Identification of Pathogenic DNA Sequence variants Underlying Clouston Syndrome

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Dedication

To my parents and Late Grand Mother for their continuous support, love, care, encouragement throughout my life, their efforts and struggles have helped me pursue my academic goals and push very hard through my bad times

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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Name: Asad Ullah

Dated _____

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LIST OF ABBREVIATIONS

%	Percentage
µl	Microliter
µg	Microgram
µM	MicroMolar
○ C	Degree Celsius
ATP	Adenosine triphosphate
Bp	Base pair
C	Cytosine
CCE	Cornified cell envelope
DNA	Deoxy ribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
G	Guanine
HED2	Hidrotic ectodermal dysplasia 2
mA	Milliampere
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
Pro	Proline
Q	Long arm of chromosome
RPM	Revolution per minute
SC	Stratum corneum
Sec	Second
SNP	Single nucleotide polymorphism

T	Thymine
Taq	Thermus aquaticus
TBE	Tris borate EDTA
TE	Tris EDTA
TGase	Transglutaminase

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Abstract

Hidrotic ectodermal dysplasia type 2 also known as Clouston syndrome is a rare genetic disease of skin having both autosomal recessive and dominant pattern of inheritance. The clinical manifestation of the Clouston syndrome includes normal teeth, sweat glands, and palmoplantar keratoderma (PPK), nail dystrophy (micronychia or anonychia) and hair loss. The disease is caused due to the mutations in *GJB6* gene located on chromosome 13q12 pericentromeric region encoding for Connexin 30 (Cx30) also known as Beta 6 Gap junction proteins. GJB6 express in different organ of the body such as esophagus, bone marrow, brain, and skin. Connexin proteins make up the hemichannel influence, selectivity of the gap junction. This gap junction connects cells by transmitting nutrients, ions, and signaling chemicals that coordinate cell activity. The GJB6 mutation results in a gap junction defect and is linked to Hidrotic ED. Four transmembrane segments, two extracellular loops, a cytoplasmic loop created by the two inner transmembrane segments, and the N- and C-termini, which are all in the cytoplasm, are all present in each connexin protein. Connexin 30 (Cx30), is encoded by the human GJB6 gene and has 261 amino acids. Five mutations, G11R, V37E, D50N, A88V, and N14S, have so far been identified in individuals with Clouston syndrome; they are all associated with nonsynonymous mutations. Moreover, the Clouston syndrome is also associated with mutations in the genes GJA1 (V41L) and GJB2 (R127H). This study was aimed to properly diagnose an autosomal recessive pattern of HED2 (Clouston syndrome). Illumina HiSeq 400 (Illumine San Diego, CA, USA) sequencing technology was performed for the screening of mutations in our selected family of HED2. Already reported missense mutation (c.209C>T in GJB6 gene) was identified in the proband and will further be validated by Sanger sequencing. In silico analysis shows that this variant is the likely cause of HED2. However, protein expression studies should be carried out to further explore the effect of this mutation accordance to their functional and structural role in disease pathogenicity.

Chapter 1

Introduction

Single human largest organ is the skin, Having a $2m^2$ surface area of and weight of 3.6 kg of an adult. Along with cytokines, hormones and neuropeptides that have effects biologically both locally and systemically on the skin across the entire body, it also generates antimicrobial peptides that fight infections. The underlying mesenchyme and the surface ectoderm work together to create the skin system. It is composed up of the appendages of skin, which have inclusion of sweat glands, nails, hair follicles and sebaceous glands. Skin is composition of three layers, epidermis layer, dermis, hypodermis layer. It performs vital tasks including protection, the preservation of electrolytes and water, control of temperature, water storage and fat acting as a life-support linkage between the body and environment.

1.THE EPIDERMIS

The skin's outermost layer, the epidermis, has a total thickness that can range from 0.5 millimeters (eyelid) to 1.5 millimeter's (palms and soles). Stratified squamous epithelial layer create this, and is mostly made up of binary systems of keratinocytes and melanocytes [1]. The stratum Basale, which is the epidermis' innermost layer, as well as the stratum spinosum, stratum granulosum, stratum lucidum, and stratum corneum are among the epidermis' layers (the most superficial portion of the epidermis). The epidermis is made up of several layers, including the stratum corneum, the stratum spinosum, the stratum granulosum, and the stratum basale, which is the deepest part of the epidermis (the most superficial portion of the epidermis).

1. a Basal stratum:

The stratum germinativum, also known as the lowest layer, is linked by hemi-desmosomes to the basement membrane and is separated by the (basal lamina) basement membrane from the dermis. This stratum contains mitotically active stem cells from columnar to cuboidal that constantly produce keratinocytes. This stratum also contains melanocytes.

1.b Spinosum stratum:

The prickle cell layer, which is composed of 8–10 number of cell layers, is composed of polyhedral cells of irregular with cytoplasmic extensions, often known as "spines," that reach out and contact neighbouring cells through desmosomes. Dendritic cells can be found in this stratum.

1.c Granulomatous stratum:

Diamond-shaped cells with lamellar and keratohyalin granules are found; there are three to five cell layers. Keratin precursors are present in keratohyalin granules, which eventually get form bundles and crosslink. The lamellar granules contain the glycolipids that are discharged to the surface of the cells and act as a binder to hold the cells together.

1.d Lucidum stratum:

Keratohyalin, produces a transformation product Eleidin, is present in a thin, transparent layer between two to three cell layers of the thick skin seen on the soles and palms.

1.e Corneum stratum:

Nucleated squamous cells and top layer is composed of 20–30 cell layers and is composed of horny scales and keratin compose of inactive keratinocytes. This layer's thickness varies the most, especially in callused skin. First line of our immunological defense is composed up of defensins, which are released by dead keratinocytes in this layer.

1.1.1 The epidermis' cells

- Keratinocytes epidermal cells
- Melanocytes epidermal cells
- Langerhans’ epidermal cells
- Merkel’s epidermal cell

1.1.1a Keratinocytes epidermal cells

The most of the epidermis's cells, known as keratinocytes, are found in the basal layer. In addition to producing keratin, keratinocytes also secrete and create lipids that help to build the epidermal water barrier. Keratinocytes regulate calcium absorption by triggering the production of vitamin D from the activation of precursors of cholesterol by UVB light.

1.1.1b Melanocytes epidermal cells

Skin gets its color from melanin which is the main product of melanocytes and descended from neural crest cells. They generate melanin and are situated between basal stratum cells. Production of melanin increases the UVB light, which act as a natural sunscreen by protecting from UV rays. Tyrosine converts to DOPA by an enzyme tyrosinase, which causes the production of melanin protein. The lengthy processes connecting the melanocytes to the nearby epidermal cells are then used to transport melanin from one cell to the next. Basal keratinocytes get melanin granules from melanocytes through protracted procedures. Melanin is delivered to neighbouring keratinocytes by "pigment donation," which involves keratinocytes phagocytosing the tips of melanocyte processes.

1.1.1c Langerhans' epidermal Cells

The skin's initial line of defense is made up of Langerhans cells, also called dendritic cells, which are important for presentation of antigen. These cells, require specific stains to be seen which mostly present in the stratum spinosum, these mesenchymal stem cells from the bone marrow's CD34-positive stem cells are a part of the mononuclear phagocytic system. They include Birbeck granules, cytoplasmic organelles resembling tennis rackets. These cells express MHCI and MHCII molecules, and they also take up cutaneous antigens and transport them to the lymph node.

1.1.1d Merkel epidermal Cells

Merkel cells are oval-shaped modified epidermal cells in the stratum Basale, on the top of the basement membrane. Although they may also be present in the soles, palms, vaginal mucosa and oral. these cells have a sensory role for mild touch as mechanoreceptors and mostly concentrated in the fingers. Their membranes communicate with skin's free nerve endings, and desmosomes link them to nearby keratinocytes. They also have keratin filaments in the middle.

1.2 Dermis

The papillary and reticular layers of connective tissue, which mix without a distinct boundary to form the dermis, are attached at the level of the basement membrane to the epidermis. Thin layer of loose connective tissue on top that contacts the epidermis is known as the **papillary layer**. Layer that is less cellular thicker and deeper known **reticular layer**, it is composed up of thick connective tissue and collagen fiber bundles. The dermis is where the hair follicles, muscles, sensory neurons, sweat glands and blood vessels are all found [2]. An interconnected system of filamentous, fibrous, and amorphous connective tissue makes up the dermis, that allows for stimulus-induced entrance of fibroblasts, mast cells, macrophages, neurons and vascular networks. Other blood-borne cells including plasma cells, lymphocytes, and other leukocytes can also trigger their way into the dermis. Much of the skin is made up of the dermis, which gives the skin its elasticity, tensile strength, and pliability. It protects the body from physical harm, helps regulate body temperature, attracts water, and has receptors for sensory inputs. To preserve the characteristics of both tissues, the dermis and epidermis interact. The two areas work together to form the epidermal appendages and dermal-epidermal junction throughout development. They also communicate to repair and rebuild the skin as wounds heal. The form and organization are predictable of the connective tissue components in a depth-dependent way, However, unlike the epidermis, the dermis does not go through an obvious differentiation sequence. Collagen and connective elastic tissue are two parts of the matrix, also depth-dependently variable and go through turnover and remodeling in healthy skin, pathologic processes, and in reaction to environmental stimulant. Apart from melanocytes like nerves, are derived from the neural crest, the components of the dermis are mesodermal in origin. The dermis is nothing more than a collection of dendritic-shaped cells that are rich in acid-mucopolysaccharides and act as fibroblast precursors up until the sixth week of fetal development. By the 12th week, reticulum fibers, elastic fibers, and collagen are being actively synthesized by fibroblasts. By the 24th week, a vascular network has developed and the fat cells under the dermis may be seen. The adult dermis has bigger collagen bundles than does the newborn dermis, which is made up of smaller collagen bundles. Infant dermis has many fibroblasts, but only a small number live until maturity. The primary element of the dermis is collagen, a fibrous family of proteins having at least 15 genetically distinct varieties in human skin. ligaments, Tendons, the dermis, and the lining of bones all contain collagen, a vital structural protein for the whole body. The main substance in the skin that is resistant to stress is collagen. On the other hand, elastic

fibers help keep the skin's suppleness but do very little to prevent ripping and distortion. Spare collagenases, proteolytic enzymes that break down collagen and replace it with new fibers, keep the collagen fibers moving constantly, 70% of the dry weight of the skin is made up of collagen. A particular helical polypeptide chain, which is procollagen molecule, incorporated by fibroblasts. The fibroblasts are then secreted by the cell, where they come together to form collagen fibrils. Collagen is greatly enriched by the amino acids glycine, hydroxyproline, and hydroxylysine. The primary category and most prevalent proteins in the body are the fibrillar collagens, which are present in the skin. Type I collagen is the dermis' main building block. Papillary and adventitial dermis have loosely positioned collagen fibers, but the reticular dermis has substantial collagen bundles. Collagen Type IV is present in the basement membrane zone, while collagen type VII, produced in large amounts by keratinocytes, is a primary structural element of anchoring fibrils. The elastic fiber is composed of two elements: protein filaments and the amorphous protein elastin and varies from collagen both structurally and chemically. Elastic fiber and the glycosaminoglycan-based extracellular matrix of the dermis are fused together by the fibroblast. In the reticular dermis, the fibers are coarse, they are fine in the papillary dermis though. Hyaluronic acid is the major mucopolysaccharide that accumulates in pathologic situations, although making up a minor part of the dermis in a healthy individual [3].

1.3 Hypodermis

The hypodermis, often known as subcutaneous tissue, is not regarded as skin. Its main purposes are to insulate the body from heat, guard against mechanical trauma, and store energy. Through this layer, the main blood vessels and nerves enter the skin. It mostly comprises of adipose, or fat, cells and irregularly organized fibers that attach to the dermis. Between this layer and the dermis, there is no distinct dividing line. The flexible link between the skin and the other inside soft tissues is provided by the hypodermis. Vertical fibrous septae that connect the hypodermis to the dermis loosely allow for some skin movement across most of the body. To draw a wound together and seal it with sutures, these septae frequently need to be sliced during surgery. Adipose tissue's lobules or chambers are divided by collagen fiber threads called septae. The connection sites between the dermis and hypodermis are more noticeable when these chambers are too packed with fat because the skin's surface has a bumpy appearance. The term "cellulite" or "gynoid lipodystrophy" refers to this look. The hypodermis thickness varies across the body, being thinnest

on the eyelids and thickest on the abdomen [4]. The disordered arrangement of fibers is most visible at the hypodermis' deepest extent, which is mostly free of fat. Compression, expansion, shearing, and stretching may be shown to cause it to consolidate and split to generate new vacuoles. These vacuoles' polyhedral form has been shown to be the most effective 3D structure for preserving space and fiber confirmation. The gelatinous fibers create polyhedral gaps, which scale down movement to each individual microvacuole, distributing the force into minute changes in sliding tissue movement. This allows for huge excursions to occur. The "microvacuolar" tissue of the hypodermis acts as an active reservoir for interstitial fluid, which may dynamically alter the structural stiffness of the tissue. The hyaluronan, glycosaminoglycan, and proteoglycan makeup of the matrix serves as a sponge for interstitial fluid when the capillary osmotic pressures are exceeded, such as during inflammation. The tissue's fibrous components, which have been proven to actively contribute to either raising or lowering the interstitial compartment pressure through cytoskeletal strain that the cells impose on the collagen fibrils, serve to restrict tissue swelling. As a result, the body's homeostasis and fluid distribution depend on the integrity of the microvacuolar tissue. It has been suggested that the fundamental components of biological form are microvacuolar units [5].

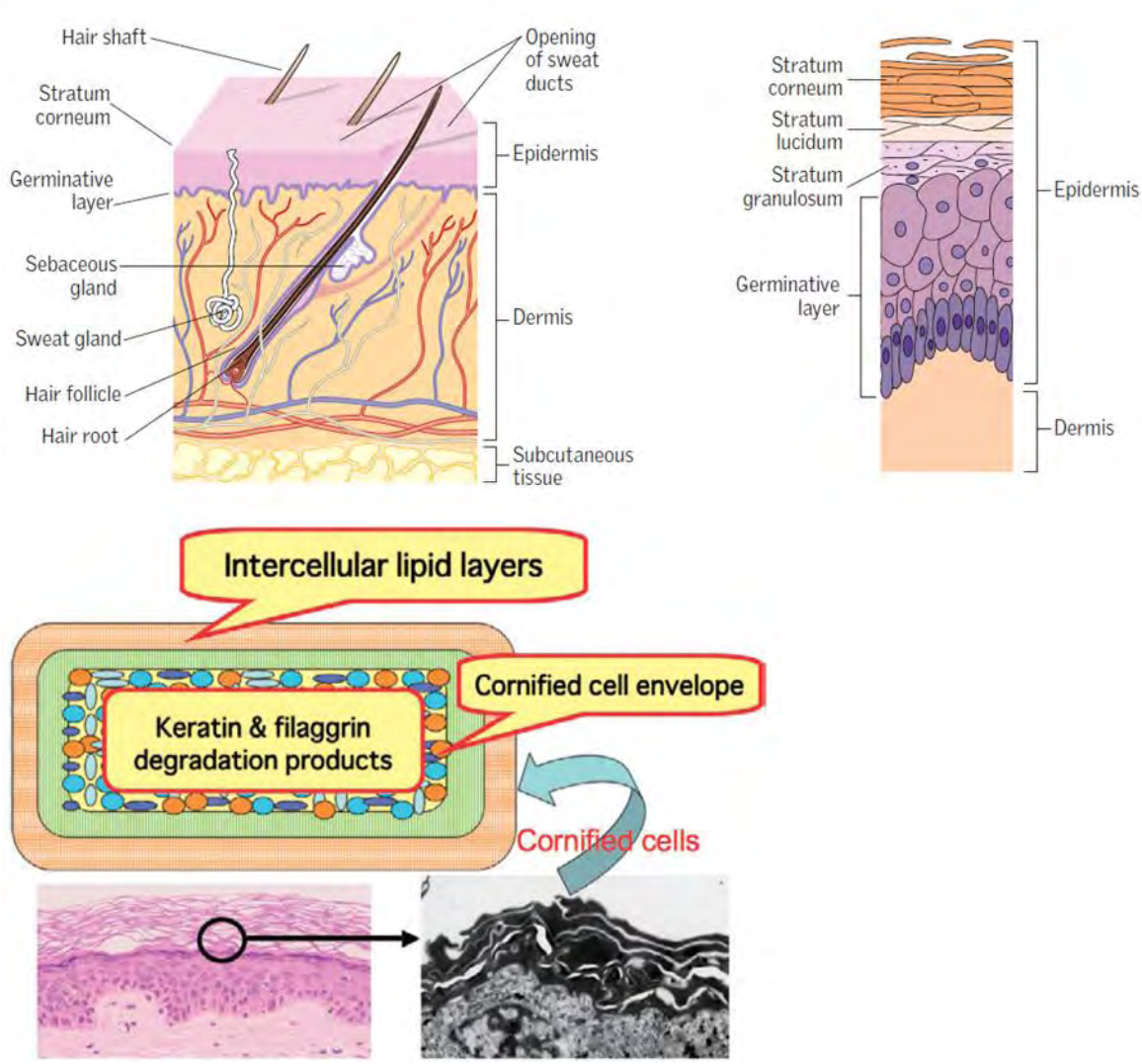

Figure 1.1: Structure of the human skin consisting of three layers i.e., Epidermis, dermis, and Hypodermis layer [6].

2. Genetics

It is well knowledge that the area of human genetics saw a tremendous expansion in the 20th century. However, less is known about the fact that over two millennia ago, people had understood some of the fundamental ideas underlying the molecular foundation of life, as well as the fundamental principles governing heredity. Some remarkable individuals throughout the *Hellenistic* and *Roman* eras were influenced by *Epicurean* philosophy, which helped them examine human nature and make some suggestions that have just lately come to be accepted as truths. "Plants give birth to plants and people give birth to humans from substance corresponding to each

creature," observed by *Aristotle*. *Stagirite* rejected the idea that females supplied genetic material since he believed that only males did so through sperm, while females provided the environment essential for the growth of the fetus, analogous to the seed and the field, respectively. *Epicurus* studied families and deduced that males and females equally supplied genetic material to their kids, in contrast to *Aristotle* who thought that only males contributed to heredity. *Epicurus* was therefore able to explain dominant, recessive, and co-dominant patterns of heredity in 1949. Human genetics is a relatively new field of study, according to *Curt Stern*, a human geneticist who worked at the *Kaiser-Wilhelm* Institute for Biology in Berlin-Dahlem until 1933. Plants and animals immediately contributed to the contemporary study of heredity that started around 1900 [7].

The quick implementation in the clinic of the revolutionary genomic technologies that spurred original research findings has been a defining feature of medical genetics, particularly as it relates to uncommon disorders. For almost all clinical manifestations caused by large-impact alleles, there are now focused genetic tests available, coupled with more thorough genome-sequencing assays that, when required, allow the investigation of a broader list of important genes. In many medical professions, Genetic testing is frequently used for symptomatic individuals and at-risk families. Parallel to the introduction of medicines that target mutational events into clinical practice, the somatic cancer diagnostics use has expanded (these developments are reviewed elsewhere).

Patients frequently prefer targeted panels as their platform of choice whose symptoms suggest a monogenic aetiology is most probable (such as retinal degeneration, cardiomyopathy, or hearing loss), however they are increasingly carried out on a more comprehensive sequence backbone. Testing has shifted toward the early adoption of genome and exome-sequencing plan that offer quick decision of for more complicated phenotypes—those that lack a clear match to a single syndrome, such neurodevelopmental disorders, and several congenital anomalies—diagnosis has historically been a horrific ordeal. For people who appear with monogenic neurological defects and alarmingly unwell babies, the value of genomic diagnosis is particularly evident. Sequencing of the triad of parents and children can find unusual phase biallelic de novo variations in recessive diseases and de novo variations in dominant disorders.

Recursive reanalysis, which may include reinterpreting certain sequences based on later findings on causative disease alleles and their phenotypic effects, is made possible by the switch from focused gene assays to genome sequencing. To reliably identify a portion of genetic illnesses, such as those caused by triplet repeats and complicated rearrangements, new molecular diagnostics are needed. Deep sequencing of affected tissues for mosaic variants and the use of RNA sequencing to find noncoding mutations that induce early-onset disease (for example, through effects on splicing) provide new fronts for clinical diagnosis.

Recessive carrier panels and non-invasive prenatal testing (more than ten million tests by 2018 across multiple countries) are two options for couples considering getting pregnant are two other instances of the new genomic technologies being quickly adopted. Many nations now require newborn screening; but only for ailments that can be identified with high throughput, low cost, and efficient early intervention (such as diet restrictions or enzyme replacement). As a follow-up test after a defected (for instance, metabolic) screening test, genetic diagnostics are being increasingly used in newborn screening. The range of illnesses detected by newborn prenatal and screening testing is projected to significantly increase during the following ten years. Although prenatal testing may be more successful in preventing illness, there are more complicated ethical concerns.

The application of genetic data in other areas of healthcare has lagged, despite the rapid expansion of genetic testing for cancer and uncommon illnesses. The implementation of pharmacogenetics, for instance, has been impeded by several factors, notwithstanding several instances of clinically important genetic indicators associated to drug effectiveness and side-effect profile, there is no clinical decision support in electronic medical systems to guide the doctor's selection of medicine or dose. Challenges with diagnostic testing have made this situation worse: to find all clinically significant variations, genome sequencing or specialized targeted group are required due to structural variants and complicated haplotype structures at several important drug metabolism sites.

Translational research for common disorders is being focused on the therapeutic potential of polygenic risk scores. Robust polygenic scores have been developed for a variety of prevalent

illnesses as a result of improved algorithms for aggregating data across millions of single-nucleotide polymorphisms, bigger GWAS datasets, and large-scale biobanks that facilitate score validation [8].

2.1 Medical Genetics

Around 1960 or a bit earlier, or around 63 years ago, medical genetics started to take shape as a distinct field, just like human genetics did 15 years earlier from more general genetics. Medical genetics did not evolve uniformly or according to a predetermined plan, but by the early 1980s, it had become a well-established and fast expanding area of medicine with presence at several medical facilities across North America and Europe. However, we must first review a few definitions that we touched on in the beginning.

In contrast to human genetics, which is described here as the science of human inheritance, medical genetics is the study of genetic problems as a component of medicine. Inherited diseases represent a continuum between the two that has never been clearly defined [9]. Over the past few decades, medical genetics and genomics have seen increased usage in clinical practice along with our understanding of the human genome [10]. Despite these developments, a few obstacles still stand in the way of fully integrating medical genomics and genetics into standard medical practice. These obstacles include ethical doubts, patient worry, insurance discrimination, lack of access to genetics knowledge, and lack of time for practitioners to stay current with developments.

These barriers could make it less feasible to apply medical genetics and genomics in the clinic, which would be detrimental to patients. As more clinical genetic and genomic tests become available on the market each year, the danger of advantages being lost will increase. The fields of genetics and genomics have an influence on almost all medical professions. Since 10% of Americans have a rare condition and up to 80% of them are treatable, it is ideal for doctors in all specialties to feel at ease using medical genetics and genomics in patient treatment [11].

Personalized medicine, which applies medical genetics and genomics to the risk assessment, diagnosis, and therapy of common disorders, has the potential to significantly alter medical

practice and enhance patient outcomes. This field of study goes beyond uncommon diseases [11-14]. To extend the use of personalized medicine techniques, it is urgently necessary to teach present and future physicians in the use of medical genetics and genomics. This requirement is made even more urgent by the fact that there are not many doctors who are board certified in these fields [11, 12, 15-17].

2.1.1 Principles of Medical Genetics

Guidelines for reporting secondary discoveries in the context of clinical exome and genome sequencing (ES/GS) were previously published by the American College of Medical Genetics and Genomics (ACMG) in 2013 and 2017 [18-20]. The ACMG Board of Directors (BOD) convened the Secondary Findings Maintenance Working Group (SFWG) to assess the need for a minimum list of genes that should be evaluated in patients undergoing clinical ES/GS based on the medical action ability of the associated condition. The SFWG developed these recommendations. In the past, lists of the genes to be examined and policy suggestions for the kinds of variations to return were also present.

The ACMG, SFWG, and BOD have decided that the list of suggested genes should henceforth be revised yearly considering the rise in the use of clinical ES/GS. Updates to the policy governing the goal, reach, and procedure for maintaining the ACMG Secondary Findings List are being made available separately [21]. Monogenic diseases are frequently transmitted through the germline and are widespread in human populations. Unfortunately, there are typically few therapeutic options available once the mutation has been passed on to a kid.

However, methods for treating gametes or preimplantation embryos with nuclear and mitochondrial DNA abnormalities that because illness have recently been established and are known as germline gene therapy (GGT). We will talk about these cutting-edge tactics and lay out a future course for safe, high-efficiency GGT that could offer a potential new paradigm for stopping the transmission of harmful genes from one generation to the next [22].

3.Common heritable monogenic disorders

There are five basic inheritance patterns for germline mutations: autosomal dominant, autosomal recessive, X-linked recessive, or mitochondrial maternal, X-linked dominant. There are examples of every in ***Table 1***. Each of the 22 pairs of autosomal chromosomes, apart from the X and Y sex chromosomes, may only contain one autosomal mutation. Only one copy of the mutant gene is required for a carrier to be affected if autosomal dominant mutations is there, while both the copies must have mutations if the illness is autosomal recessive. Males with X-linked mutations usually have problems.

Only if females acquired two copies of the defective X chromosome would they be susceptible to the effects of an X-linked recessive mutation. Homozygous carriers of a recessive harmful mutation frequently pass away extremely young. However, certain dominant monogenic diseases—known as "late-onset adult manifestation diseases"—affect people later in life. Examples include Huntington's disease, hypertrophic cardiomyopathy, and malignancies brought in the *BRCA1/2* genes by mutations.

Many dominant mutations are successfully able to circumvent natural selection by remaining in the following generation when disease first manifests after sexual maturity. Mutations in the (mtDNA) mitochondrial gene are considered to as maternally inherited if they solely develop through the maternal line. Consequently, if a man has an mtDNA mutation, his children won't catch the disease from him. Sickle Cell Anemia, Cystic Fibrosis, Huntington's Disease, Marfan's Syndrome, Tay-Sachs Disease, Hemochromatosis, Spinal Muscular, thalassemia, Atrophy Type 1, and Color Blindness are a few well-known hereditary genetic illnesses that affect people ***Table 1***.

The most common deadly genetic autosomal recessive condition in the white population is thought to be cystic fibrosis. Cystic fibrosis-causing gene mutations affect *1/2,500* individuals, has a *1/25* heterozygous carrier frequency. The disease is caused by a mutation in the CFTR gene, which controls the function of the membrane conductance regulator in the body. For this condition alone, nearly 2,000 different CFTR mutations have been documented.

Missense mutations are the most prevalent mutations, although there have also been reports of splicing, frameshifts, insertions, in-frame deletions, and nonsense mutations. The F508 del variation, a 3-bp loss that inhibits normal protein folding, is the most prevalent CFTR mutation. Due to the great prevalence of CFTR mutations globally, heterozygous carriers, or those who contain at least one copy of a pathogenic gene, number in the hundreds of millions and are all capable of passing the mutation to their progeny [22-25].

Table 1.1 Examples of monogenic genetic disorders that can be treated with GGT

Disease	**Inheritance pattern**	**Location or Gene**	**A projected frequency.**	**The disease's onset age**
Cystic fibrosis [26]	AR	CFTR	1 in 2,500	Newly born
Tay–Sachs disease [27]	AR	HEXA	Rare in Ashkenazi Jews (1 out of 3,600)	3–6 months
Huntington's disease [28]	AD	HTT	4–15/100,000	30–50 years age
Marfan syndrome [29]	AD	FBN1	1 in 5,000–10,000	Teen - adults
Ovary and breast cancers that are genetically induced [30]	AD	BRCA1&2	Over 2% Ashkenazi Jews have	Adults
Cardiomyopathy with hypertrophy [31]	AD	MYBPC3	1 in 500	Teen - adults

Color blindness to red and green [32]	XR	OPN1LW, OPN1MW, and OPN1SW	1% of women up to 7–10% of men	Childhood
Leigh syndrome [33, 34]	Maternal	MtDNA	1 in 40,000	1 year to adults
Leber's Genetical optic neuropathy [35]	Maternal	mtDNA	1/ out of 50,000 in Finland	Younger adult
Beta-thalassemia [36, 37]	AR	HBB	1 in 100,000	In first 2 years
Muscle atrophy of the spine [38]	AR	SMN1	1 in 8,000–10,000	Newborn

4.Inherited skin disorders

A hereditary condition known as genodermatosis manifests as multisystem involvement. Please contribute to the identification of widespread mutations and horrifying recessive inherited skin conditions. Given how common genetic diversity is, molecular diagnosis requires a lot of work [39].

An inherited skin disorder which is associated with structure and function of is refers to genodermatosis. Research Center of the genetics is persistent in its search for the underlying molecular causes of such horrifying skin conditions with recessive inheritance. 100 patients with genodermatosis were referred to the center during the years 2011–2013, Ichthyosis was the most common, followed by Cutis laxa, progeroid disorders, ectodermal dysplasia ***Table 2***, epidermolysis bullosa, albinism, and precancerous conditions *Rothmund Thomson syndrome*, dyskeratosis congenital, dyskeratosis congenita, and xeroderma pigmentosum [40].

Human genodermatosis are a diverse group of several uncommon illnesses with confluent and overlapping characteristics that can make it difficult for an individual with one of these diseases to receive an accurate diagnosis. Methods for high-throughput sequencing have accelerated the identification of novel genes and made it significantly easier to establish genetic diagnoses for such a wide range of illnesses. The precise genetic diagnosis of a skin ailment is crucial for providing patients and their families with the right counselling regarding the course of the disease, recurrence concerns and prognosis.

Grasp the illness and creating specialized, targeted, or personalized therapy methods require an understanding of the underlying pathophysiology. In order to give a thorough description of human genodermatosis and the genetic causes that have so far been identified [41]. Over the past few years, the number of genodermatosis that are known has steadily increased. In 1991, *Moss* [41, 42] reported 90 geographically specific cutaneous features; After a further 15 years, this number increased to 580 [43].

This number has grown further because of major technical advancements over the previous several years and new sequencing techniques. A specific genodermatosis can occasionally result from genetic flaws in many genes (genetic heterogeneity), much as other monogenetic disorders. As opposed to that, various disorders occasionally have corresponding underlying genic abnormalities (allelic variants). Although it appears that a single gene deficiency is responsible for more than 80% of hereditary skin disorders, more than 80% of gene faults only result in one disease [44].

However, only a small portion of the present genetic information has found its way into therapeutic practice. This may be in part because of [42], the vast quantity of genetic information, which can occasionally seem difficult to obtain, evaluate, and filter for pertinent information [43]. inconsistent terminology for things named for phenotypic vs genotypic characteristics, difficulty in making a precise clinical diagnosis since phenotypes are frequently overlap and non-specific, and the frequent absence of immediate treatment benefits following genetic testing.

An accurate genetic diagnosis, however, may be very beneficial in advising the family and patient about risks of recurrence and prognosis, and in certain circumstances, it may even direct therapy. The first step in creating individualized therapy strategies that focus on the actual molecular

deficiency is understanding the underlying genetic abnormality and its resulting patho-mechanisms. Beyond that, even knowing that a condition is now incurable and can be deadly might assist avoid needless and frequently stressful diagnostic tests as well as fruitless treatment endeavors. Thus, understanding the patient's genotype is more advantageous than a purely clinical alignment of the phenotype in terms of treatment decisions [45].

Table 1.2. Inherited disorders Ectodermal dysplasia of ectodermal appendages

Gene	Gene MIM No.	Disease	Phenotype MIM No.	Inheritance
GJB6	604418	Clouston type, Ectodermal dysplasia	129500	AD
GJB6	604418	Clouston type, Ectodermal dysplasia 2	129500	AR

4.1 Classification

- Chromosomal
- Single gene
- Polygenetic [39, 46]

4.1.1 Ectodermal Dysplasia

A collection of hereditary diseases. At least two anatomical structures that are derived from the ectoderm fail to form. inheriting in one of the following ways: Autosomal recessive or dominant X-linked.

4.1.2 Ectodermal dysplasia Hypohidrotic

Reduced expression of the epidermal growth factor receptor in the vicinity of the band Xq-12-q13.1's long arm, Gene ED1 is incharge. Phenotypically identical to the X-linked type; autosomal recessive where the gene is found the (downless) DL locus [47, 48].

4.1.3 Hidrotic ectodermal dysplasia

The causal gene is GJB6. This is situated in the pericentromeric region of chromosome 13q and encodes for connexin 30. Patients with the cleft lip and palate mutation PVRL 1, which codes for a cell-to-cell adhesion molecule or herpes virus receptor, have fewer sebaceous glands, hair follicles, and sweat glands. Ectasia of ducts and inflammatory alterations may be seen in the salivary glands [49].

4.1.4 White sponge nevus

A mutation in keratin-4 or keratin-13 that impairs the oral mucosa's natural keratinization is inherited as an autosomal dominant trait, high penetrability and varying expressivity [46].

4.1.5 Genetic, benign, intraepithelial-dyskeratosis

A triracial anomaly (native American, white, and black). autosomal dominant transmission. Triple alleles for two connected markers are produced by the duplication of a region of DNA at 4q35, which suggests that the illness first manifests in childhood due to gene duplication. The mouth sores resemble white sponge nevi in appearance. Leukoedema's characteristic opalescent look can be seen in milder forms. a secondary candida infection. develop throughout the early years. The oral lesions resemble white sponge nevi in appearance. Leukoedema's characteristic opalescent look can be seen in milder forms. extravagance of a candida infection [50].

4.1.6 Pachyonychia congenital

As an autosomal dominant characteristic, inheritance. specific keratin sixteen gene mutations (Jadassohn–Lewandowsky type) The *Jackso-Lawler* type has keratin 17 gene mutations related with it. The *Jadassohn-Lawandowsky* variant of the oral lesions can be noticed. Cheek mucosa and white plaques on the tongue. There is also perinuclear clearance of epithelial cells, hyperplasia, and keratoses. The raised free edges of the nails are caused by a deposit of keratinaceous material in the nail beds. The plantar surfaces and palmar, which results in dense, callous-like sores. Punctate papules, which are a keratin accumulation that is abnormal in the follicles of hair are visible on the remaining skin. after a short while of walking in warm weather, On the bottoms of

the feet, painful blisters are frequently developed. the removal of the epithelial cells' perinuclear, there is significant hyperplasia, keratoses, and acanthosis [39, 51].

4.1.7 Dyskeratosis congenital

It is an X-linked recessive disorder that is inherited, Noticeable preference towards men. Less frequently seen are AR and AD types. Gene changes in the DKC1 protein the mutant gene seems to interfere with telomerase's regular maintenance. The affected regions are neck, upper chest, and face by the development of skin hyperpigmentation. Bullae form on dysplastic alterations of the buccal mucosa, intraoral nails, and tongue; they are then followed by abrasion and, ultimately, leukoplastic lesions. Lesions that are leukoplakic thought to be precancerous. The first hematologic issue to manifest itself is often thrombocytopenia. Then comes anemia, Aplastic anemia eventually sets in. epithelium atrophy together with hyperorthokeratosis. Epithelial dysplasia grows as the lesions advance, eventually developing into frank squamous cell carcinoma [52].

4.1.8 Xeroderma pigmentosum

Autosomal recessive traits are inherited. caused by a variety of flaws in the DNA's excision repair and/or post-replication repair processes. the epithelium cells' incapacity to heal UV light-induced damage, greater propensity for sunburn. Soon after, Atrophy, freckly pigmentation, and patchy depigmentation are all present. Actinic keratosis first manifests in infancy.

Lesions develop into squamous cell carcinoma quite fast. Skin cancers such as melanoma, non-melanoma, and basal cell carcinoma, also appear before the age of 20. Carcinoma Squamous cell development on the lower lip and the tip of the tongue Skin cancers and premalignant lesions that are not particular are identical to those found in healthy individuals under the microscope [53].

4.1.9 Incontinentia pigmenti

A dominant X-linked characteristic that is inherited. For most guys, an unpaired gene is fatal on the X chromosome. Patients that are affected exhibit chromosomal instability. mostly impacting

oral tissues, eyes, skin and the (CNS) central nervous system begin during the vesicular period of infancy; lesions that are vesiculobullous develop on the limbs and trunk of the skin of the trunk. Within 4 months, spontaneously settling takes place. Verrucous stage, the limbs are affected by the development of verrucous cutaneous plaques.

They are obvious by the age of six months. Stage of hyperpigmentation: macular, dark skin lesions that have an odd swirling pattern start to emerge. Stage of atrophy and depigmentation: Ultimately, the skin experiences depigmentation and atrophy. Central nervous system abnormalities Some of the linked symptoms include mental retardation, seizure disorders, motor problems, strabismus, cataracts, abnormalities of the oligodontia (hypodontia), optic nerve atrophy, hypoplasia of the teeth, retinal blood vessels and delayed eruption. Permanent and the primary both dentitions are impacted by the tiny, conical teeth. Histopathology: vesicular stage; eosinophil-filled intraepithelial clefts are seen. Hyperkeratosis, acanthosis, and papillomatosis are seen at the verrucous stage. The sub-epithelial connective tissue exhibits a large number of macrophages containing melanin (melanin incontinence) during the hyperpigmentation stage [54].

4.1.10 Keratosis follicularis

Defects in the central nervous system (CNS) like strabismus, mental retardation, seizure disorders, cataracts, motor challenges, anomalies of the retinal blood vessels, delayed eruption, atrophy of the optic nerve, oligodontia (hypodontia), and teeth hypoplasia. Both the primary and permanent dentitions are impacted; the teeth are cone shaped and tiny. Vesicular stage in histopathology; eosinophil-filled intraepithelial clefts are seen.

At the verrucous stage, papillomatosis, acanthosis, and hyperkeratosis are noticed. In the subepithelial connective tissue, the hyperpigmentation stage is evident by the large number of melanin-containing macrophages. The sole and palm have pits and keratosis, and the offensive odor is brought on by bacteria that break down the keratin. Nails have ridges, longitudinal lines, or excruciating cracks. because of greater perspiration or susceptibility to UV radiation in the summer, which results in more epithelial clefting, cobblestone [55].

4.1.11 Warty dyskeratoma

The same histo-pathologically as Darier's illness. The lesion is therefore referred to as an individual case of *Darier's* illness. It shows up on the skin as a single, umbilicated papule with no symptoms. Patients above the age of 40 might also develop intraoral lesions. over the keratinized mucosa, notably the and the alveolar ridge and hard palate, a white or pink, umbilicated papule, is the outward sign of an intraoral warty dyskeratoma, a suprabasilar cleft as well as dyskeratosis. Corps ronds and grains are forming, although not especially visibly [56].

5. *Clouston Syndrome* or Hidrotic ectodermal dysplasia 2 (HED2)

Hidrotic ectodermal dysplasia 2 clinical manifestations, also known as *Clouston syndrome* or HED2, include partial to full baldness, nail dystrophy, and palmoplantar hyperkeratosis. There is generally no dental anomaly and sweat is maintained. Early in life, dysplastic nails and sparse scalp hair are seen. Scalp hair is fragile, thin, and scant throughout infancy. By the time a child reaches adolescence, complete alopecia may have developed.

Nails may be milky white in Early childhood, with time, they slowly swell, become dense (in around 30% of afflicted individuals), and detach from the nail bed. Although not always present, palmoplantar keratoderma (also known as hyperkeratosis of the palms and soles) can start in childhood and get worse as people age. Skinny hyperpigmentation (especially around the joints) and finger clubbing are possible companion characteristics. Even within the same family, the clinical symptoms might vary greatly. HED2 is passed down by autosomal dominant inheritance.

Although de novo pathogenic mutations have also been found, most people with HED2 had an afflicted parent. Affected persons' offspring have a 50% probability of inheriting the virulent mutation and developing the condition. If the causative *GJB6* pathogenic variant has been identified in an affected family member, preimplantation genetic testing for HED2 and prenatal testing for a baby at increased risk are also alternatives [57].

Thurman was first to described HED in 1848, and later by *Darwin* in 19th century. It was assigned by *Thadani* to the X chromosome in 1921 [58-60]. Who subsequently revealed that carrier females

might show symptoms of the illness. It wasn't until *Mary Lyon* proposed that each female cell randomly inactivates one X chromosome early in embryogenesis that the full implications of this was understood [61, 62]. All racial groups and regions of the world are affected by HED. *Stevenson* and *Kerr* calculated the incidence at birth as *1 per 100 000* births based on the frequency of HED in *Oxfordshire*, This assessment would be supported by personal experience [63].

5.1 Differential Diagnosis

There are several varieties of hidrotic ectodermal dysplasia, and more varieties are probably to be discovered [64]. It's important to distinguish between Hidrotic Ectodermal Dysplasia 2 (HED2) and other Ectodermal Dysplasia's that might damage hair and nails.

Table 1.3. ED in the Differential Diagnosis of HED 2

Gene(s)	Disorder	MOI	Clinical Characteristics	Comment / Distinguishing Features
EDARADD EDAR WNT10A EDA	(HED) 1 Ectodermal dysplasia Hypohidrotic	AD XL AR	• Hypotrichosis: thin, lightly pigmented, slow-growing scalp hair. • Hypohidrosis: deficient sweating w/ episodes of hyperthermia • Hypodontia: few & abnormally formed teeth erupt, later than average	• Hypohidrosis & dental abnormalities are the major distinguishing features. • Eyelid papules may develop in WNT10A-HED.
GJB2	Keratitis-ichthyosisdeafness syndrome	AD	• Sensorineural deafness • Photophobia, corneal ulceration & scarring Progressive hyperkeratotic plaques & palmoplantar hyperkeratosis.	• Sensorineural deafness & ocular changes are the major differentiating features.

			• Sparse hair & nail dystrophy: less pronounced than in HED2	
HOXC13	Ectodermal dysplasia 9, hair/nail type	AR	• Generalized congenital atrichia. • Nail dystrophy	• Absent palmoplantar keratoderma
KRT6A KRT6B KRT6C KRT16 KRT17	Pachyonychia congenita (PC)	AD	• Hypertrophic nail dystrophy w/ subungual hyperkeratosis • Painful focal palmoplantar keratoderma & blistering • Variably present: oral leukokeratosis, pilosebaceous cysts, palmoplantar hyperhidrosis, follicular keratoses on the trunk & extremities, natal teeth	• Absence of hypotrichosis or atrichia is the main distinguishing feature. • Palmoplantar keratoderma is focal in PC (vs diffuse in most cases of HED2).
KRT74	Ectodermal dysplasia 7, hair/nail type	AR	• Generalized hypotrichosis or atrichia. • Nail dystrophy	• Absent palmoplantar keratoderma
KRT85	Ectodermal dysplasia 4, hair/nail type	AR	• Sparse or absent scalp hair • Absent eyebrows, eyelashes, pubic & axillary hair • Nail dystrophy	• Absent palmoplantar keratoderma

AD = autosomal dominant; AR = autosomal recessive; MOI = mode of inheritance; XL = X-linked

1. EDA pathogenic variants are associated with X-linked HED. *EDAR*, *EDARADD*, and *WNT10A* pathogenic variants are associated with autosomal dominant and autosomal recessive HED [65].

5.2 History

Having grown up in the little *Quebec* town of *Huntingdon*, which is roughly 50 miles from *Montreal*, Dr. *Howard Rae Clouston* took over his father's practice there. He was a great illustration of the contributions a general practitioner may bring to academic medicine. He was familiar with the families affected by the condition known as *Clouston's* Hidrotic ectodermal dysplasia when he was a little lad in Huntingdon (HED).

Later, he encountered several of them as his patients and learned of related families residing in *Montreal* and other communities close to the state of *New York*. His fascination in them turned into a pastime, and he kept a three-ring loose-leaf journal where he kept track of his findings. The notebook was a gift from Dr. *Clouston's* son, *James MacRae Clouston*, to *F. Clarke Fraser*. It is an extraordinary paper. On August 29, 2000, *FCF* and *V.M. der Kaloustian* gave it to the *Osler* Library of the History of Medicine at *McGill University*.

The book, *Lead Kindly Light*, was written and published by *Dr. Clouston's* daughter, *Marjorie Clouston Dale*, who kindly sent us a copy. The book has further information on *Dr. Clouston*. The notebook contains *Dr. Clouston's* observations on his patients as well as his notes on the ectodermal dysplasia-related literature he read. The potential syndromologist was *Dr. Clouston*. He mentions aberrant hair slopes, vertex whorls, upturned noses with upturned nares, protuberant jaws, clubbing of the terminal phalanges, and delayed epiphyseal unions in his descriptions of the patients [49, 66].

6. Aetiology and Molecular Mechanism of *Clouston syndrome*

During the crucial time windows for the ectoderm, a set of illnesses known as ectodermal dysplasia’s develop abnormally in the ectodermal structures. The link between disease-related and embryonic development-related genes is revealed by the embryonic development network. Nearly every month, new ectodermal dysplasia’s-related disorders are reported, while there may be issues with the ichthyosis group or the epidermolysis bullosa group, it is frequently thought that if more than two ectodermal appendages are damaged, the symptom complex should be automatically classed as ectodermal dysplasia.

In monogenic conditions, the skin frequently suffers. According to the *WHO*, there are over 10,000 monogenic disorders, about 3,000 genes have been cloned or isolated, and molecular causes of

about 1,000 diseases are known for more than one third of all *Mendelian diseases*, which are characterized by abnormalities or malformations of the skin and their appendages [67-71].

Spemann's organizer hypothesis states that even the endoderm contributes to skin formation. Only information exchange and interference between intracellular and intercellular structures of the mesoderm and ectoderm may lead to orchestrated skin growth. To induce skin appendages, mesodermal signaling pathways like wingless (*Wnt*) are essential. Mesenchymal and epithelial cells are both influenced by *Wnt* signaling. Ectodermal and mesodermal development are also a part of the ectodysplasin pathway; mesoderm induces the placodes of adnexal structures, and ectoderm develops into the epidermis [72, 73].

Genetic abnormalities in signaling networks, including the *p53* and ectodysplasin signaling pathways Factors that interfere with how the ectoderm and mesoderm interact lead to ectodermal dysplasia's. The *Wnt* system, which contains an inductive signal and originates from the mesoderm, is another mechanism that controls the development of ectodermal appendages. In contrast, the ectodermal dysplasin signaling pathway is crucial for maintaining placodes [74].

Mutations in the *TNF signaling* route can cause incontinentia pigmenti, anhidrotic ectodermal dysplasia's with and without immunodeficiency, and cylindromatosis because the *TNF signaling* system plays a significant morphogenetic function for ectodermal structures. Mutations in the ectodysplasin-A gene (EDA) can cause selective tooth agenesis. EDA mutations and other mutations in crucial signaling pathways for the ectoderm may not always cause syndromic ectodermal dysplasia diseases, but they can also cause non-syndromic ectodermal abnormalities [75-77].

Ectodermal dysplasia, according to *Priolo*, should be divided into two groups: group 1 exhibits aplasia of ectodermal structures, whereas group 2 exhibits changes in tissue homeostasis and developmental regulation [78]. Mutations in the genes responsible for the development of the feotal ectoderm cause the illness and other ailments of varied degrees of severity [79]. Only 30 of the approximately 200 distinct forms of ED that have been discovered thus far have been molecularly identified. X-linked recessive Hypohidrotic ectodermal dysplasia (HED) is the most prevalent and severe form of ED name as *Christ-Siemens-Touraine* Syndrome [80, 81].

Hidrotic ED (Clouston syndrome), which is often inherited as autosomal dominant but can also be inherited as recessive autosomal recessive, is the second most prevalent kind. Teeth, hair, and nails are the only organs affected by the disease, which is characterized by a reduction in sweat glands. Although the clinical characteristics of the HED type are identical, the severity varies across men and women. In certain instances, ED is obvious.

However, in other instances, parents and medical professionals may identify ED when the tooth is atypical (after 15 months). Identification of the affected body parts and evaluation of their development, growth, and function will enable the diagnosis of ED. Currently 30% of the ED-related genes, including *ED1, EDA, EDAR, GJB6, A88V EDARADD*, and *WNT10A*, have been found. Genetic testing is only accessible for a small subset of ED patients [82-84].

EDA, EDAR, and *EDARADD* gene mutations, where changes in one gene can result in many symptoms and changes in other downstream genes in the same signaling pathway might entirely change the phenotype, The *EDAR* gene gives instructions for producing the *EDAR* protein. This receptor is a component of a signaling system that is crucial for foetal growth. It is essential for the communication between the ectoderm and mesoderm, two layers of embryonic cells. These cell layers (ectoderm and mesoderm) are created in the early embryo. In this route, mutated genes mostly affect signaling, cell adhesion, and cellular control [83, 85].

Beta 6 Gap Junction Protein (also known as *ED2, EDH, HED*, and *CX30*) is the acronym for this protein. It has six exons and is found on 13q12.11. The esophagus, bone marrow, brain, and skin all express *GJB6*. Connexin 30, a protein that is encoded by *GJB6*, connects cells to one another via gap junctions. Four transmembrane segments, two extracellular loops, a cytoplasmic loop created by the two inner transmembrane segments, and the N- and C-termini, which are all in the cytoplasm, are all present in each connexin protein.

Which connexin proteins make up the hemichannel influence the selectivity of the gap junction. This gap junction connects cells by transmitting nutrients, ions, and signaling chemicals that coordinate cell activity. The *GJB6* mutation results in a gap junction defect and is linked to hidrotic ED (also known as *Clouston syndrome*), a condition that includes normal teeth, sweat glands, and palmoplantar keratoderma (PPK), but has clinical symptoms such nail dystrophy (micronychia or anonychia) and hair loss [86-88].

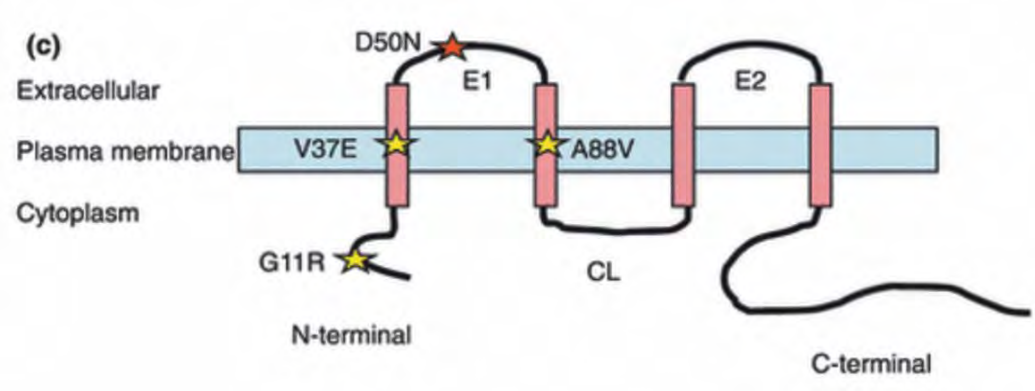

Figure 2.1 Structure of *GJB6* connexin 30 protein

Connexin 30 (*Cx30*), which creates gap junctions between neighboring cells to let chemicals and ions to move from one to the other, is encoded by the human *GJB6* gene and has 261 amino acids. Five mutations, *G11R, V37E, D50N, A88V*, and *N14S*, have so far been identified in individuals with *Clouston syndrome*; they are all associated with nonsynonymous amino acid changes. Moreover, the *Clouston syndrome* is also associated with mutations in the genes *GJA1 (V41L)* and *GJB2 (R127H)* [89-92].

7. Aims of the study:

The study was aimed to clinically diagnose an HED2 *Clouston* disorder in a Pakistani family using whole exome sequencing WES and to find out whether the parents are segregating, or the disease has been caused by a de-novo variant so that an effective and timely therapeutic action could be taken. Moreover, preventive measures such as genetic counselling, and prenatal diagnosis can be considered to prevent another affected child.

Chapter 2

2-MATERIAL AND METHODS

2.1 Sample Description:

The *Clouston syndrome*-affected family *DER124* samples were gathered. The concerned family is from *Pakistan's Khyber Pakhtunkhwa* province, *Malakand Division*, *Dargai* tehsil. Accordingly, blood samples, histories, photos, and pedigrees were taken.

2.2 Pedigree Construction:

Using various shapes and designs as symbols to represent gender, relationships, and illness problems, Pedigree was created using https://www.biorender.com/. A circle was used to symbolize women, and a square was used to represent men. Like this, blank forms were used to depict healthy family members while full shapes were used to represent those who were unwell. Crossed shapes and an arrowhead, respectively, were used to represent the departed members and the culprit. The spouses' consanguinity was shown by two horizontal lines.

2.3 Consent and Sample (Blood) Collection:

After the patient or guardian gave their agreement, blood was drawn in 3mL BD vacutainer tubes with Ethylene Diamine Tetra Acetate in 5mL and 3mL syringes from the afflicted and normal people (EDTA). The samples were stored in a 4°C refrigerator.

2.4 Genomic DNA Extraction:

The organic/phenol-chloroform extraction technique was utilized to isolate the family *DER124* complete genomic DNA from their blood samples.

2.4.1 Compositions of solutions:

The following four solutions were used to extract DNA using the phenol-chloroform technique.

They are listed in ***Table 2.1.***

SOLUTIONS	COMPOSITION	QUANTITY
SOLUTION A	Sucrose (0.3M) Tri- HCL 5mM $MgCl_2$ Triton X-10 Distilled water	54.7g 5ml 2.5ml 5ml 400ml
SOLUTION B	NaCl (400mM) Tris-HCL (10mM) EDTA (2mM) Distilled Water	40ml 5ml 1ml Make total volume of 500ml
SOLUTION C	Phenol Hydroxyquinoline Tris-HCL B-mercaptoethanol	100ml 10mg 10ml
SOLUTION D	Chloroform Isoamyl Alcohol	24ml 1ml

Table 2.4. Chemical composition of solution used in the extraction of DNA.

2.4.2 Phenol-Chloroform method of extraction or Organic method:

- The blood samples from the specified family were stored in racks for 30 minutes at 37 C in a resting posture.
- The following procedures were followed to extract DNA:

- Approximately 750µl of solution A and blood were combined in 1.5mL microcentrifuge tubes. The tubes were maintained at room temperature for 10 to 15 minutes before being centrifuged for one minute at 13000 rpm.
- The extra fluid was dumped. The microcentrifuge tube containing the pellet received around 400µl of solution A. Following tapping and vortexing to dissolve the pellet, centrifugation at 13000 rpm for 1 minute completed the process.
- The pellet was re-suspended in 400µl of solution B, 5-8µl of proteinase K, and 25µl of 10% Sodium Dodecyl Sulfate after the supernatant was discarded (SDS).
- The pellet was dissolved via tapping and incubated overnight at 37°C.
- Equivalent volumes of 500µl of solutions C and D were added to the incubated tubes, and they were centrifuged for 10 minutes at a speed of 13000 rpm.
- Fresh Eppendorf tubes were used to capture the top layer.
- A 500µl amount of solution D was added to this Eppendorf tube holding the aqueous layer, and the tube was centrifuged for 10 minutes at 13000 rpm.
- The top layer was collected in fresh microcentrifuge tubes after centrifugation.
- The tubes were filled with 55µl of sodium acetate and 500µl of cold isopropanol (-20 °C) to precipitate the DNA. The tubes were then repeatedly turned upside down and centrifuged for 10 minutes at 13000 rpm.
- About 200µL of 70% cooled ethanol (-20 °C) was added, and after carefully discarding the supernatant to prevent damaging the DNA pellet, it was centrifuged at 13000 rpm for 7 minutes.
- The tube was kept open in the concentrator for 10 min at 45°C and the ethanol was carefully disposed to avoid disruptions in the removal of residuals and downstream processing.
- DNA that had been precipitated was dissolved in 80–100µl of TE or deionized water and incubated for 24 hours at room temperature.

2.5 Molecular Analysis of Extracted DNA:

Agarose gel electrophoresis was used to analyze the extracted DNA. The instructions were carried out:

- A graduated cylinder or flask containing 0.5g of agarose, 5ml of 10x Tris-borate EDTA buffer (TBE), and 45 ml of distilled water was combined and microwaved for a short period of time to create a clear solution to create 1% agarose gel. After that, the mixture was allowed to cool at ambient temperature.
- The cooled solution was mixed with 3µl of ethidium bromide before being poured into the casting tray set with the necessary combs inserted and allowed to set for 15 to 20 minutes at 37 °C.
- The gel tank was filled with the casting tray containing the gel, and 1x TBE buffer (which serves as the electrolyte) was poured on top of it.
- The loading dye, bromophenol blue, and the DNA sample, a volume of 2µl each, were combined and put into the wells, and the gel was operated at 120 volts for 30 minutes (400 amps).
- It was possible to see the bands and intensities of DNA samples using a UV-Trans Illuminator (*Wealtec, Taiwan*).
- The final 40ng/L diluted form of DNA was created by combining 20µl of extracted DNA with 60µl of PCR water.

2.6. DNA SEQUENCING:

2.6.1. WHOLE EXOME SEQUENCING:

The DNA of the proband was sequenced using whole exome sequencing (WES), which employed the Sure Select V5-post kit from Agilent Technologies, *Santa Clara, California, USA*, to collect exome libraries. The target area was sequenced using the Illumina HiSeq 400 (*Illumine San Diego, CA, USA*); the average sequencing depth was 142X for termination reads of 150 bp. The *Burrows-Wheeler alignment (BWA)* program (BWA-mem; http://bio-bwa.sourceforge.net/bwa.shtml; accessed on February 5, 2023) was used to map the resulting reads (in relation to the human genome), and the Genome Analysis Toolkit (*GATK 3.v4*) was used to name variants. Variant alleles and the Haplotype Caller pipeline were annotated in standard VCF format using SnpEff (*SnpEff v4.1*). Whole Exome Sequencing used the suggested process for variant filtering.

- The *Clouston syndrome* candidate gene's variations were prioritized.
- Splice site, non-synonymous, frame shift, and gain or deletion of stop and start codons were some of the several types chosen.
- Variants with an allele frequency of 1% were chosen from the AD-genome aggregate database (https://gnomad.broadinstitute.org; accessed on February 10, 2022).
- Numerous methods, including *Polyphen2*, Mutation Taster, and SIFT, were utilized to score, and forecast variations for the prediction of pathogenicity.
- Different variations were discovered in the probe based on filtering.
- Sanger sequencing will be used to validate a particular disease-causing variation.
- The afflicted person must possess the candidate gene in hemizygous form (*GJB6*).
- Obligate carriers should show heterozygous peaks.
- Exons or splice junctions of the targeted gene's coding regions should have variations.
- A stop codon must be gained or lost, or the mutation must be missense, nonsense, or both.
- To determine if a variation is pathogenic or detrimental, in-silico methods should be used.
- The pathogenicity of a variation is rated at less than 0.01 (minor allele frequency), according to the 1000 Genome Project (http://www.1000genomes.org/).

2.6.2. PRIMER DESIGNING:

The necessary primers were manually created for the target exon to be amplified by PCR. The target gene sequence was retrieved from the Ensemble Genome Browser (*http://www.ensembl.org/*), and Oligo Calc was used to assess the features of the primers. BLAST was then used to check the homology and single hits of the primers. Furthermore, Insilco PCR, also known as virtual PCR, was carried out for the product size and position. The chosen primers were from *Macrogen South Korea* (shown in Table 2.2).

EXON NO.	TYPE F/R	PRIMER SEQUENCE 5' TO 3'	PRIMER LENGTH (bp)	T_m (C)	GC content (%)	Product size (bp)
5	F	GTATCACAGCCTACCTTAAA	20	56	40	642
5	R	GGGTCAATCCCACATTTCAA	20	58	45	642
5	F	GACGTACACCAGCAGCATC	19	58	60	484
5	R	CAAACTCTTCAGGCTACAG	19	56	47	484

Table 2.2. Designed primers for GJB6 Gene

2.6.3 POLYMERASE CHAIN REACTION:

The amplification of the *Clouston syndrome* gene's exon was carried out using a 50µL PCR reaction mixture in 0.2mL PCR tubes (*Axygen, USA*). DNA was used from both the affected and healthy people. The following elements were included in the reaction mixture (***Tables 2.3***):

S.NO	COMPONENT	CONCERNTRATION
1	Concentrated DNA	3µL
2	Forward Primer	2µL

3	Reverse Primer	2µL
4	PCR Water	18µL
5	Master Mix (Promega)	25µL

Table 2.5: Components and concentrations used in the PCR.

In the PCR tube, all these components were mixed via tapping. The PCR tubes were placed in a Thermocycler (*Biometra, Germany*), and the program set for the amplification reaction is mentioned below in ***Table 2-4.***

STAGES	TEMPERATURE	DURATION
Initial denaturation	96℃	4 min
Denaturation	94℃	30 sec
Primer annealing	56℃	35 sec
Extension	72℃	35 sec
Final extension	72℃	4 min

Table 2.6. Program set for PCR amplification.

A total of 36 cycles of the PCR amplification reaction were performed. The amplified PCR products that were produced were then examined on a 2% agarose gel. The gel was positioned in the running buffer (1x TBE), then amplified products and a 1kb ladder were put into the wells (Thermo Fischer). Gel was operated at 120 volts for 30 to 35 minutes. The bands were seen using a UV-Trans illuminator, and particular bands were chosen for purification.

2.6.4. Purification of PCR products:

The kit manual was adhered to remove contaminants (enzymes, primers, dNTPs, salts, and nucleotides) from the PCR products (*Gene Jet PCR* Purification, *lot 00129472*, *Thermo Scientific USA*).

2.6.4.1. Gene Jet PCR purification Kit:

Following protocol was followed which consists of the given steps.

- Equal amounts of binding buffer and PCR product were combined, and they were then incubated at 37°C for 3-5 minutes.
- In the collection tubes, the mixture for filtration was poured onto the membrane of mini columns. It was kept in the resting position for 1-2 minutes, followed by centrifugation at 13000 rpm for 1 minute.
- The mixture was then given 700µL of washing buffer before being centrifuged one more at 13000 rpm for a minute. The flow-through was after then thrown away.
- The columns were then put in a 1.5mL Eppendorf tube and allowed to air dry for 15 to 20 minutes at room temperature.
- Finally, 7-9µL of elution buffer were precisely poured to the membrane of the small column and centrifuged for a few minutes at 13000 rpm to get the pure product. This process was carried out twice.
- 1.5µL of the purified PCR product was loaded and tested on a 2% agarose gel with a 1kb ladder to test the product's specificity.

2.6.5. VALIDATION OF EXOME SEQUECNING BY SANGER SEQUENCING METHOD:

Sanger sequencing results will be interpreted by using *BioEdit* tool to validate for the whole exome based detected variant.

Table 2.1 Preparation and composition of solutions

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

3-Results

3.1 DER124 Analysis based on pedigree:

DER124 family was recruited from *Pukhtoon khwa Malakand Division, Dargai* tehsil *Pakistan* and the pedigree constructed with one consanguineous marriage at generation 2nd and 3rd. The one individual in generation 4th was affected in the pattern of recessive form of inheritance. Phenotypic and clinical descriptions was supportive of *Clouston syndrome* or disorder of Ectodermal dysplasia type 2 (Hidrotic). The proband *DER124-4* was diagnosed as developmental Palmar keratoderma at the age of 1 while *DER124*-4, aged 15 Months was considered normal till 6 months and eventually developed related symptoms as no change in behavioral, emotional, and physical development were observed with many changes like delayed teeth growth, sparse hair and PK. Both the parents and 2 siblings were phenotypically normal, the proband show symptoms of Hidrotic ectodermal dysplasia (*Clouston Syndrome*) at the time of sampling as it is a progressive disease, but the clinical report of the doctor suspects the proband with *Clouston syndrome*. But when she was 15 months old, she experienced plantar keratoderma. The parents of the patients and other siblings had normal phenotypic expression and the pattern of inheritance for the disease was assumed to be autosomal recessive. (***Figure 3-1***).

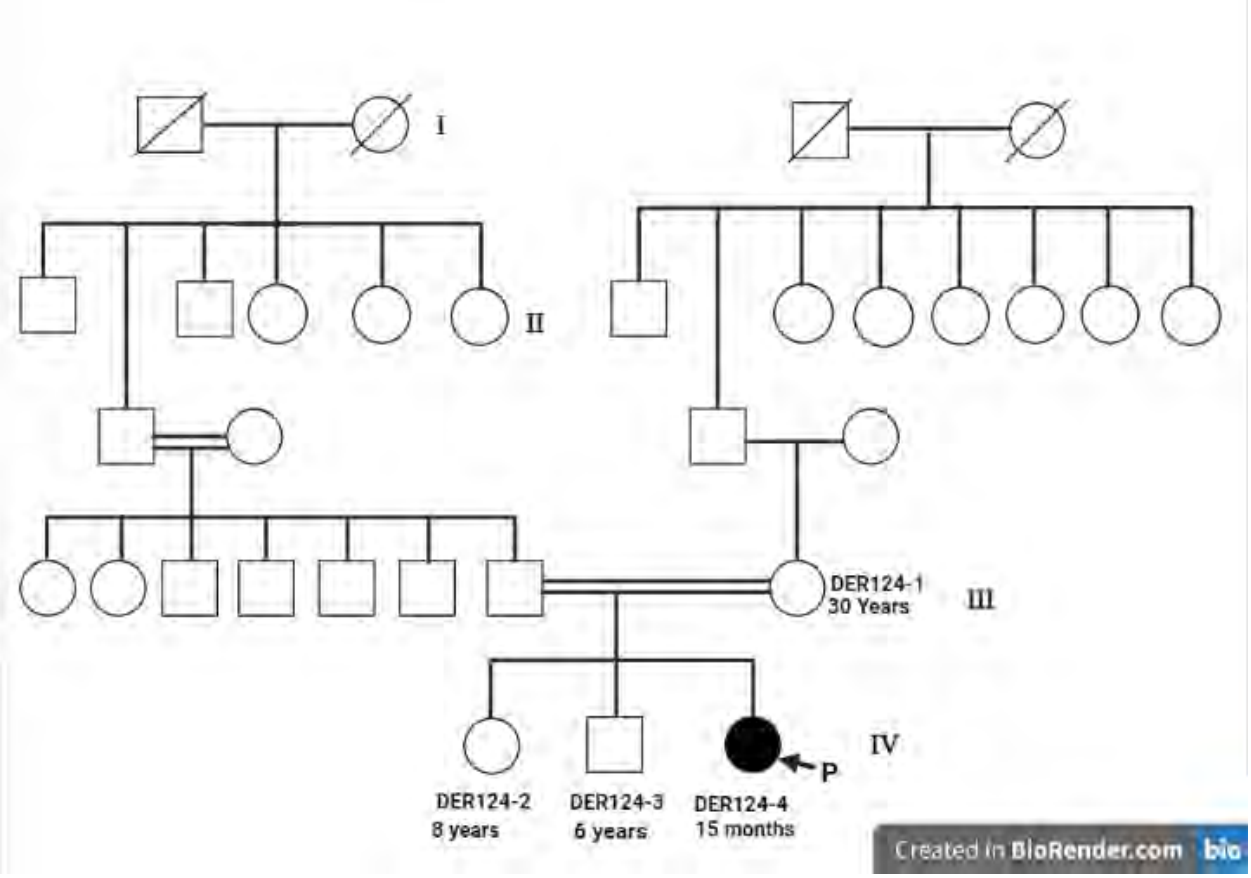

Figure 3.2: Pedigree of family *DER124*. Consanguinity shown in the 4th generation and one female in generation 4th is affected. Circles in the pedigree shows females and squares represent males. Shaded shapes represent the affected individuals. The shapes with a cut in middle show the deceased members of the family. The shape with the arrow represents proband. One individual in 2nd and 3rd generation was affected.

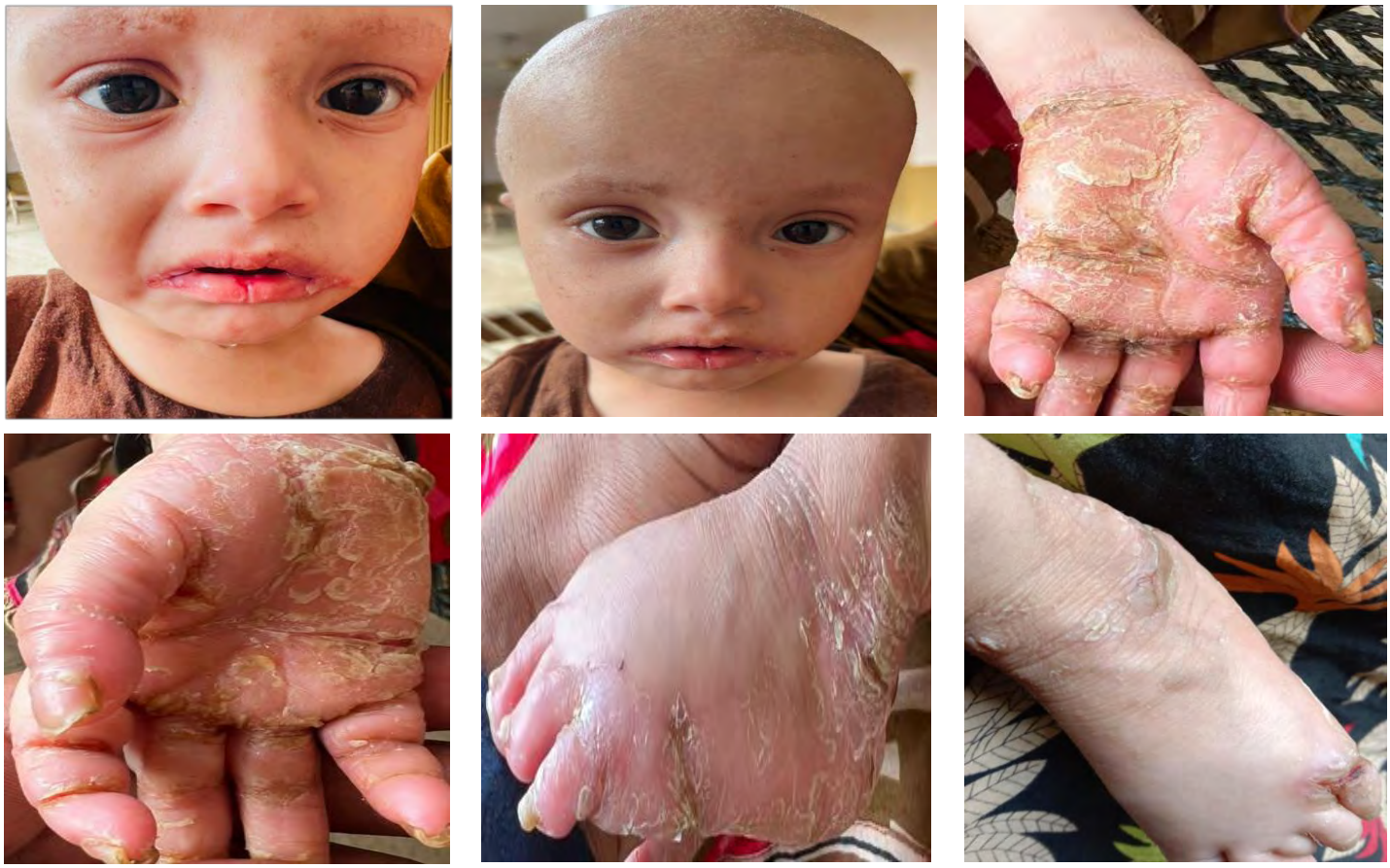

Figure 3.2 Affected individual of *DER124-4* effected female with ED, Plantar keratoderma, hypodontia, Alopecia and delayed teeth.

3.2 Gel Electrophoresis:

To check the concentration of DNA extracted from *DER124* family, it was analyzed via gel electrophoresis (***Figure 3.2***). The bands shown on the gel was indication of good quality and suitable to be used for the PCR reaction and were free from contamination of protein.

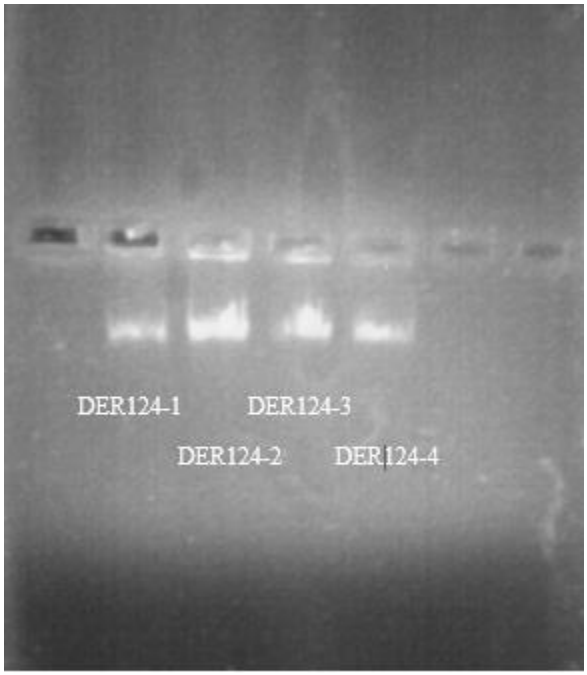

Figure 3.3. From right to left, *DER124-1* to *4* are labeled, displaying the concentration of extracted DNA of family *DER124*.

3.3 DNA sequencing

3.3.1. Whole exome sequencing:

Whole Exome Sequencing of the patient's DNA disclosed 91,253 variants. The variants were narrowed down to one variant most likely to be causing the disease by using various filters to cut off variants which were synonymous or irrelevant to the disease of interest.

3.3.1.1. Variant filtration:

The total variants reduced to 15043 upon applying filter for synonymous and intronic region variants. Several variants were excluded due to their heterozygous nature. The number reduced to 2345 variants after excluding all the variants which had a minor allele frequency (MAF) greater than 0.01 in the general human population. The number reduced to 45 after excluding variants with lower likeliness of being pathogenic upon Insilico analysis. After applying various filters to the Whole Exome data, most pathogenic one according to phenotypes the variants were narrowed

down to 6 in which 1 variant has been targeted showing higher chances of pathogenicity. Already reported mutation c.209C>T in *GJB6* gene. The mutation occurs in the fifth exon of *GJB6* gene. The variant is Present in *HGMD, Clinvar* and *gnomAD*. But present at a very low frequency (0.00001651) in *ExAC* in Asian and African population data base.

Table 3.1 Variant filtration data

Filter applied	**No. of variants**
Initial variants	56203
Variants potentially affecting protein were kept	15043
Homozygous variants were kept	7590
Variants with MAF >0.01 in the 1000 genome, *ExAC* or *gnomAD* were excluded	2345
Insilco tools based filtration	45
Most pathogenic one according to phenotypes	6
One variant was targeted for further validation	1

Table 3.2. Identified variant from exome data.

Exon	**Mutation**	**Sequence Change**	**Mutation Type**	**Protein Variation**
5	c.209C>T	C**C**G>C**T**G	Missense	p.P70L

3.3.2 POLYMERASE CHAIN REACTION (PCR):

Along with 1kb ladder 2µL of PCR products were also visualized using (2% agarose gel). The specific bands shown on the gel were of required size and had good quality. A mix of purified products and loading dye of equal volumes 1.5µL each, were also run on 2% agarose gel along with 1kb ladder. The bands analyzed were specific and ready to be used for Sanger-sequencing (***Figure 3.4***).

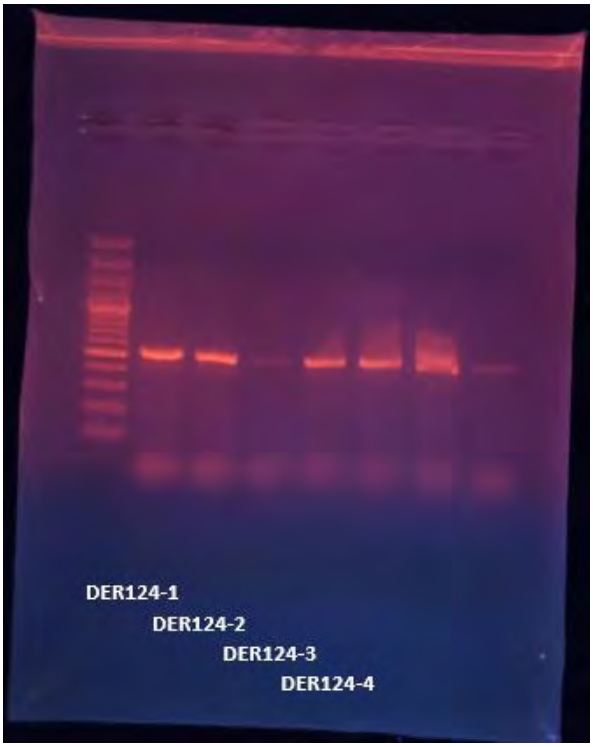

Figure 3.4. PCR products of family DER124 (1 to 4) are shown from left to right, along with loading dye and 1kb ladder on the left side

Chapter 4

4) Discussion

Skin is the largest human organ having $2m^2$ surface area and weigh 3.6kg. It consists of three different layers, I. Epidermis, ii. Dermis and iii. Hypodermis, there are five basic inheritance patterns for germline mutations: autosomal dominant, autosomal recessive, X-linked recessive, or mitochondrial maternal, X-linked dominant. Each of the 22 pairs of autosomal chromosomes, apart from the X and Y sex chromosomes, may only contain one autosomal mutation.

Ectodermal dysplasia with Hidrotic dysplasia 2 or *Clouston syndrome* is an autosomal recessive and autosomal dominant disorder that arises due to mutation in *GJB6* gene. The *GJB6* gene has been mapped on the pericentromeric region of 13q12 chromosome which has 6 exons and encodes for Connexin 30. Beta 6 Gap Junction Protein (also known as *ED2, EDH, HED*, the esophagus, bone marrow, brain, and skin all express *GJB6*.

(*Cx30*) protein that codes a long chain of 261 amino acids. Connexin (*Cx30*) has Jap junctions which attach cells through these junctions to transmit nutrients, ions, chemical to each other as well as signaling chemicals that coordinate cellular activities.

The patients with the defects of ED 2 normally have mutations in *GJB6* gene Jap Junction and is linked to hidrotic ED (also known as *Clouston syndrome*), a condition that includes normal teeth but delayed in growth, sweat glands, and palmoplantar keratoderma (PPK), but has clinical symptoms such nail dystrophy (micronychia or anonychia) and hair loss are reported in the patients.

ED that has been discovered thus far have been molecularly identified, X-linked recessive hypohidrotic ectodermal dysplasia (HED) is the most prevalent and severe form of ED. Hidrotic ED (*Clouston syndrome*), which is often inherited as autosomal dominant but can also be inherited as recessive autosomal recessive, is the second most prevalent kind.

Although the clinical characteristics of the HED type are identical, the severity varies across men and women. In certain instances, ED is obvious. Ectodermal dysplasia, according to *Priolo*, should be divided into two groups: group 1 exhibits aplasia of ectodermal structures, whereas group 2

exhibits change in tissue homeostasis and developmental regulation. Only 30 of the approximately 200 distinct forms of ED that have been discovered thus far have been molecularly identified.

Thurman was first to described HED in 1848, and later by *Darwin* in 19th century. It was assigned by *Thadani* to the X chromosome in 1921, *Stevenson* and *Kerr* calculated the incidence at birth as *1 per 100 000 births* based on the frequency of HED in *Oxfordshire*, while *Dr. Clouston* discovered Clouston hidrotic ectodermal dysplasia when he was normally practicing in his hometown and observe a population of same phenotypic characteristics [1, 20-24, 48, 59, 62, 80, 88-91, 93-96].

HED 2 is a genetical disease with a very few cases reported in literature, while few cases are reported as syndromic and non-syndromic. Till date all mutations found related to *Clouston syndrome* to be nonsynonymous amino acid changes. On the basis of clinical features, the disease shows similarity with ED2 *Clouston Syndrome* autosomal recessive caused by the mutations in the *GJB6* gene.

In this study, a patient of Pakistani family *DER124*, has been investigated to search for the present cause of manifestations based on clinical observations and to help in the proper diagnosis of the disorder. The Proband is a 15 months' female at the time of sampling to consanguineous parents presenting the features of the disease. The symptoms were first started at the age of 6 months with severe Palmar keratoderma on palms and continued to mouth sore and nail dystrophy. The family history revealed that the Proband has other 2 siblings who were phenotypically normal including their parents.

Several variants revealed in the exome by applying whole exome sequencing to the patient DNA. The variants were then filtered by using various web-based filtration tool to the most likely variant, to be pathogenic and relevant to the disease conditions. The possibility of the *Clouston syndrome* was ruled by the pedigree of family following an Autosomal recessive inheritance and it has been further confirmed with the whole exome sequencing results showing already reported mutation in the candidate gene, *GJB6* for *Clouston syndrome*.

Already reported missense mutation, c.209C>T was detected, mutation occurs in the fifth exon of *GJB6* gene. The variant lies in the highly conserved region CCG site predicting a high probability of missense changes to the features of protein. *GnomAD, Clinvar* and *HGMD* has the variant. After

Exome sequencing, PCR carried for the DNA samples which shows good band and analyzed samples were ready to be used for Sanger sequencing.

Conclusion

In this study, a Pakistani family affected with *Clouston syndrome* was screened by whole exome sequencing to detect the underlying genetic cause. Missense mutation (c.209C>T or p.P70L) in the *GJB6* gene on chromosome 13 was detected which is previously reported. No effective therapy is existing for this disease so diagnosis mostly relies on the clinical manifestation. The rapid and growing development of NGS technology made it possible to explore the genetic mechanism for such type of inherited disorders. History of the family and pedigree used in this research is suggestive of autosomal recessive pattern of inheritance which will further be confirmed using sanger sequencing to check for the heterozygosity in the parents. Additionally, protein expression studies should be carried out to understand the possible effects of the variant and the region where it lies, on the protein structure and function. The study can be helpful in a better understanding of the functional role of protein, the associated disease and hence help in finding better therapeutic options.

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