilled water and 5ml of 10X TBE buffer and 0.5g agarose were mixed in graduated cylinder/ flask and then melted it in a microwave oven for 2-3 minutes. After 5-7 minutes, 5 µl of ethidium bromide was added and the mixture was poured down in a gel casting tray. Desired combs were used to make wells in the gel. For solidification of gel, it was left for 15-20 minutes at room temperature. The DNA sample and bromophenol blue (bromophenol blue 0.25%, sucrose 40%) were mixed. It was used as a loading dye to indicate the movement of DNA in the gel. Equal amount (3µl DNA, 3µl bromophenol blue) was loaded into the wells. In gel tank, 1X TBE buffer was used as electrolyte and gel was run for 25-30 minutes at 115 volts. UV-Trans illuminator (Wealtec, Taiwan) was used to show the bands of DNA in gel and also its intensity. For dilution of DNA, 20 µl of extracted DNA and 80 µl of PCR water were mixed to get a final concentration of 40ng/µl.

2.6 DNA sequencing

2.6.1 Primer designing

For the exons of *TGM1* primers were designed manually while the sequence of this gene was downloaded from UCSC genome browser (http://genome.ucsc.edu/). The homology of primers was checked by BLAST (http://www.ensembl.org/) and single hit primers were selected and purchased from MACROGEN (Korea). The primer sequences of *TGM1* (Table 2.2) were used to amplify each exon.

2.6.2 Polymerase chain reaction

50 µL of amplification reaction was performed in a 0.2ml PCR tube (Axygen, USA) for each exon. DNA of affected and normal individuals of each family was used during PCR reaction. The reaction was prepared by adding the following composition;

Components	Concentration
DNA dilution	3 µL
Forward Primer	2 µL
Reverse primer	2 µL
PCR water	18 µL
PCR Mix (Promega)	25 µL

In PCR tube, all these contents were mixed and vortexed before placing in thermocycler, T-Personal Thermo-cycler (Biometra, Germany). The following program was set for the amplification;

Stage	Duration		Temperature
Initial denaturation	5 minutes		95°C
Denaturation	1 minutes		94°C
Primer annealing	45 Seconds	→ 40 cycles	56 °C
Extension	30 Seconds		72 °C
Final extension	4 minutes		72 °C

PCR products were analyzed on 2 % agarose gel after the amplification. The gel was placed in 1X TBE buffer and PCR products were loaded into the wells. Electrophoresis was carried out at voltage of 90-125V for 20-30 minutes. DNA bands were confirmed inside UV transilluminator and specific bands were selected for purification purposes.

2.6.3 Purification of PCR products

Two kits (Silica Bead DNA gel extraction (Lot 00206349) Thermo Scientific, USA. and Gene Jet PCR purification (Lot 00129472) Thermo Scientific, USA) manuals were followed to purify the selected PCR products.

2.6.4 Gene jet PCR purification Kit

For the removal of different impurities (enzymes, primers, dNTPs, salts, and nucleotides) from PCR reaction, this method was used to purify specific amplified exon. This type of purification method consisted of following five steps.

1. Equal volume of binding buffer and PCR product were mixed and allowed to incubate at room temperature for 2-3 minutes.
2. Mixture was filtered through a column and centrifuged at 13000 rpm for 1 minute.
3. Before discarding the flow-through, washing buffer (700 µL) was added to the column and centrifuged at 13000 rpm for 1 minute. Then, after centrifugation, the flow-through was discarded.
4. The filter was transferred to a new Eppendorf tube and kept open at room temperature for air drying.
5. Finally, 14-15 µL of elution buffer was added and centrifuged at 13000 rpm for 1-2 minutes to get final volume of purified product.

2.6.5 Silica bead DNA gel extraction kit

This type of purification method was used for non-specific amplified products of PCR reaction. Proteins, agarose, primers, nucleotides, salts, ethidium bromide and primer-dimers and additional impurities were removed through this method because it was designed for non-specific binding in PCR. The protocol was followed during purification process and the specific products sizes were then checked in 2% agarose gel. After confirmation, samples were stored at -20°C.

2.6.6 Analysis of sequencing results

BioEdit software (version 7.2.0) was used to analyze the chromatogram of affected and normal individuals. The sequence of *TGM1* was saved from Ensemble and imported to BioEdit. Changes in the sequence of each exon were confirmed with the help of ClusterW Multiple Alignment, an option in BioEdit to align both reference sequence of a gene and affected individuals as well.

Table 2.1 Preparation and composition of solutions

Solution A	**Ingredient**	**Quantity**
	Sucrose (0.3M)	54.7 g
	MgCl2 (5mM)	2.5 ml
	Tris HCL (5ml)	5 ml
	Triton X-10	5 ml
	Distilled water	400 ml
Solution B	NaCl (400Mm)	40 ml
	Tris HCL (10mM)	5 ml
	EDTA (2mM)	1 ml
	Distilled water	Make total volume 500ml
Solution C	Phenol	100 ml
	Hydroxyquiniline	10 mg
	Tris HCl	10 ml
Solution D	Chloroform	24 ml
	Isoamyl alcohol	1 ml

Table 2.2: List of primers sequence for *TGM1* gene amplification.

Exon No	F/R	Sequence	Primer length	Tm (ºC)	GC content	Product size
		5' > 3'	bp	ºC	%	Bp
2	F	GGTGGGATTGTTTCGGTCATC	21	64	52	584
	R	GCAAGGACACAGCTCCGTGTC	21	68	61	
3	F	CTGGAGATGGCACCATCCGAG	21	68	61	600
	R	GGAGTACTCTGAGACCGATG	20	62	55	
4	F	GCTCTAAGGGAGACTGTCAG	20	62	55	573
	R	CTGGGCTGGCCACCTTTCTG	20	66	65	
5	F	CGTGTCCTCCCACAGCAGTC	20	66	65	410
	R	CAGCACCCCGTGGTCAAACTG	21	68	61	
6	F	GTGAGCGGACCTGGAACTACG	21	68	61	417
	R	GAAGAGGAGGGAGACCCCTTC	21	68	61	
7	F	GTCTGGATCCTGAGGCATTTAG	22	66	50	514
	R	CTGGCTCAGTCCTTGCCTGTC	21	68	61	
8	F	CAAGTGCTCTGTAGCCCAAAG	21	66	50	470
	R	CGTTCCACACATGGAAGTTC	20	60	50	
9	F	CTGAACCATGATTCTGTCTG	20	58	45	407
	R	CTGACAAACCCGTTTAAGAAGC	22	64	45	
10	F	GTGTTGCACTGGCTGTCAAC	20	62	55	455
	R	CTGATCCCGAGTATAGGAAGC	21	64	52	
11	F	CGTCTGGCTCGTCTTGGAAAG	22	68	54	451
	R	CTTCCGCTCTGCGTCTGAGC	20	66	65	
12	F	CCTCTATAAGCACCCAGAAG	20	60	50	552
	R	GAAGAGGCATGGCGTCACTG	20	66	65	
13	F	CCAGGGGCCTGTAAGTGCTC	20	66	65	440
	R	GACCTTCTCCTACAAAGGCTC	21	66	52	
14	F	CTTATGTCTCTGGTTCTGTGAC	22	64	45	533
	R	CAAGAGGTCTCCCTAAAGGC	20	62	55	
15	F	GTGGAAATTTCAGGTAAGCCTG	22	64	45	691
	R	CTCTCTGGTGAGGGACACCAG	21	68	61	

Chapter 3

RESULTS AND DISCUSSION

In this research study, three families (DER58, DER49, and DER48) were screened for *TGM1* because mutations in this gene are associated with subtypes of ARCI including CIE and bathing suit ichthyosis.

3.1 Human subjects

3.1.1 Family DER49

Four generation pedigree (Figure 3.1) was constructed, one consanguinity in the third generation was observed. All the three individuals in the fourth generation (IV: 1, IV: 2 and IV: 3) were affected. The inheritance pattern of the pedigree to be recessive. Phenotypic examination and clinical description of all affected individuals (Figure 3.2) of this family were strongly suggestive of CIE. These patients presented collodion membrane at birth, mild erythema, prominent palmoplantar keratoderma, hyperkeratosis (on dorsal surfaces of hands and feet) and scalp abnormalities along with fine, whitish fish-like scales all over the body (forehead, face, neck, chest, abdomen, back, arms, forearms, legs).

3.1.2 Family DER58

Family DER58 was also recruited from Khyber Pakhtunkhwa, Pakistan. Fourth generation pedigree (Figure 3.3) was constructed with no consanguinity. Total two individuals (IV: 1 and IV: 5) were affected while other members (IV: 2, IV: 3 and IV: 4) and parents were normal. Both affected individuals presented clinical features of bathing suit ichthyosis (Figure 3.4).

These two patients presented collodion membrane at birth, dark and brownish or grey fish-like scales over parts of the body such as groins, legs, neck, chest and hands. Scaling of bathing suit area showed severity in summer and its severity reduced in winter. Palms, soles and face were found normal.

3.1.3 Family DER48

Four generation pedigree (Figure 3.5) was constructed and one consanguinity in the third generation was observed. All the individuals in the fourth generation (IV: 1, IV: 2, IV: 3 and IV:

4) were affected. The inheritance pattern of the pedigree to be recessive. Phenotypic examination and clinical description of all affected individuals (Figure 3.6) of this family were strongly suggestive of CIE. These patients presented collodion membrane at birth, mild erythema and mild hyperkeratosis (on dorsal surfaces of hands and feet) along with fine, whitish fish-like scales all over the body (neck, chest, abdomen, back, arms, forearms, legs). Legs, groins and hands were prominently affected with whitish scaling while palms, soles, and face were observed normal.

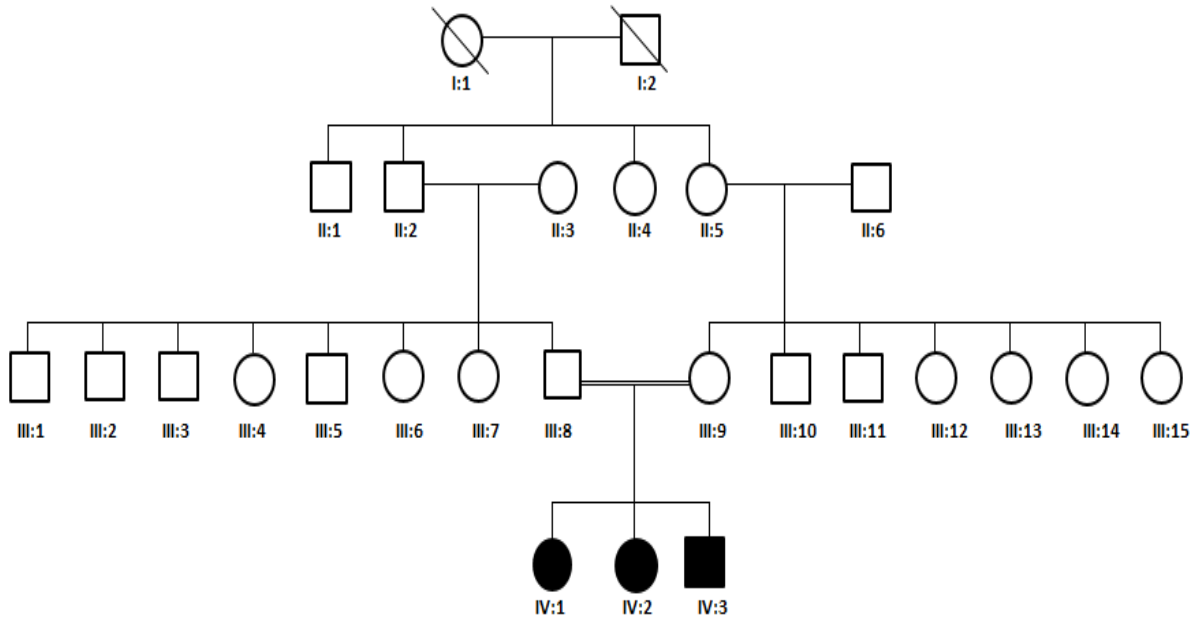
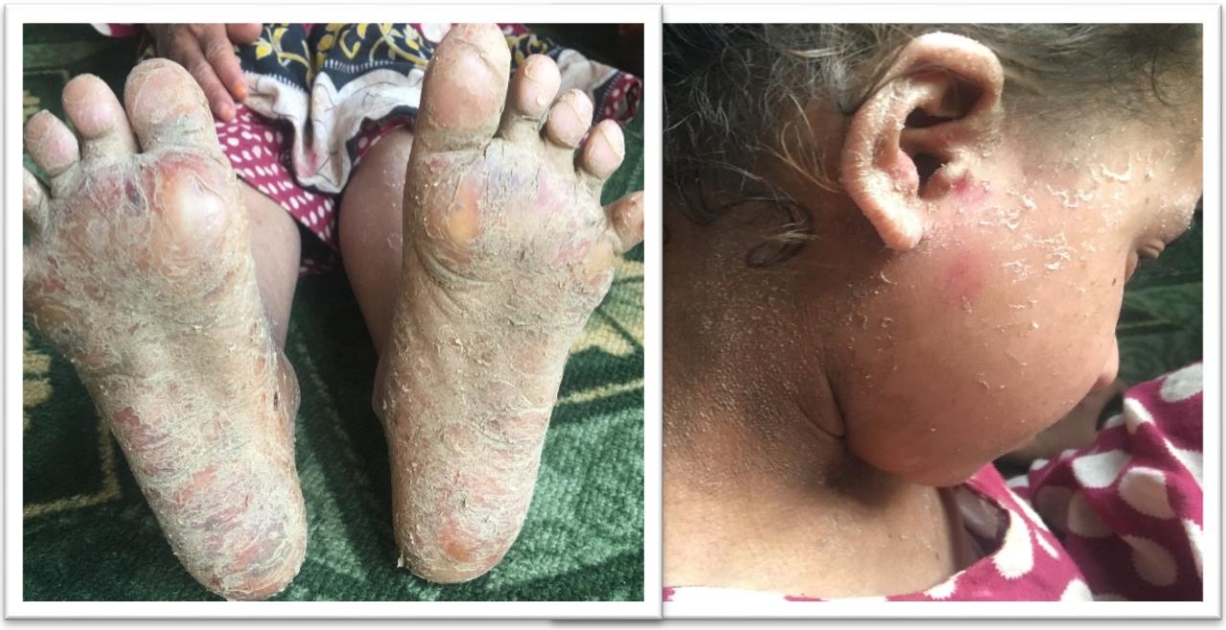


Figure 3.1: Pedigree of DER49 family. All females are represented with circles and males with square. The empty symbols represent unaffected individuals while filled symbols denote affected individuals. Double line in the fourth generation represents consanguinity.



(a) (b)

Figure 3.2. Affected individuals of DER49. (a) Affected female (IV: 1) with CIE and (b) Affected female (IV: 2) with CIE.

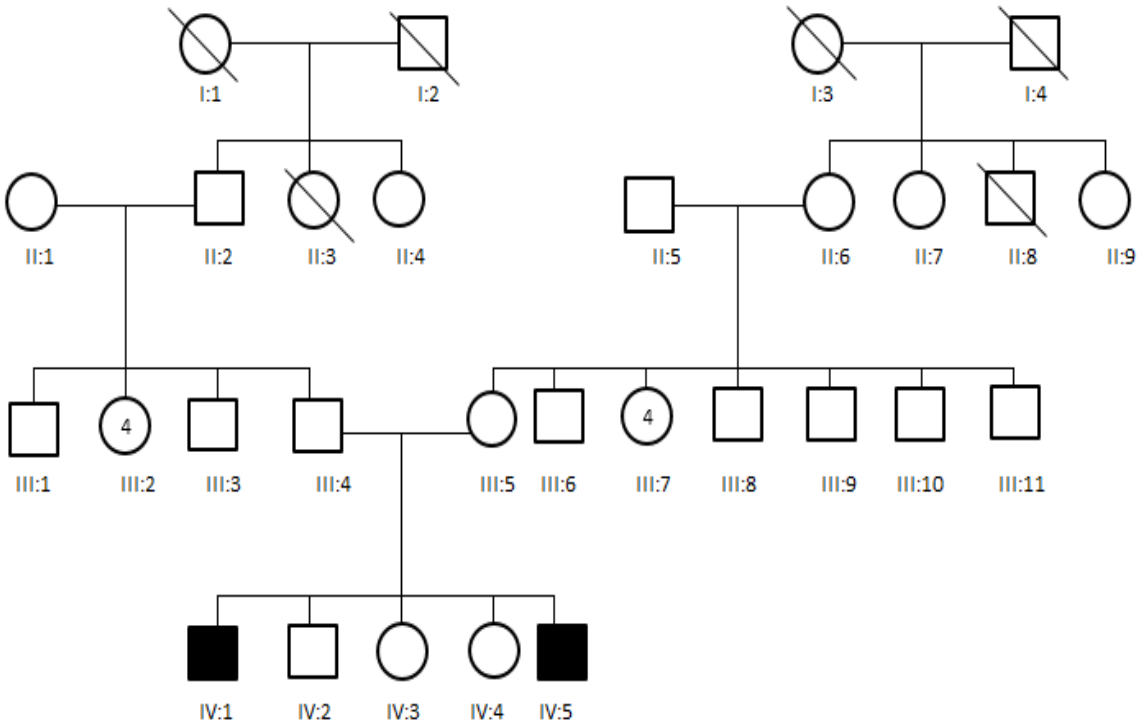
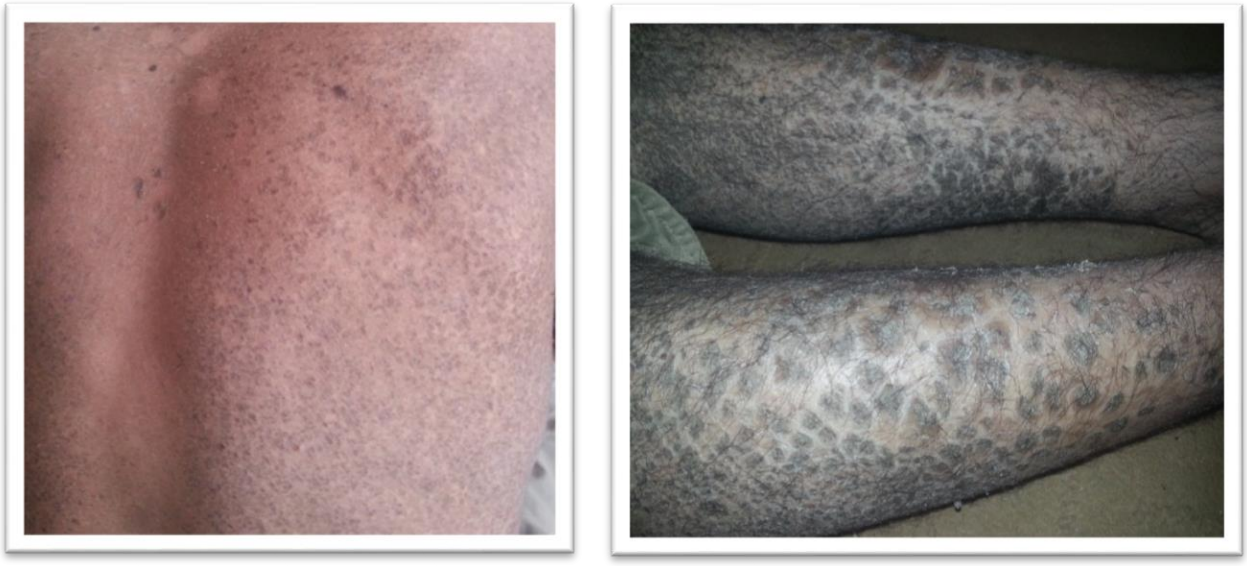


Figure 3.3: Pedigree of DER58, all males have been represented with squares and females with circles. Unfilled symbols represent the unaffected and filled symbols represents affected individuals. No consanguinity was observed in these three generation.



(a) **(b)**

Figure 3.4 Affected individuals of DER58 family. (a, b) Affected male (IV: 1) with bathing suit ichthyosis.

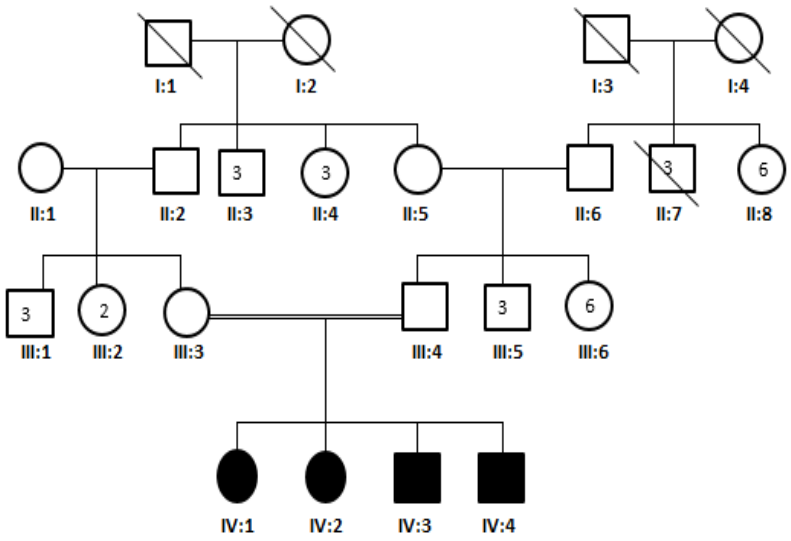
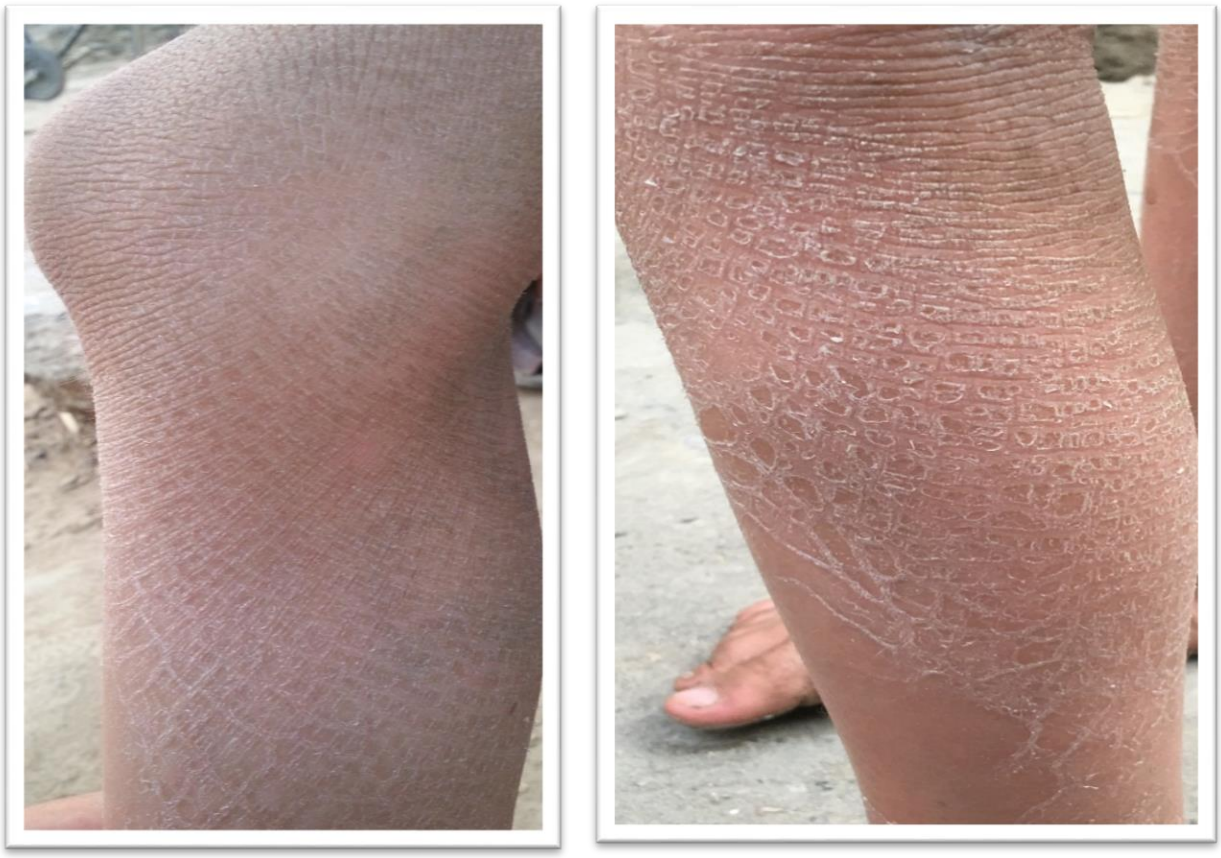


Figure 3.5: Pedigree of DER48 family. All males are represented with square and females with circles. The empty symbols represent unaffected individuals while filled symbols denote affected individuals. Double line in the fourth generation represent consanguinity. Blood samples were collected from individuals III: 3, III: 4, IV: 1, IV: 2, IV: 3 and IV: 4.



(a) (b)

Figure 3.6 Phenotype of DER48. (a) Affected female (IV: 1) with CIE and (b) Affected female (IV: 2) with CIE.

3.2 TGM1 sequencing

All the 14 exons were found identical to the wild-type sequence and no pathogenic mutations were observed in both families DER49 and DER58. For Bathing suit ichthyosis only this gene is responsible but the phenotypic manifestations of DER58 also overlapped with lamellar ichthyosis, for which other genes are also included such as *ALOX12B, ALOXE3, NIPAL4, ABCA12, CERS3, CYP4F22, LIPN* and *PNPLA1*.

In DER48, the *TGM1* of the patient (IV: 1) contained two heterozygous mutations. One previously reported missense mutation (c.550 C>T) was found in exon 4. The other novel, mutation was observed in splice site (IVS5-1G>A) (Figure 3.7). The splice site (IVS5-1G>A) and missense mutation (c.550 C>T) were confirmed in mother and father, respectively (Figure 3.8).

Autosomal recessive congenital ichthyosis is a diverse group of non-syndromic ichthyosis in which keratinization and cornification processes are disturbed to make skin scaly and dry. It is characterized by the presence of collodion membrane at birth, palmar plantar, hyperkeratosis, alopecia, ectropion, eclabium, hypohidrosis, erythema and epidermal scaling of the skin (Herman et al., 2009; Oji et al., 2010; Pagon et al., 2012). Major three phenotypes of ARCI include HI, CIE and LI while other minor phenotypes are bathing suit ichthyosis (BSI), self-healing collodion baby (SHCB), acral self-healing collodion baby (ASHCB) (Takeichi and Akiyama, 2016). The following 11 genes *TGM1, ALOXE3, ALOX12B, NIPAL4, ABCA12, CYP4F22, LIPN, ST14, PNPLA1, CERS3* and *SDR9C7* have been reported for phenotypic manifestation of ARCI (Shigehara et al., 2016).

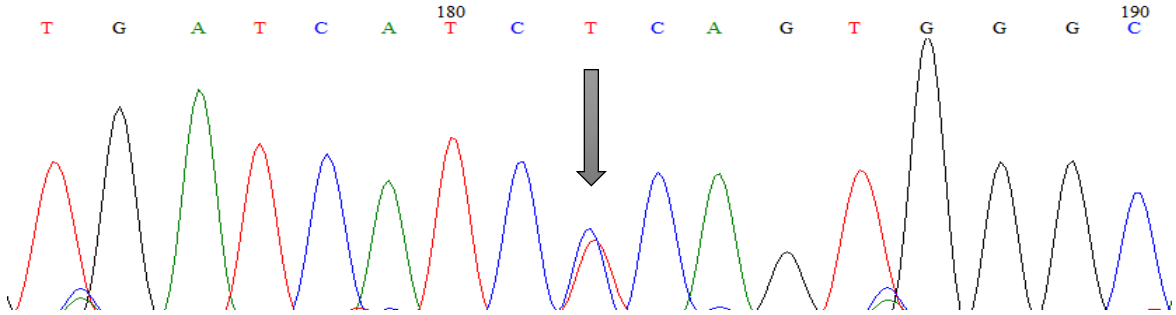
3.2.1 Family DER48 and DER49

CIE is a subtype of ARCI, characterized by the presence of fish-like fine, whitish scales along with erythemic background on the entire body and palms and soles are severely affected (Williams and Elias, 1985). Other clinical features have been reported which are collodion membrane at birth, contractures of the digits, painful fissures while in severe form, loss of eyebrows, eclabium and ectropion have been reported. Collodion membrane of infants with the passage of time changes into fine whitish scales and generalized erythema. Mode of inheritance of this disorder is

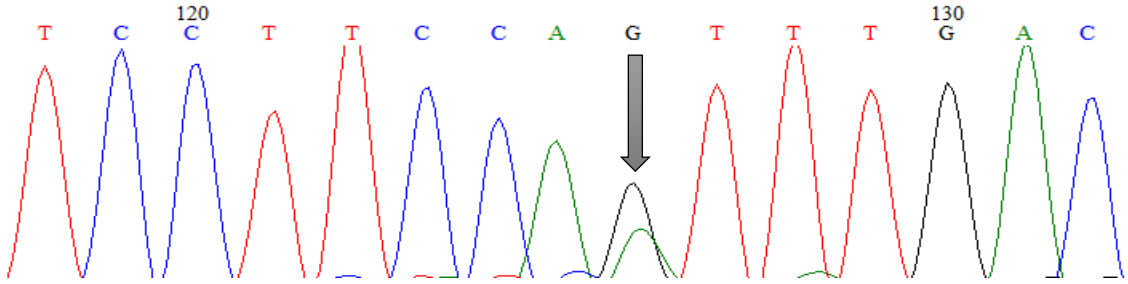
autosomal recessive and affects 1-200,000 individuals. Too much production of epidermal cells occurs than its normal level in short period of time. Normally, these epidermal cells take fourteen days to reach the SC while when overproduction occur it takes four to five days. SC and other layers start to expand and as a result scales appear because the shedding rate of cells are faster than production rate (Richard and Bale, 2014). All the genes, namely, *TGM1, PNPLA1, NIPAL4, CYP4F22, CERS3, ALOXE3, ALOX12B* and *ABCA1* are the cause of CIE. These genes encode for various proteins to produce stratum corneum or to maintain its structure and function for the barrier function of the skin (Oji et al., 2010; Grall et al., 2012; Radner et al., 2013). Approximately, 55% of mutations in ARCI are due to *TGM1* (Farasat et al., 2009). It encodes transglutaminase 1 (TGas-1) enzyme that catalyzes the cross linking between precursor proteins in cornified envelope (Fischer, 2009). Skin cells are covered by the cornified cell envelope and protect the body from different types of infections as well as dehydration. Basically, for the formation of cornified cell envelope, crosslinking of different proteins are essential, however, this crosslinking mediated by TGas-1 (Avrahami et al., 2008).

In this study, we investigated two mutations in *TGM1* in one family, DER48, by genetic analysis. All the patients from family DER48 were presenting the clinical features of CIE. All the affected individuals were born as collodion babies and fine whitish scales along with erythroderma on the whole body (neck, chest, abdomen, back, arms, forearms and legs) of each patient was observed. Legs, groins and hands were prominently affected with whitish scaling while palms, soles, and face were observed normal. The parents of these patients were unaffected. Two heterozygous mutations were observed in *TGM1*; One missense mutation (c.550 C>T) was found in exon 4. This mutation was already reported by Numata et al in 2015 for CIE phenotype. The other novel, mutation was observed in splice site (IVS5-1G>A) in the fifth intron of the *TGM1*.

The second family in the present study, DER49, presents all the clinical manifestations of CIE. All patients present collodion membrane at birth, mild erythema, prominent palmoplantar keratoderma, hyperkeratosis (on dorsal surfaces of hands and feet) and scalp abnormalities along with fine, whitish fish-like scales all over the body (forehead, face, neck, chest, abdomen, back, arms, forearms, legs). All the exons of this gene in patient IV: 1 were found alike to wild-type sequence.

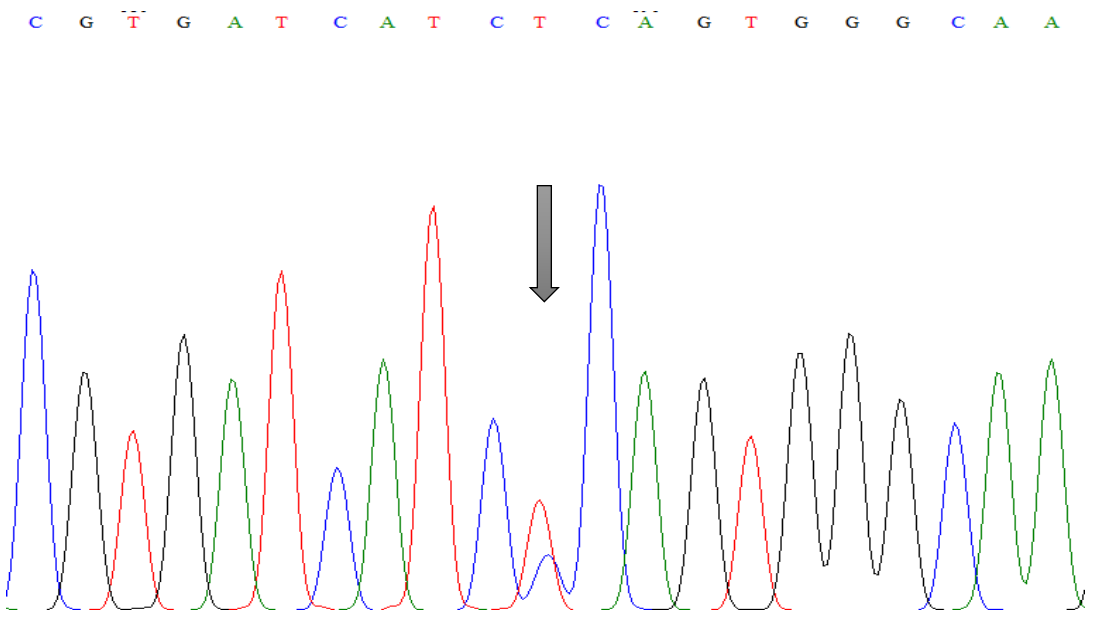


(a)

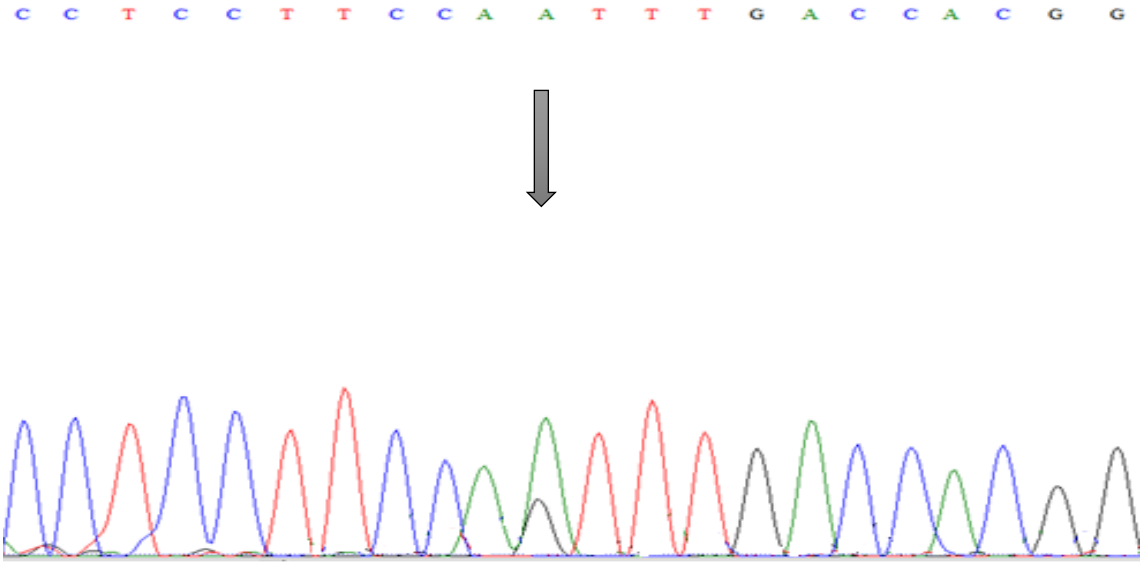


(b)

Figure 3.7 Chromatogram of *TGM1* in family DER48 indicating reported missense mutation (c.550C>T) at nucleotide position c.550 and novel splice site mutation (IVS5-1G>A). (a) Heterozygous peak was found in patient (IV: 1), presenting cytosine to thymine mutation that substitutes proline into serine amino acid at position 184. (b) Heterozygous peak was found in same patient (IV: 1), presenting guanine to adenine mutation (IVS5-1G>A) in fifth intron.



(a)



(b)

Figure 3.8 Sequence chromatogram of *TGM1* in parents of affected individual of DER48. (a) Heterozygous peak was found in father (III: 4), presenting cytosine to thymine mutation that substitutes proline into serine amino acid at position 184. (b) Heterozygous peak was found in mother (III: 3), indicating novel splice site mutation (IVS5-1G>A) in fifth intron.

3.2.2 Family DER58

BSI is a minor subtype of ARCI characterized by collodion membrane covering the entire body and develop brownish or dark scaling limited to bathing suit areas while the face and other areas of the body are almost normal (Schultz, 1994).Three conditions are essential for the accurate diagnosis of BSI: (1) Lamellar ichthyosis with brownish or dark grey scales on the trunk while face and extremities should be normal. (2) Phenotypic manifestations of ARCI in family history. (3) The manifestations should be limited to the skin (non-syndromic ichthyosis) (Jacyk, 2005).

In 2006, Oji et al confirmed that *TGM1* is a causative gene for BSI, through genetic, biochemical and ultrastructural studies. He also explained that BSI is a temperature-sensitive phenotype because through *in situ* transglutaminases testing in the skin of BSI patients, it was revealed that increase in temperature causes decrease in transglutaminase 1 activity.

In 2010, Hackett et al reported pathogenic mutations for three BSI phenotypes. These phenotypes present collodion membrane at birth, thick dark scales or lamellar scales on the scalp and trunk with sparing of the limbs and central face. One patient shows prominent scaling in the axillae and also in auditory canals because of too much desquamation within it. These both parts of the body have a higher temperature so the functional activity of transglutaminase 1 in these parts is reduced.

In our study, two individuals were affected in DER58. Both patients presented collodion membrane at birth, dark and brownish or grey fish-like scales over the parts of the body such as groins, legs, neck, chest and hands. The scaling of bathing suit area shows severity in summer while it reduces in winter. Palms, soles and face were found normal in both affected individuals. We screened all the exons of *TGM1* for mutations in this family but all the exons were found similar to wild-type sequence and no pathogenic mutations was found.

3.3 Conclusion

In this study three unrelated Pakistani families, DER48, DER49 and DER58 affected with ARCI were checked for mutations in *TGM1*. Two compound heterozygous mutation (one previously reported and novel splice site mutation) were found in the affected individuals of DER48 which are the likely cause of ARCI in this family. No *TGM1* mutation was identified in DER49 and

DER58. Therefore, whole exome sequencing of patients, of DER49 and DER58 is suggested to find out the causative variants.

Table 3.1 Mutations identified in family DER48

	Exon	Mutation	Sequence change	Mutation type	Protein variation	
	4	c.550C>T	**C**CA/**T**CA	Missense Heterozygous	p.Pro184Ser	
	5* Splice site	IVS5-1G>A	A**G**/A**A**	Heterozygous	-	

Chapter 3

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3	Matthew L. Herman. "Transglutaminase-1 gene mutations in autosomal recessive congenital ichthyosis: Summary of mutations (including 23 novel) and modeling of TGase-1", Human Mutation, 04/2009 Publication	<%1
4	Liu, J.-J., Y.-Y. Yuan, X.-Q. Zhang, Z.-M. Li, Y.-S. Xu, S.-M. Gao, J.-F. Cai, X.-H. Shao, X.-H. Lin, and B.-X. Li. "Mutations of transglutaminase-1 in Chinese patients with autosomal recessive congenital ichthyosis: a case report with clinical and genetic analysis of Chinese cases reported in literature", Clinical and Experimental Dermatology, 2014. Publication	<%1

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6	B.C. Hackett. "Genotype-phenotype correlations with *TGM1*: clustering of mutations in the bathing suit ichthyosis and self-healing collodion baby variants of lamellar ichthyosis", British Journal of Dermatology, 02/2010 Publication	<%1
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8	Takeichi, Takuya, and Masashi Akiyama. "Inherited ichthyosis: Non-syndromic forms", The Journal of Dermatology, 2016. Publication	<%1
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10	Yanagi, T.. "DNA-based prenatal exclusion of harlequin ichthyosis", Journal of the American Academy of Dermatology, 200804 Publication	<%1
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