IDENTIFICATION OF GENES INVOLVED IN ATOPIC DERMATITIS.

Submitted by

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151-FBAS/MSBT/F14

Supervised by
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Department of Bioinformatics and Biotechnology
Faculty of Basic and Applied Sciences
International Islamic University Islamabad

(2017)

Atopic dermatitis

Eczema

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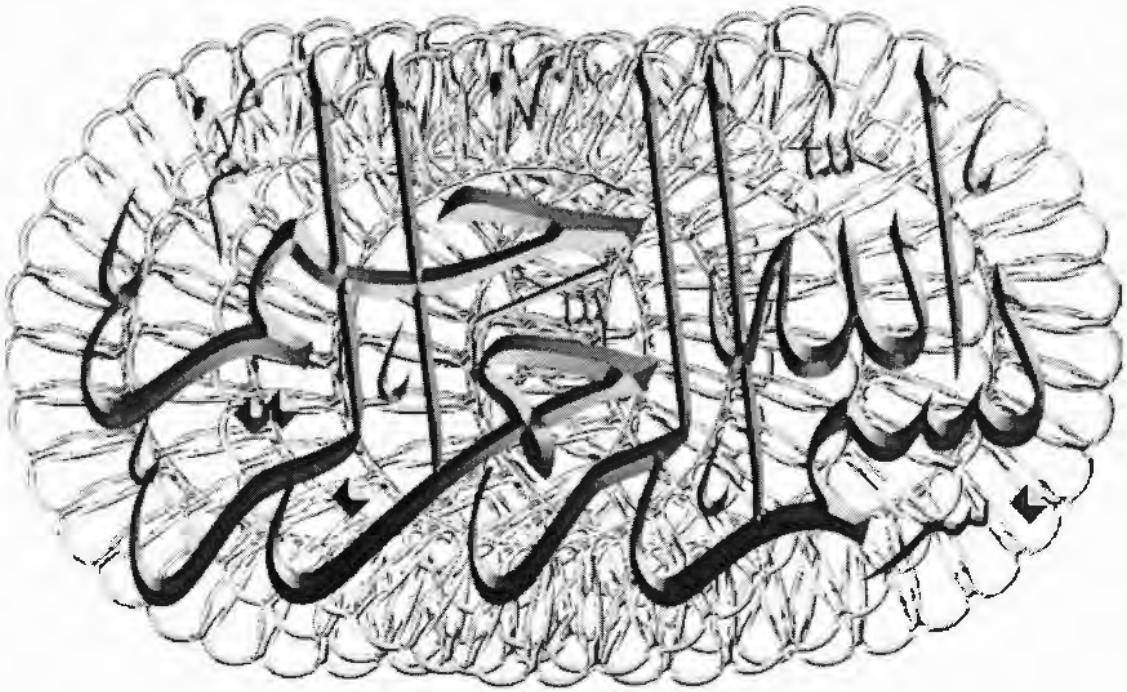
بسم الله الرحمن الرحيم

Department of Bioinformatics and Biotechnology

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Dated: 15-2-17.

FINAL APPROVAL

It is certified that we have read the thesis submitted by Nabeela Ashraf and it is our judgment that this project is of sufficient standard to warrant its acceptance by the International Islamic University, Islamabad for MS degree in Biotechnology.

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A dissertation submitted to Department of Bioinformatics and Biotechnology, Faculty of Basic and Applied Sciences International Islamic University, Islamabad as a partial Fulfillment of requirement for the award of the degree of MS Biotechnology

DEDICATION

I dedicate this thesis, with all

My heart, to my beloved

mother, Father And Eldest Sister

AND

ALL MY FAMILY

For their unforgettable prayers, love and Support.

DECLARATION

I hereby declare that the work presented in the following thesis is my own effort, except where otherwise acknowledged and that the thesis is my own composition.

No part of the thesis has been previously presented for any other degree.

Date _____

_____

NABEELA ASHRAF

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Glory be to Allah and praise be to Him equivalent to the number of His creation andequivalent to what pleases His Beings and equivalent to the weight of His throne andequivalent to the ink of His words. HE is the Lord of the worlds and without HIS willnothing would happen. My foremost duty is to acknowledge omnipresent kindness andlove of Almighty ALLAH who blessed me with good health and conductive environment to accomplish my research work.

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In the end I would like to offer special thanks to all those families who agreed for their participation in my research work.

For you all, a thousand times over!

Nabila.

LIST OF ABBREVIATIONS

AD	Atopic Dermatitis
MM	Master mix
SNP	Single nucleotidepolymorphisn
Taq	Thermus Aquaticus
TBE	Tris base EDTA
TE	Tris EDTA
UV	UltraViolet
TH2 cell	T helper cell Type
AMP	Antimicrobial peptides
SP	Serine protease
SC	Stratum Corneum
Cer	Ceramide
FDA	Food and Drug Administration
TJs	Tight junctions
IgE	Immunoglobin E
Dc	Dendritic cell
FLG	Fillagrin
TYK2	Tyrosine Kinase
DOCK8	Dedicator of Cytokinesis
PGM3	Phosphoglucomutase
CCl11	Chemokine cc motif,ligand
ZNF750	Zinc finger Protein 750
Il12B	Interleukin 12B
HAVCR2	Hepatitis A virus cellular receptor

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Abstract

Skin act as an effective barrier and protectant to foreign pathogens and chemicals. The proper functioning of skin as a protective layer is therefore necessary to avoid any kind of allergic disease like Atopic Dermatitis. Atopic Dermatitis is a skin disorder in which keratin matrix of skin ,which is important to maintain skin functioning especially its moisture content, is disturbed due to genetic and environmental factors. Molecular genetics study of this disease reveal that gene mutations are the primary cause of Atopic dermatitis and the problem is aggravated by unfavorable environmental conditions. In the present study, three consanguineous Pakistani families (A, B, C) with autosomal recessive Atopic dermatitis, were genetically evaluated.

Homozygosity mapping with highly polymorphic microsatellite markers were typed in the region of candidate genes. Genotyping studies in all three families showed no linkage to any of the known candidate regions. Genotyping analysis in these families revealed that affected individuals were heterozygous for different combination of parental alleles, therefore linkage in these families was excluded to the known Atopic dermatitis candidate regions. These results show that a novel gene is responsible for Atopic Dermatitis (AD) in these families

Introduction

Atopic dermatitis (AD) is a severe inflammatory skin problem which is linked with cutaneous hyperactivity to environmental triggers (Howell *et al.,* 2004). Patients must have an itchy skin condition with three or more of the following symptoms: antiquity of flexural involvement, asthma/hay fever, dry skin, beginning of rash under the age of two years, visible flexural dermatitis (Williams *et al.,* 2006).

Clinical characteristics of AD:

With both age and disease period, clinical characteristics of AD change. During early age, clinical symptoms are characterized by minute vesicles, eczematous change, along withstrapping itch. Age-related change proceeds with clinical features. It has been observed that astrogliosis in spinal dorsal horn affects chronic itch in AD-like model animals, and that the processing of the sensation of itch, that is characteristic in AD, occurs in the central nerves (Elias,2014).

After childhood, skin appearance transform into symmetrically localized widespread dermatitis with lichenification and this skin condition occurs repeatedly where the itch initially appeared.Moreover, itch caused by AD is intensified by, sweating, high temperature, woollen fabric, stress, many food types, alcohol, and even by the flu.(Baker, 2006).

Major itch inducing factors and their ligands:

Some important itch-inducing factors in AD are Histamine, Bradykinin. Endothelin. Acetylcholine,TSLP,PAF,LTB4 having receptors Histamine receptor, radykinin

receptors, Endothelin receptors, TSLP receptor adn LTB4 receptor respectively. (Murota and Katayama *et al.*, 2016)

Classification of AD:

Depending on co-existence with allergic features; AD can be classified into either intrinsic or extrinsic AD. Penetration of foreign allergens of environment and food are strongly associated with aggravation of extrinsic AD barrier dysfunction. Allergen penetrates through skin barrier and bind to an epidermal dendritic cell (DC) to start cutaneous inflammation 13 and activate Th2 polarization damaged in case of an acute stage.(Lee *et al.*,2016). Disease is classified on the basis of pathogenesis. Primary defect in skin is known as intrinsic AD, whereas extrinsic AD is skin defect with allergy. In both conditions, genes involved in lipid metabolisim are upregulated, differences between the two types is due to differences in their cytokine pathways for example, decreased IL37 appearance in intrinsic AD.(Henkes *et al.*,2014).AD patients have immunological abnormalities but in intrinsic or non allergic AD normal IgE levels are seen in blood n skin of patients but exact mechanism causing this type is unclear (Natalija *et al.*, 2002). About 20% of AD patients have Intrinsic type of AD. Extrinsic AD has positive link with environmental and food allergens whereas Intrinsic type shows no positive response to these allergens.(Yang *et al.*, 2003).Skin barrier is puturbed in Extrinsic but not in Intrinsic AD. (Tokura, 2010)

Prevalance:

According to a study 20% of children are affected by AD worldwide but percentage varies in some countries. Children between the age of 6-7 years revealed prevalence of AD is 0.9 % in India to 22.5 % in Ecuador with high values in Asia and Latin America. Children from 13-14 years showed prevalence values ranging from 2 % in China to 24.6% in Columbia. A prevalence above 15% was noticed in 4-9 regions including South Africa, Latin America, Europe and Oceania (Williams *et al.*, 2009). Highest prevalence noticed in U.K and New Zealand. Moreover, its observed that AD risk is increasing in low income countries (Cookson *et al.*, 2008). Atopic manifestations are more dominant among boys during childhood but in adulthood girls become more symptomatic (Thomson, 2015). It mostly occurs during childhood and early infancy, but it can persist or even start in adult hood. The life time prevalence of atopic dermatitis (AD) is 10 to 20 % in children and 1 to 3% in adults. In industrialized world its prevalence has increased two to three folds during past three decades (Leung *et al.*, 2004). AD has increased in frequency in recent decades (Palmer *et al.*, 2006). Children having atopic dermatitis are at a strong risk of allergic asthma and allergic rhinitis. Those with AD during first two years of life, 50% will have asthma in future life (Robertson *et al.*, 2006). The disease is strongly heritable as shown by twin and family studies (Kwiatkowski *et al.*, 2006)

Genes involved in AD:

Genetic studies associate 81 genes, 46 amongst them showed at least one positive link with AD. Linkage at epidermal differentiation complex (EDC) locus on chromosome 1q21 and on 17q21 were screened. Thirteen genes (*FLG, IL4, IL4RA, SPINK5,CMA1, RANTES, CD14, DEFB1, GSTP1, IL18,NODI, TIM1*) are positively linked with atleast

one another independent study with filaggrin protein ,associated with AD the highest number of studies (Barnes, 2010). A genome wide linkage study showed strong proof for linkage on chromosome 3q21.This locus also provided proof for linkage of allergic sensitization under the supposition of paternal imprinting supporting atopy gene presence in this region (Kehrt *et al.*, 2000). In a genome wide screen for AD, linkage on chromosome 1q21, 17q25,20p is identified. The results indicate that AD is linked with genes with general effects on skin inflammation and immunity (Lawrence *et al.*, 2001). A genome wide association study of AD in Chinese population identifies previously undescribed susceptibility loci at 5q22.1,20q13.33, later also showing association for in German sample. Linkage to AD is found on chromosome 1, 3, 4, 5, 11, 13, 15, 17, 18, 19, 20 with only 3p24 locus showing significant replication. Genome wide linkage approach identifies few candidate genes linked with AD none has pinpointed a specific locus or gene (Barnese, 2010). Complete Sequencing of *FLG* gene has showed many other polymorphisms with varying frequency across the ethnic groups (Hull *et al.*, 2007). AD is triggered by complex association between environmental agents and genes. Loss of function mutations to Filaggrin-2 (*FLG2*) were linked with atopic dermatitis in African American children in which various external factors (environmental) and internal factors (Genes) influence immune cells and speed up abnormal immune response (Yang *et al.*, 2014). Two independent losses of function genetic alternates (R510X and 2282del4) in filaggrin encoding gene are very strong predepositing factors for atopic dermatitis(David *et al.*,2014)

Following are the genes involved in AD, having autosomal recessive mode of inheritance.

1: Interleukin12B. (*IL12B*)

High number of IL-12 mRNA-positive cells in chronic skin lesions was seen as compared to acute lesions and normal skin from patients with AD. It shows that acute AD skin lesions are linked with an increased expression of IL-13 mRNA. On the contrary, the relative boost in IL-12 mRNA in chronic AD skin lesions advocate a possible role for IL-12–producing cells in regulating chronic inflammation. (Hamid *et al.*, 1996)

IL12B SNP is related to susceptibility to AD,by influencing the Th1/Th2 balance. The IL-12 p40 subunit gene (*IL12B*) is present at chromosome 5q31-33 and linkage discovery of AD on 5q31 was seen. A single nucleotide polymorphism (SNP) (1188A/C) of *IL12B* has been reported recently. This SNP is it has been reported to be associated with *IL12B* mRNA expression levels. Interleukin-12 (IL-12) is thought to play an central role in bringing Th1-type cytokine profiles. Psoriasis vulgaris (PsV) and atopic dermatitis (AD) are known to be Th2 and Th1 type disease.(Sekiya *et al.*, 2002)

2: Tyrosine Kinase 2. (*TYK2*)

Tyrosine kinase 2 (*Tyk2)* is a noreceptor tyrosine kinase which belongs to the Janus kinase (Jak) family. *Tyk2* insufficiency is likely to account for the patient's multipart clinical symptoms, that includes the phenotype of impaired T helper 1 (Th1) differentiation and accelerated Th2 differentiation. The study identifies human *Tyk2* deficiency and exhibit that *Tyk2* plays essential roles in multiple cytokine signals, which are involved in innate and acquired immunity of human beings, (Bunkyo and yushima 2009) Hyper-IgE syudrome (HIES) is a complex primary immunodeficiency characterized by atopic dermatitis. It is associated with very high serum IgE levels and resultant susceptibility to infections of extracellular bacteria. Cause of this disorder is a

null mutation in the tyrosine kinase 2 (*TYK2*) gene leads to an autosomal recessive HIES linked with mycobacterial and viral infections.(Aral *et al.*, 2006).

Hyper-IgE syndrome is a rare Autosomal recessive combined immunodeficiency syndrome characterized by susceptibility to atopic eczema,viral infections, defective T cell activation and high serum IgE. The genetic etiologies of the diease are diverse. Null mutations in *TYK2* and *DOCK8* are responsible for various such cases. (Alsum *et al.*, 2013)

3:Dedicator of cytokinesis 8 (*DOCK 8*)

Autosomal recessive hyper-IgE syndrome is a combined immunodeficiency disorder. It is characterized by very high morbidity and mortality rate. More severe phenotypes seen in AD disease can be explained by environmental factors and different genetic background. Most common identified genetic cause is *DOCK8*mutation. (Alsum *et al.*, 2013)

DOCK8 Autosomal-recessive mutations are liable for many cases of recessive autosomal hyper-IgE syndrome. *DOCK8*, its disruption is associated with a phenotype of severe cellular immunodeficiency that is characterized by susceptibility to viral infections and atopic eczema (AD). The deleted interval involved dedicator of cytokinesis 8 *(DOCK8)*, that encodes a protein concerned in the regulation of the actin cytoskeleton. Distinct homozygous mutations revealed in sequencing of patients without large deletions *DOCK8* causing premature termination, splice site disruption, frameshift, single exon deletions and microdeletions. (Winkler *et al.*, 2009).

Atopic Dermatitis and Hyper-IgE syndrome patients showed unusual sensitization pattern of serum IgE corresponding to the allergic disease symptoms and Th-cell subset data, it is suggesting that *Dock 8*has a key role in the development of allergy. (Hagl *et al.*,2014)

4: Filaggrin (*FLG*)

A strong risk factor to develop atopic dermatitis (AD)is a group of filaggrin (*FLG*) mutations (Leeflang *et al.*, 2016). It is confirmed in various studies that FLG mutations are risk factors for AD (Kiiski *et al.*,2016). *FLG* mutations lead to modifications in epidermal eicosanoid metabolism that could act as an autocrine trigger of impaired epidermal, inflammation aand late differentiation in AD. Ichthyosis vulgaris (IV) is caused by a loss-of-function mutations in the filaggrin (*FLG*) gene and represent the chief predisposing genetic risk factor for atopic dermatitis (AD). (Blunder *et al.*, 2016)

Within the granular layer keratinocytes of the epidermis is the giant, repetitive polyprotein profilaggrin it is the major proteinaceous constituent of the keratohyalin granules. When granular layer cells move to terminal differentiation to form the stratum corneum, It is rapidly broken into many copies of the 37 k Da filaggrin units, which join keratin cytoskeleton, and condenses it, so enabling cellular compression. Filaggrin is cleaved to form natural moisturising factors within the stratum corneum, of amino acids and theire derivatives and that give multiple effects.*FLG* mutations are different in different geographical regions.(Park *et al* .,2016)

Several patterns of *FLG* mutations are noticed in different geographical regions. For instance, c.3321delA was noticed as a pan-Asian mutation, found in all communities. some mutations proved south-to-north (or north-to-south) distribution pattern, detailed

analysis of the genetic diversity of *FLG* mutations for East Asian lineage isnot confirmed till now (Kapsok *et al.*,2016)

At least three descents contributed to the current genetic pattern of East Asian patients suffering fromAD: north-to-south south-to-north and Japanese-specific lineages. The three lineages were multiplied in other genetic data, such as Y chromosomal SNP and autosomal SNP data set. (Hammer *et al.*, 2006)

5:Phosphoglucomutase (*PGM3*)

Glycans are essential regulators of antibody actions and half-lives. The most heavily glycosylated antibody is IgE, the site specific glycosylation of IgE from a patient with a newl hyper IgE syndrome was related to mutations in *PGM3,* an enzyme responsible for synthesizing UDP-GlcNAc, a sugar donor direly needed for glycosylation. Damage of *PGM3* function leads to a novel inborn mistake of development and immunity because biallelic hypomorphic mutations are linked with faulty glycosylation and a hyper-IgE-like phenotype.(Haslam*et al.*, 2014)Autosomal recessive hypomorphic *PGM3* mutations cause a disorder of strong atopy, autoimmunity, intellectual disability ,immune deficiency and hypomyelination (Zhang *et al.*, 2014) The recognition of hyper IgE syndromes linked mutations in *PGM3* give the basis for future studies on the molecular mechanisms and pathophysiology of eczema, increased susceptibility to infections and IgE dysregulation.(Yang *et al.*,2014).

6: Hepatitis A Virus Cellular receptor 2 (*HAVCR2*)

Polymorphisms in) in Tim3 (*Havcr2) and* Tim 1 (T cell immunoglobulin mucin) 1 (*Havcr1*) members of a new gene family, found within the *Tapr* locus, (T cell and airway

phenotype regulator) were proved to linked with Th2 cytokine production.(Shang *et al.*,2007). Mutations in *HAVCR2*in atopic disease have been seen in other human asthma disease studies. (Graves *et al.*,2005). Genetic association studies revealed that polymorphisms in *HAVCR* correlate with the growth of atopic disease. (Barlow *et al.*,2011). *HAVCR2* have been shown to strongly order adaptive immunity. (Umetsu *et al.*,2008).

7: Chemokine cc motif, ligand (*CCL11*)

Gene expression for *CCL24*, *CCL26* *CCL11*, is seen frequently in skin changes of AD patients than in normal skin. The higher level of *CCL26* in skin diseasess, shows its role in their in AD. (Paplinska *et al.*,2010) Expression of *CCL11* mRNA, *CCL1*, *CCL3*, *CCL4*, was higher in people with acute AD as compared to patients with chronic AD. A rise in the bounty of CCLs after allergen application leads eczema development. (Paplinska *et al.*,2010). Furthermore, the profile of increased expression of cytokines and chemokines in skin of AD patients, known to be involved in human AD pathogen (Teletin *et al.*, 2005)

8: Zinc finger protein 750 (*ZNF750*)

ZNF750 is involved in many skin problems development. Mutations in *ZNF750* found to be linked with AD symptoms development. *ZNF750* is protein found in nucleous posing a functional C-terminal nuclear localization signal. *ZNF750* was explicitly articulated with the epidermal supra basal sediments and its expression was improved during differentiation, in human skin as well as in-vitrosss.(Cohen *et al.*, 2012)

Pathogenesis:

The pathogenesis is multifactorial involving disruption of epidermal barrier, IgE dysregulation, defects in skin mediated immune response and genetic factor. Desaturase activity is deficient in atopic dermatitis which leads to decreased linoleic and linolenic acid metabolites and results in AD (Piliang , 2009). Atopic dermatitis has genetic basis but no specific gene to date has been identified as a unique marker for AD. Increased expression of cytokines produced by distinct T cells is seen in AD patients. Th2 cells circulating in blood of AD patients results increase in IgE and eisonophil levels. Th2 dominant cytokines down regulate terminal differentiations proteins (TDP) and production antimicrobial peptides (AMP) in keratinocytes. The inhibition of TDP and AMP ultimately contribute to barrier deficiency and increased skin infections. Mutations in *FLG* disturb epidermal differenciation and results in AD (Rosso *et al.*, 2013).The latest identification of loss of function mutation in filaggrin protein puts light on disease mechanisms in eczema (Mclean *et al.*, 2016). The exact contribution of *FLG* to atopic dermatitis, factors modifying *FLG* expression and barrier protein role is yet to be delineated (Sandilands *et al.*, 2008). The clinical phenotype that identifies atopic dermatitis is the sum of associations between genes, the environment, abnormal skin barrier function and immunologic responses (Leung *et al.*, 2004).Protease, filaggrin. specific lipids and serine in the structural and functional integrity of stratum corneum has important roles in disease (Friedlander and Rosso, 2013). Stratum Cornuem is a barrier in the uppermost layer of skin which is a matrix made up of lipid and protein. Filaggrin protein is present in stratunm Corneum. Filaggrin is made by conversion of profilaggrin in a multistep process. This profilaggrin has no Keratin binding activity but filaggrin has.

Free fillagrin binds to Keratin intermediate filaments resulting into their aggregation in to microfibrils in which the intermediate filaments are aligned in strongly packed parallel arrays. This results into strong cross linking of keratin intermediate filaments to form highly insouluble keratin matrix. This matrix act as a protein scaffold for attachment of protein and lipids that make a barrier in upper layer of skin, Stratun corneum. Filaggrin gene which is located in the epidermal differentiation complex on chromosome 1q21 and encode profilaggrin. Therefore any loss of functional mutation in *FLG* gene results in disturbance of whole process mentioned above . Filaggrin therefore has defensive role against environmental substances or allergins. Loss of Filaggrin or Profillaggrin results in to poorly formed stratum corneum which ultimately results in to water loss from skin (Xerosis) and perturbation of skin barrier leads to enhanced percutaneous transfer of allergins (Sandilands *et al.*, 2009).

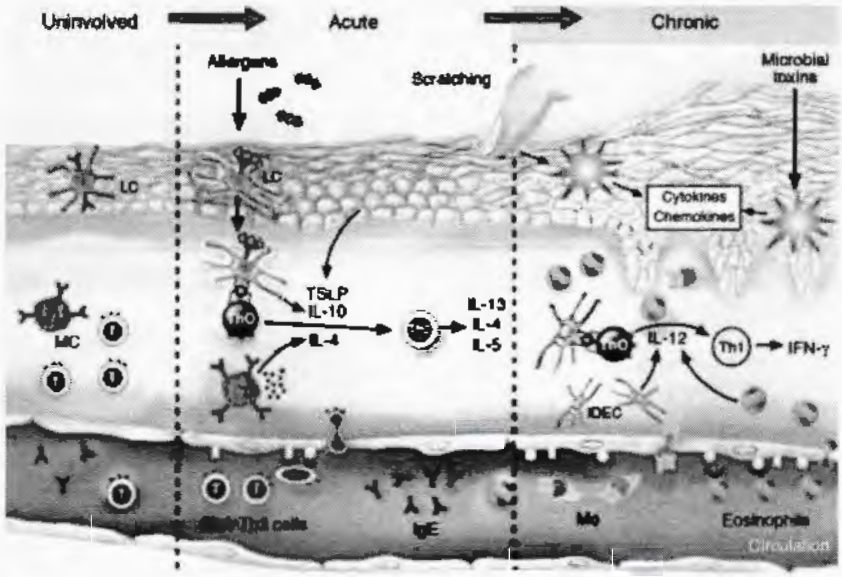


Fig B:**Immunologic pathways in AD**. Th2 cells moving in the peripheral blood of AD patients result in increased serum IgE and eosinophils. These T cells state the skin

homing receptor, CLA, and circulate again through unaltered AD skin where they can engage allergen-triggered IgE^+ LCs and mast cells (MCs) that help to Th2 cell development. Skin wound by environmental agents, injury, or microbial toxins activates keratinocytes to discharge proinflammatory cytokines and chemokines that bring the expression of adhesion molecules on vascular endothelium and help the extravasation of inflammatory cells into the skin. Keratinocyte-derived thymic stromal lymphopoietin (TSLP) and DC-derived IL-10 also increase Th2 cell differentiation. AD inflammation is associated with increased Th2 cells in acute skin lesions, but chronic AD results in the infiltration of inflammatory IDECs, macrophages (Mφ), and eosinophils. IL-12 production by these various cell types results in the switch to a Th1-type cytokine milieu associated with increased IFN-γ expression (Leung *et al.*, 2000).

Causes of AD:

The epidermis generates various protective or defensive functions which are mediated by its distinctive differentiation end-product, the stratum corneum (Elias, 2005)Propensity to develop secondary infections (Baker, 2006) and defective epidermal permeability barrier (Patrick, 2010) are well-recognized features of AD, the barrier abnormalities in AD are not just epiphenomena, but to a certain extent the 'driver' of disease activity (i.e., an 'outside-to-inside' view of disease pathogenesis. (Elias, 2014).

1) Deficiency of Filaggrin and other S100 Proteins

The strongest proof that a primary structural deformity underlies the pathogenesis of AD is derived from the latest work that connects loss-of-function mutations in the gene

encoding, filament aggregating protein (filaggrin, *FLG*) in humans with AD.(Irvine and Mclean, 2006)

Almost 50% of northern hemisphere European and American children with AD shows either double allele or single mutations in the gene encoding for *FLG* and it is found in the differentiation complex on chromosome 1q21 (Cole *et al.*,2014).

2) Protease-Anti-Protease Expression

Inherited abnormalities in either serine protease (SP) or anti-protease expression lead to defects in the structure and function of the SC, and lead to AD (*Lee et al.*,2010).

3) Ceramide deficiency:

A repeatedly-noted, selective reduction in Cer content (Vertz *et al.*, 1998)is the most impressive indication of human AD, however, various mechanisms appear to contribute to the reducion in Cer. First, the pH-induced increase in kallikrein (KLK) activity and barrier-related increase in pH, that results in malfunctioning, and finally in accelerated degradation of the Cergenerating enzymes, β-glucocerabrosidase and acidic sphingomylinase,showed most dramatically in patients with AD (Hachem *et al.*, 2006).

4:Low sphingoid base content:

Finallyimbalance in the ratio of sphingoid bases, specifically sphinganine and sphingosine seems to put remarkable effects on lamellar membrane permeability in AD patients [Obata *et al.*, 2013).

5:Th2 derived cytokines

Thi /th2 cell dysregulation:

Amplified production of the Th2-derived cytokines, IL-4 and IL-13, are otherimportant feature that results to lower Cer in AD(Hatano *et al.*, 2013) . IL-4 downregulates not only serine palmitoyltransferase, that is the rate-limiting enzyme of ceramide synthesis. It also diminishes the possible beneficial effects of Th1-derived TNF-α on ceramide-generating enzymes. Whereas Th1 cytokines instead upregulate Cer production (Sawada *et al.*,2012)

6:Antimicrobial barrier dysfunction:

Streptococcus aureus is a gram-positive bacterium that can produce a remarkable array of cell-surface and secreted virulence factors to aid causation of disease, antimicrobial resistance developed speedily, as fast as new therapeutic agents are produced(Sugarman *et al.*, 2003).In AD,Antimicrobial barrier is also compromised. One common, often disease-precipitating feature of AD is colonization by S. aureus, while colonization is maximum on lesional skin of AD patients, colony count is often high on clinically healthy skin of AD patients (Baker, 2006).

Ultimately non-toxigenic strains of S. aureus which colonize AD can be replaced by enterotoxin-generating strains (Lomholt *et al.*,2005), which sequentially, could intensify AD by at least three different mechanisms: 1) toxigenic strains are more liable to induce clinical infections than those of non-toxigenic strains. Few toxins stimulate pruritus and formation of specific IgE(Wehner *et al.*, 2001).

Reduced environmental humidity:

A well-known risk factor for AD is exposure to a reduced environmental humidity for a longer time, as occurs commonly in radiant-heated homes during the winter in temperate climates,(Langan and Irvine, 2013), transcutaneous water loss especially under these conditions would speed up across a defective SC, that aggravates the underlying permeability barrier abnormality, whileintensifying cytokine signaling of inflammation. Continued reductions in environmental relative humidity could further drain residual *FLG* in single-allele *FLG*-deficient patients with AD since*FLG* proteolysis is regulated by changes in external humidity (Scot and harding., 1986) prolongedemotional stress intensifies permeability barrier function in normal human beings (Garg *et al.*, 2001)

Lipid-Based Therapeutic treatments in Atopic Dermatitis together, describe a strong rationale (Bieber *et al.*, 2012]. Lipid-supplemented moisturizers are commonly used in AD (Loden, 2003). Evening primrose oil, a rich source of both linoleic acid and α-linolenic acid was prompted as early attempts to treat these AD patients with prominent barrier abnormality and inflammation (Senapati *et al.*,2008). Atopic children may take benefit from other dietary and extra dietary interventions which include: 1) reducing intake of trans-FA, thus increasing levels of arachidonic acid in infants' serum. 2) use of dietary sphingolipids – such as glucosylceramides andceramides that may be helpful (Duan *et al.*, 2012) ; 3) intake of reducing agents, including N-acetylcysteine and ascorbic acid; 4) topical or dietary niacinamide, which stimulates Cer assembly in vitro; and 5) dietary vitamin D. Dietary vitamin D which as compared to topical vitamin D seems to improve AD (Amestejani *et al.*, 2012). Dramatic improvements in permeability, clinical activity, barrier function, and SC integrity was shown during An open-label

study, when an commonly available version of this technology (TriCeram®) was replaced for standard moisturizers in children with severe, recalcitrant AD (Chamlin *et al.*, 2002). Even recently, a higher-strength, prescription formulation approved by FDA(EpiCeram cream, PuraCap Pharmaceutical) demonstrated effectiveness that was comparable to a mid-potency steroid (fluticasone, Cutivate cream) in an, multicenter investigator-blinded clinical trial of pediatric patients who were suffering from moderate-to-severe AD (Sugarman *et al.*, 2008).Phototherapy is generally a part of the entire treatment plan for the management of atopic dermatitis patients (AD) (Karolina *et al.*, 2016). Ultraviolet A1 (UVA1) is quite effective in healing flares, while narrow-band UVB has been suggested for chronic-moderate forms of the disease (Patrick et al., 2010). Interleukin-8 is an important mediator of chemotaxis (Breuckmann *et al.*, 2002).Tacrolimus (FK 506) is an immunosuppressant drug for avoiding organ rejection after transplantation and is effective for treatment of atopic dermatitis too (Rubins *et al.*, 1997).

2. Materials and Methods

2.1. Families identification and diagnosis

After the authorization from Institutional Review Board of International Islamic University, Islamabad, Pakistan, the study was started.

Families were identified on the basis of phenotype from different areas of Pakistan.In current work we acknowledged two families (A, B, C) having hereditary Skin disease AD. All families were living in Pakistan. The society where these families were living, cousin marriages are common. To collect blood samples the families were visited at the place where they reside. Moreover, the ample data of the family history, onset and severity of the disease was obtained. Photographs of the affected members were taken. Written consent forms were filled from all the members participating in this study. Clinical diagnosis compatible with moderate to severe Atopic dermatitis will be done to confirm our investigations.

2.2. Pedigree Analysis

An extensive pedigree was constructed based on the standard method elucidated by (Bennet *et al.*, 1995) after collecting data about the thorough medical history of the families. On the basis of material obtained by questioning the elder members of the families Pedigrees were constructed. The purpose of constructing pedigrees was to understand the mode of inheritance of the disease and genetic relationships among the family members. To symbolize males and females, Squares and circles were used, respectively. Unfilled circles or squares represent the normal individuals whereas filled circles and squares were used to represent the affected ones. To show the late females and males in the pedigree crossed circles and squares were used. respectively. Roman

numerals in descending order shows the Generation Numbers while individuals within a generation were represented by Arabic numerals in left to right order. To indicate the consanguineous marriages between the couples double line in the pedigree was used.

2.3. Blood Sampling

Blood samples as of all accessible normal and affected members were taken from peripheral venous route through 10 ml sanitized syringes (BD 0.8 mm x 38 mm 21 G x 1 ½ TW, Franklin lakes, USA). Blood samples from kids (less than 2 years of age) were collected by using special vacutainer (BD Vacutainer R safety-LokTM blood collection set with pre-attached holder 23G x 3/4" x 12". 0.6 x 19 x 305 mm). Blood samples of the individuals were transferred to the vacutainer sets having potassium EDTA (BD Vacutainer® K3 EDTA, Franklin Lakes, NJ, USA) and stored at 4°C and then processed for DNA extraction

2.4. Genomic DNA Extraction and Purification

Genomic DNA extraction was carried out by following either one of the two standard protocols:

- ✓ Standard phenol-chloroform method (Sambrook*et al.*, 1989)
- ✓ Commercially available kit method(GeneElute™Sigma-Aldrich., St. Louis, Mo.USA).

2.4.1.Organic DNA Extraction Method

Before starting genomic DNA extraction via phenol-chloroform method, blood samples stored at 4°C were incubated for one hour at room temperature. This was performed to settle all the blood components. When the blood settled 750 µl of human blood and solution A were taken in Eppendorf tubes (1.5 ml) ,then the Eppendorf tubes were inverted 4-6 times for proper mixing of

human blood and solution A(Table 3.1) and then the mixture was placed for half an hour at room temperature. The Eppendorf tubes were then centrifuged at 13,000 rpm (revolution per minutes) for 1 minute and nuclear pellet was obtained while the supernatant was discarded. Then the nuclear pellet was re suspended by adding 400 µl of solution A and again centrifuged for 1 minute at 13,000 rpm. Again the supernatant was discarded the nuclear pellet was re suspended in 400 µl of solution B, 7 µl of proteinase K (10 mg/ml stock) and 14 µl of 20% SDS. Samples were then placed at 37°C for overnight incubation or incubated at 65 °C for 3 hours. On the day second of extraction the pellet was fully dissolved, 500 µl fresh mixture prepared from equal volume of both solutions C+D (Table 3.1) was added to each of Eppendorf tubes and mixed properly. The samples were centrifuged at 13,000 for 10 minutes. As a result of centrifugation two layers were formed, upper aqueous layer was collected that contained DNA with the help of 1000 µl wide pipette set at 200 µl to reduce the risk of DNA damage in a new labelled eppendorf tube, 500 µl solution D(Table 3.1) was added to each tube and centrifuged for 10 minutes at 13,000 rpm.The separation of two layers was carried out carefully again and upper layer was transferred to new eppendorf tubes and 500 µl of iso-propanol kept at -20°C and an amount of 55 µl of sodium acetate (3 M, pH 6) was used for the precipitation of DNA.

The tubes were gently inverted several times to certify DNA precipitation and again centrifuged at 13,000 rpm for 10 minutes. DNA pellet was obtained carefully by discarding the supernatant.200 µl of chilled 70% ethanol was added to the tubes having the DNA pellet and centrifuged at 13,000 rpm for 7 minutes. Ethanol was carefully discarded to avoid detachment of the pellet, while the remaining ethanol was evaporated by keeping the tubes for 5 minutes at 45°C in a vacuum concentrator 5301 (Eppendorf, Hamburg, Germany). An appropriate amount of Tris-EDTA (TE) buffer was added to completely dissolve the DNA pellet when the residual ethanol was evaporated

completely. The tubes were kept for incubation for 24 hours so that DNA gets completely suspended in the buffer.

2.4.2. DNA Extraction Using Commercially Available Kit Method

For the extraction of genomic DNA from the blood samples, commercially available kit, Sigma's GenEluteTM Blood Genomic DNA Kit (Sigma Aldrich, USA) was used.

In this method 200 µl of whole blood, 200 µl buffer B3 (NucleoSpin®, MACHEREY-NAGEL, Duren, Germany) and lysis solution C was added in 2 ml collection tube .An amount of 10 µl proteinase K was added and thorough vortex for 15 seconds was done.At 55°C the sample was incubated for 10 minutes.

In the pre-assembled GenEluteMiniprep Binding Column 500 µl of Column Preparation Solution was added and for 1 minute centrifugation was carried out at 13,000 rpm.Flow-through liquid was discarded and 200 µl of 100% chilled ethanol (labscan analytical Sciences, Bangkok, Thailand) was added to the lysate and vortexing for 5-10 seconds was done for proper mixing.

In the whole solution was shifted to the treated column and centrifugation was carried out for 1 minute at 10,000 rpm.Flow-through liquid was discarded from the collection tubes and the column was shifted to a new 2 ml collection tube.An amount of 500 µl of Prewash solution (NucleoSpin, MACHEREY-NAGEL, and Duren, Germany) was added to the column and then the tubes were centrifuged for 1 minute at 10,000 rpm. The column was transferred to a new 2ml collection tubeafter discarding flow through liquid. The column was centrifuged at 10,000 rpm for 3 minutes after adding 500 µl Wash solution. To elute the residual ethanol, empty spin for 1 minute was done at 12.000 rpm.200 µl elution solutions was added in the centre of the column after transferring it

into a new 2 ml collection tube after discarding flow through liquid, the tube containing column was left at room temperature for 5 minutes.In order to elute DNA the column was centrifuged for 1 minute at 10,000 rpm and the collection tube containing pure genomic DNA was stored at 4°C.

2.5. Agarose Gel Electrophoresis for Extracted DNA Quantification

Quantitative analysis of extracted DNA was done by using 1% agarose gel which resolve the DNA. Gel was prepared by adding 0.5 g of agarose to the mixture of 45 ml of distilled water and 5 ml of 10 X TBE (0.89 M Tris-Borate, 0.032 M EDTA) (Table 3.2) in the flask. 0.5 g of agarose was weighed with the help of electric balance. Agarose was dissolved by heating the flask in microwave for 2 minutes. As the transparent solution was obtained, 7 µl of ethidium bromide was added to the flask. Ethidium bromide was added to stain the DNA bands. By gentle shaking the dye was thoroughly mixed in the solution and then the solution was carefully poured into the gel tank.

The gel mixture was left for 20-25 minutes at room temperature for solidification. Equal volume(5 µl) of extracted DNA was mixed with loading dye (0.25% bromophenol blue with 40% sucrose) and the mixture of DNA and dye was loaded into the gel wells. In the gel tank 1X TBE buffer was added and the gel electrophoresis was run for 30-40 minutes at 120 volts, horizontally.DNA bands were visualized under U.V Trans-illuminator (Biometra, Gottingen, Germany).To properly analyse the DNA bands photographs were taken by using Digital Camera EDAS 290 (Kodak. New York, USA).

2.6. Homozygosity Mapping/Linkage Analysis

Linkage analysis or genetic mapping or genotyping of the families with hereditary hair loss was performed by using highly polymorphic microsatellite markers for the well-known gene loci. The genetic map distance of markers was obtained from UCSC Genome browser and Rutgers's

Combined Linkage-Physical Map (Matise*et al.*, 2007).Markers were found by Invitrogen Gene link (USA).

27. Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) was carried out to amplify the specific DNA sequences. DNA sequences were subjected to genotyping by using specific polymorphic microsatellite markers (Table 3.5). 25 µl was set as the total volume of mixture using 2 µl DNA. 0.2 ml volume PCR tubes (Axygen, California, USA) were used. The mixture was prepared by adding different chemicals of different amounts and compositions for this reaction. (Table 3.3). 2.5 µl PCR buffer 10X (200 mM (NH4)2SO4, 750 mM of Tris-HCl pH 8.8, 0.1% Tween 20), a DNA sample dilution of 1.5 µl, 2 µl of MgCl2 (25 mM), forward and reverse microsatellite markers (20 ng/µl) in amount of 0.3 µl each, 0.5 unit Taq Polymerase (Perkin-Elmercetus, Ferments, Burlington, Canada) in amount of 0.3 µl and 0.5 µl of (10 mM) dNTPs (MBI Fermentas, UK) in 17.6 µl PCR water. The reaction mixture was subjected to centrifugation at 4,000 rpm in order to get thorough mixing of reaction mixture. Reactions were performed by means of T3 thermocyclers (Biometra, Gottingen, Germany) with the following conditions.

The standard conditions for thermocycler are:

- To denature the double stranded DNA the cycle of template DNA denaturation at 96°C for 7 minutes.
- Amplification cycles were kept 40 each of which included 3 sub- cycles. Each cycle included denaturation of double stranded DNA into single stranded

- DNA at 96°C for one minute, followed by cyclic "annealing" or hybridization of microsatellite markers to their complementary DNA sequences for 1 minute at a temperature of 57-60°C, 1 minute for cyclic elongation of specific DNA sequences from each primer at 72°C, also called cyclic extension.
- Final extension or polymerization was performed at 72°C for 10 minutes by Taq DNA polymerase to complete amplification of any leftover specific DNA sequences present in the reaction mixture.

With the help of PCR, specific DNA sequences were amplified a number of times.

Table 3.1 Composition of Solutions Used in Genomic DNA Extraction

Solution	**CompositionS**
Solution A	Sucrose 0.32 M MgCl2 5 mM Tris (pH 7.5) 10 mM 1 % (v/v) Triton X-100
Solution B	EDTA (pH 8.8) 2 Mm NaCl 400 mM Tris (pH 7.5) 10 mM
Solution C	Saturated phenol
Solution D	24 volumes of Chloroform : 1 volume of Isoamylalcohol i.e. 24:1
10% SDS	10 g in 50 ml water

DNA dissolving Buffer	10 mMTris (pH 8.0) 0.1 mM EDTA
Bromophenol blue	Sucrose 40 g Bromophenol Blue 0.25 g

Table 3.2 Composition of Solutions used in Gel Electrophoresis

Solution	Composition
Gel Loading Dye	40 g Sucrose 0.25 g Bromophenol Blue
10X TBE Buffer	0.89 M Tris 0.025 M Boric Acid EDTA 0.5 M (pH 8.3)

3.8. Polyacrylamide Gel Electrophoresis (PAGE)

The analysis of the amplified PCR product was carried out on 8% polyacrylamide gel performing electrophoresis. The gel composition is as follows.

The gel mixture (Table 3.4) was thoroughly mixed by inverting the measuring cylinder for two to three times. Two washed glass plates apprehended 1.5 mm apart by clips and spacers were set and the gel mixture was poured gently between the plates and the combs were inserted to make wells in the gel and for proper polymerization and solidification the plates were left for 30-40 minutes at room temperature. The amount of 6-7 µl loading dye(40% sucrose with 0.25% of bromophenol) was gently mixed with amplified PCR products and then were loaded in wells and left for 2-3 hours at 140 volts for electrophoresis in Vertical gel apparatus (Whatman, Biometra, Gottingen,

Germany). The running media used was 1X TBE buffer. The gel was taken out from the plates, stained with ethidium bromide (0.5 µg/ml), visualized by UV Trans illuminator (Biometra, Gottingen, Germany) after the gels were run enough at the edge along with the loading dye, and finally the picture of the gels were captured by using digital camera EDAS 290 (Kodak, New York, USA).

Table 3.3 Chemicals Used in Polymerase Chain Reaction

Chemical Used	**Stock Concentration**	**Amount used in PCR**
PCR Buffer	10 X (200 mM Ammonium Sulphate, 750 mMTris-HCL (pH 8.8), 0.1 %Tween 20)	2.5µl
$MgCl_2$	25 mM	2 µl
dNTPs	10 mM each dNTPs	0.5µl
Microsatellite Markers(R & F)	20 ng/µl each	0.3µl each
Taq Polymerase	0.5 U/µl	0.3µl

Table 3.4 Polyacrylamide Gel Composition

Chemicals	**Stock Concentration & Composition**	**Amount for one Gel**
30% Acrylamide	29:1 ratio of acrylamide (MERCK, Darmstadt,Germany).N,N'Methylene-bisacrylamide (BDH,Poole, England)	13.5 ml

10X TBE	Tris 0.89 M, Borate 0.89 M and EDTA 0.02M	5ml
10%APS	Ammonium per sulphate (5 g/45 ml distilled water) (Sigma-Aldrich St Louis, MO,USA)	400µl
TEMED	N, N, N', N'-Tetra methyl ethylene diamine) (Sigma-Aldrich, USA)	25µl
Distilled Water		Raise up to 50

Table 3.5 List of microsatellite markers used to investigate the linkage to knowngenes/loci.

NO.	CANDIDATE GENE/LOCI	CHROMOSOMAL LOCATION	MARKERS	DISTANCE cM	Mb+
1.	*FLG*	1q21.3	D1S2643	243 cM	22.48
			D1S2858	244cM	21.81
			D1S2709	245cM	22.85
			D1S1644	239cM	22.76
2	*IL12B*	5q33.3	D5S412	31.16 cM	46.79
			D5S527	34 cM	47.72
			D5S1386	35cM	47.78
			D5S486	38cM	48.82

3	*TYK2*	19p13.2	D19S586 D19S916 D19S884	29.1cM 27cM 23.69cM	26.54 25.95 27.41
4	*PGM3*	6q14.1	D6S1609 D6S968 D6S497	56.29cM 57 cM 55 cM	67.50 68.50 69.89
5	*HAVCR2*	5q33.3	D5S1507 D5S527 D5S2093 D5S2112	162.60cM 166.97cM 169cM 163.6	18.36 18.48 18.55 18.58
			D17S1850 D17S58	57 cM 61.81cM	183.61 185.04

6	*CCL11*	17q12	D17107	64 cM	185.76
7	*ZNF750*	17q25.3	D17S1806	125cM	125
			D17S668	133cM	173.16
			D17S1830	127cM	173.28
			D17S914	131cM	173.99
8	*DOCK8*	9p24.3	D9S1779	0.38cM	20.35
			D9S1858	0.86cM	24.62
			D9S917	0.38cM	23.43

3. Results

3.1. Families Description

3.1.1. Family A

Family A belongs to Islamabad Pakistan. Three generation pedigree was constructed after collecting appropriate information about the disorder history from elder members of this family (Figure 3.1). Pedigree investigation strongly put forward the autosomal recessive pattern of inheritance. The pedigree consists of total 10 members including one affected member; seven females and three males. The affected individual was observed carefully. Clinically investigations show that one affected male member have skin problem, i.e. dry itchy and dark skin on face arms and legs (Figure 3.2). The disease is because of genetic and environmental factors. Blood samples were taken from six participants of the family. Family samples consisting 1 affected individual (III-1) and other five members.(II-3,II-4,III-2,III-3,III-4).

3.1.3.Family B

Family B is also a consanguineous family living in Islamabad Pakistan (Golda Shareef). A three generation pedigree was constructed by gathering the correct data from the elders of the mentioned family (Figure 3.3). The pedigree consists of total 11 members out of which 2 members were affected, both were males. The affeeted individuals are thoroughly observed to know about the clinical features. Pedigree investigation strongly put forward the autosomal recessive mode of inheritance. The affected members have dry,itchy, allergic skin dark reddish in appearance(Figure 3.4). Blood samples were

collected from five members of the family. Family samples Including two affected individuals (III-2,III-3) and three normal members (I-2, II-2, II-3).

3.1.2.Family C

Family C is a wide consanguineous family which is residing in Islamabad Pakistan. A three generation pedigree was constructed by gathering the correct data from the elders of the mentioned family (Figure 3.5). The pedigree consists of total 13 members out of which 4 members were affected including three males and one female. The affected individuals are thoroughly observed to know about the clinical features. Pedigree investigation strongly put forward the autosomal recessive mode of inheritance. The affected members have dry,itchy skin reddish in colour. Blood samples were collected from nine members of the family. Family samples Including four affected individuals (II-6, III-2, III-3, III-4) and five normal members (I-2, II-1, II-2, II-3, II-4).

3.2. Genetic Mapping of Candidate Genes for Atopic dermatitis

On the basis of genetic linkage studies in autosomal recessive hereditary AD, several known candidate genes were tested for linkage in these three families. Markers flanking AD candidate genes were amplified in all members of each family and resolved on PAGE to document the allelic patterns. These allelic patterns were further analyzed to observe homozygosity at multiple markers in the affected member of the tested families. Table 3.5 summarizes the microsatellite markers in the region of known loci, which were used for candidate gene mapping.

In the present study, first family (A) was tested for linkage to the loci known for Atopic dermatitis. The candidate genes tested for linkage analysis of Atopic dermatis include,

FLG (Fillagrin), *TYK2* (Tyrosine Kinase), *DOCK8* (Dedicator of cytokinesis 8), *PGM3* (Phosphoglucomutase), *CCL11* (Chemokine, cc motif ligand), *ZNF750* (Zinc finger protein 750), *IL12B* (Interleukin 12B) and *HAVCR2* (Hepatitis A virus cellular receptor 2). Analysis of the results revealed that the family was not linked to any of the known Atopic dermatitis loci and genes (Figure 3.6-3.13), suggesting the involvement of a novel gene yet to be discovered as responsible for AD in this family.

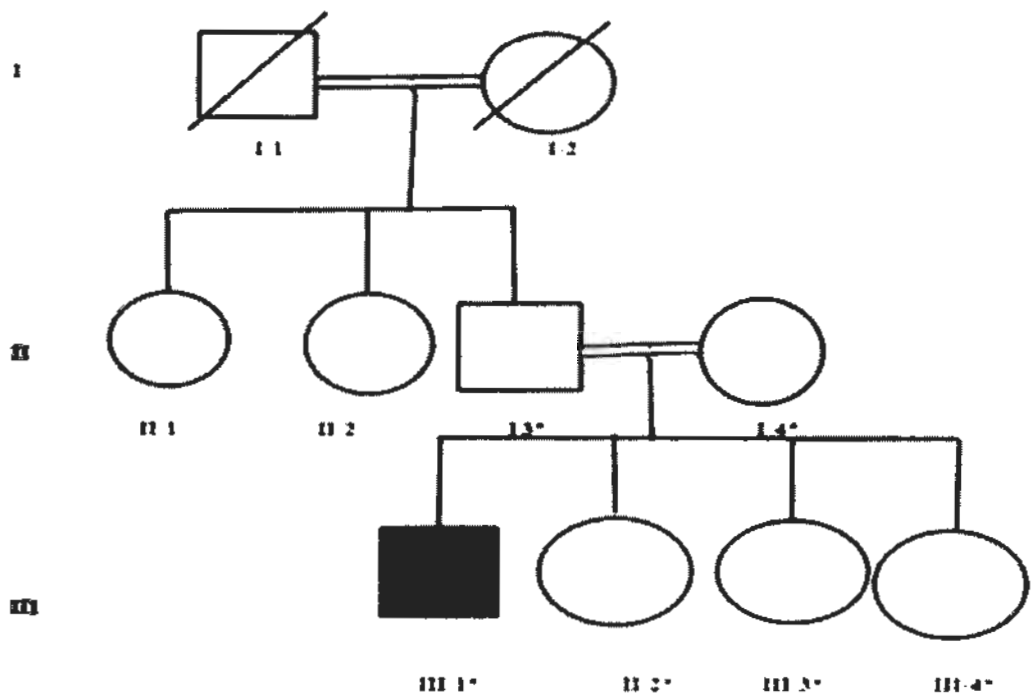
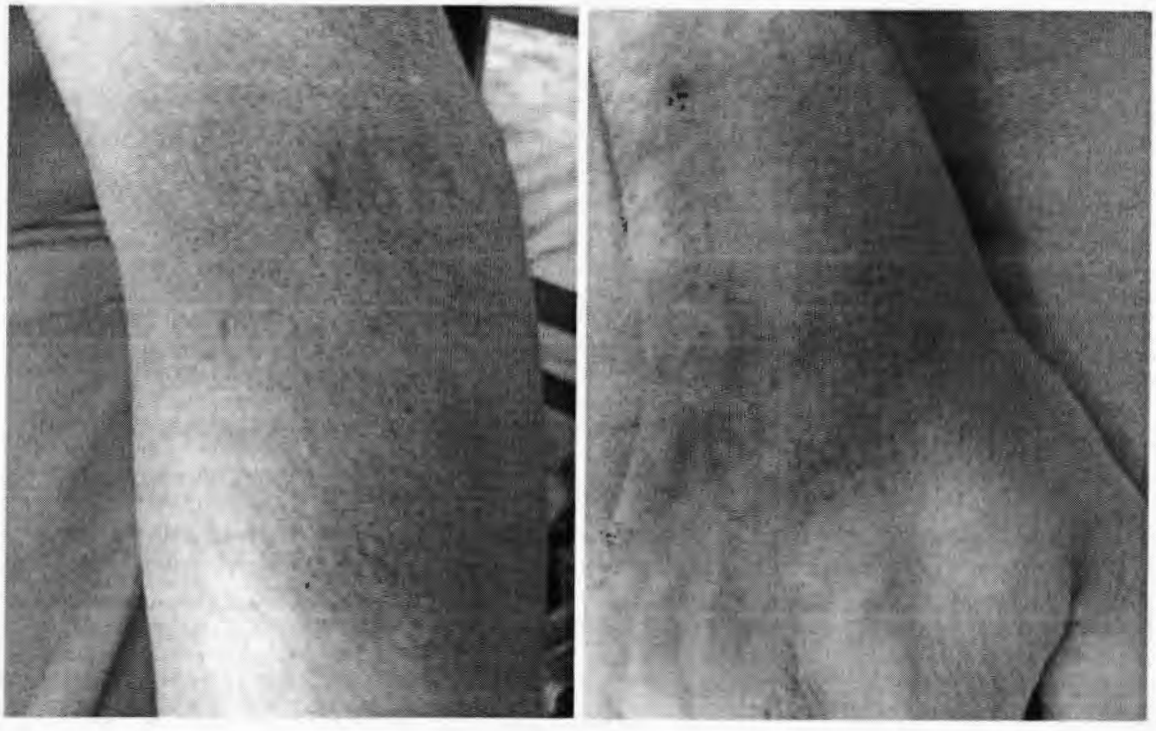


Figure 3.1 Pedigree of family A segregating autosomal recessive Atopic Dermatitis. The samples were collected from the member that are mentioned with (*) in the pedigree.



A- Patient having symptoms of Atopic dermatitis. (Black patches on skin,dryness)

Figure 3.2 Affected individual (III-1) of family A with autosomal recessive hereditary Atopic dermatitis.

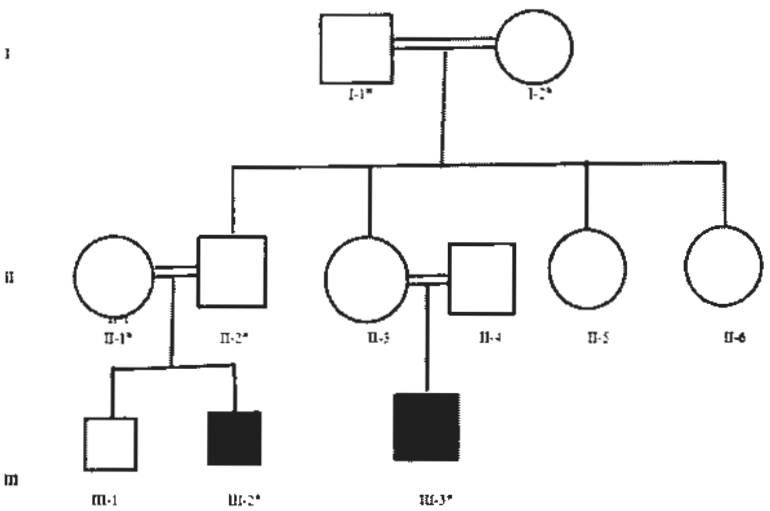


Figure 3.3 Pedigree of family B showing autosomal recessive Atopic Dermatitis. The samples were collected from the member that are mention with (*) in the pedigree.

Figure 3.4 Affected individuals (III-3) of family B with autosomal recessive disease Atopic dermatitis having reddish cheeks and chin along with extreme dryness .

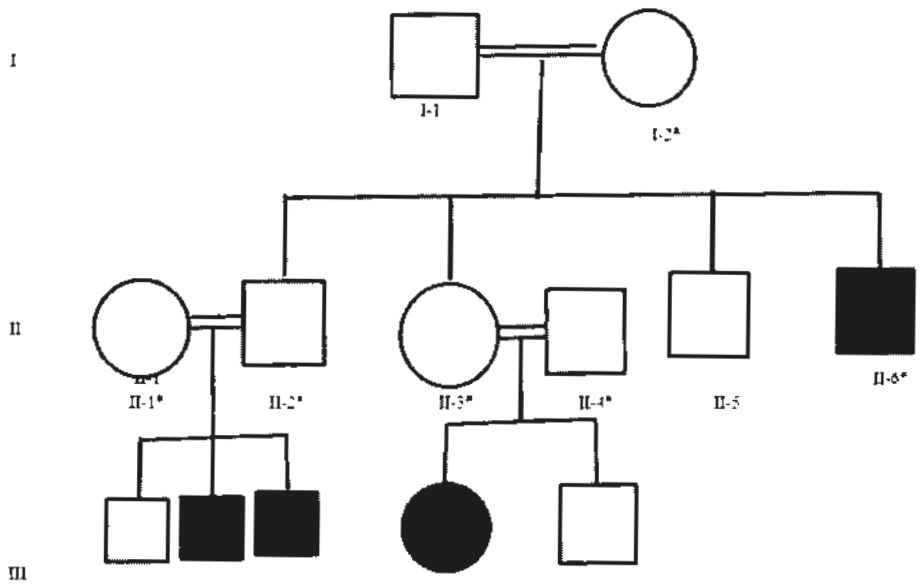
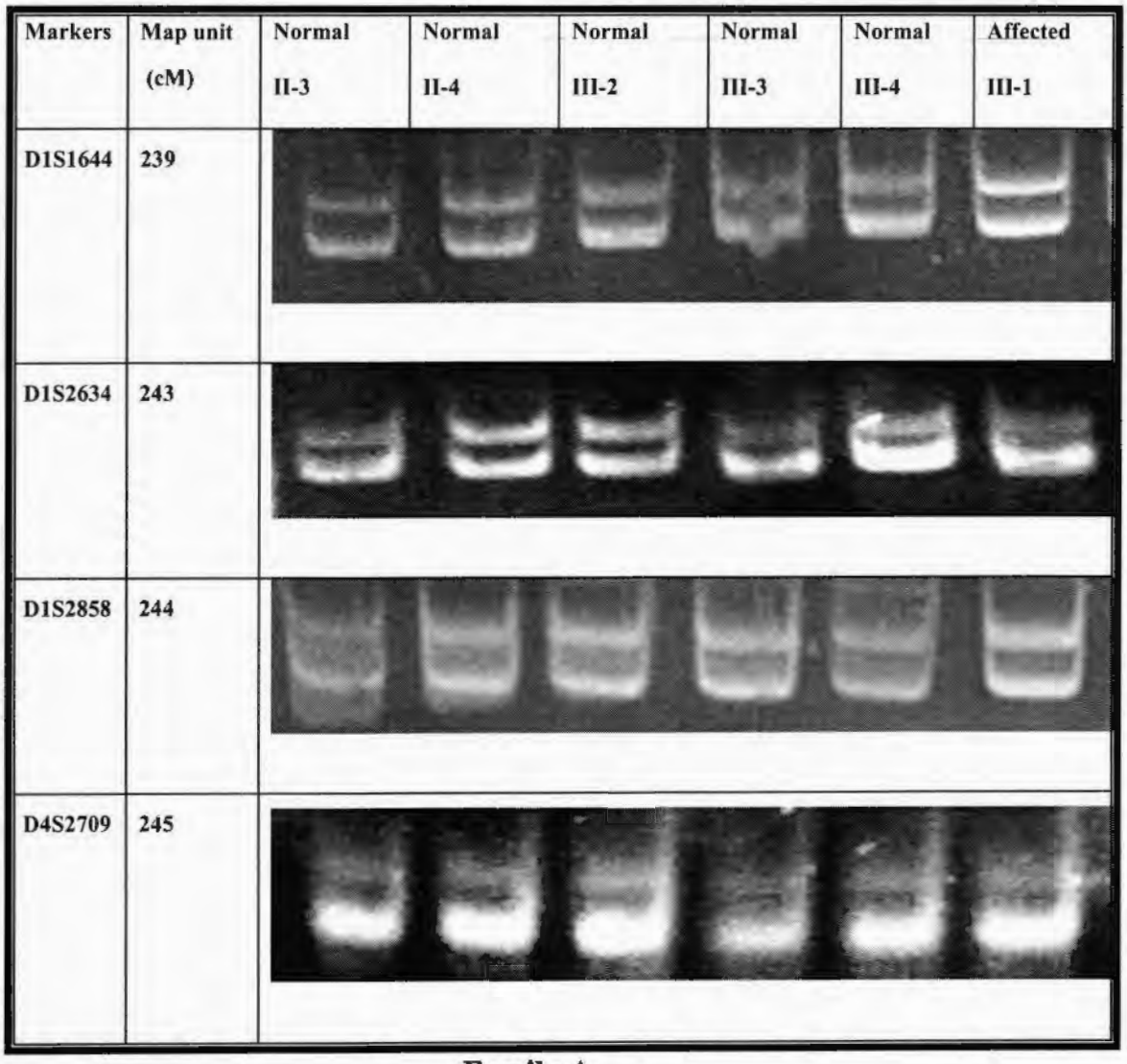


Figure 3.5. Pedigree of family C showing autosomal recessive Atopic Dermatitis. The samples were collected from the members that are mention with (*) in the pedigree.

Family B and C were also tested for linkage to the loci known for Atopic dermatitis. The candidate genes tested for linkage analysis of Atopic dermatitis for Family A and B include, *FLG* (Fillagrin), *TYK2* (Tyrosine Kinase), *DOCK8* (Dedicator of cytokinesis 8), *PGM3* (Phosphoglucomutase), CCL11 (Chemokine, cc motif ligand), *ZNF750* (Zinc finger protein 750), *IL12B* (Interleukin 12B) and *HAVCR2* (Hepatitis A virus cellular receptor 2). Whereas for family C linkage analysis include *FLG* (Fillagrin), *CCL11* (Chemokine, cc motif ligand), and *HAVCR2* (Hepatitis A virus cellular receptor 2). Analysis of the results revealed that the these families were not linked to any of the known Atopic dermatitis loci and genes (Figure 3.6-3.24), suggesting the involvement of a novel gene yet to be discovered as responsible for AD in these families (A,B,C).



Family A

Figure 3.6 Gel images showing the banding pattern of chromosome1q21.markers in six member of family A. These markers were used to test the *FLG* gene. Markers

with genetic location are mentioned on the left where as individual IDs are written above the respective lanes

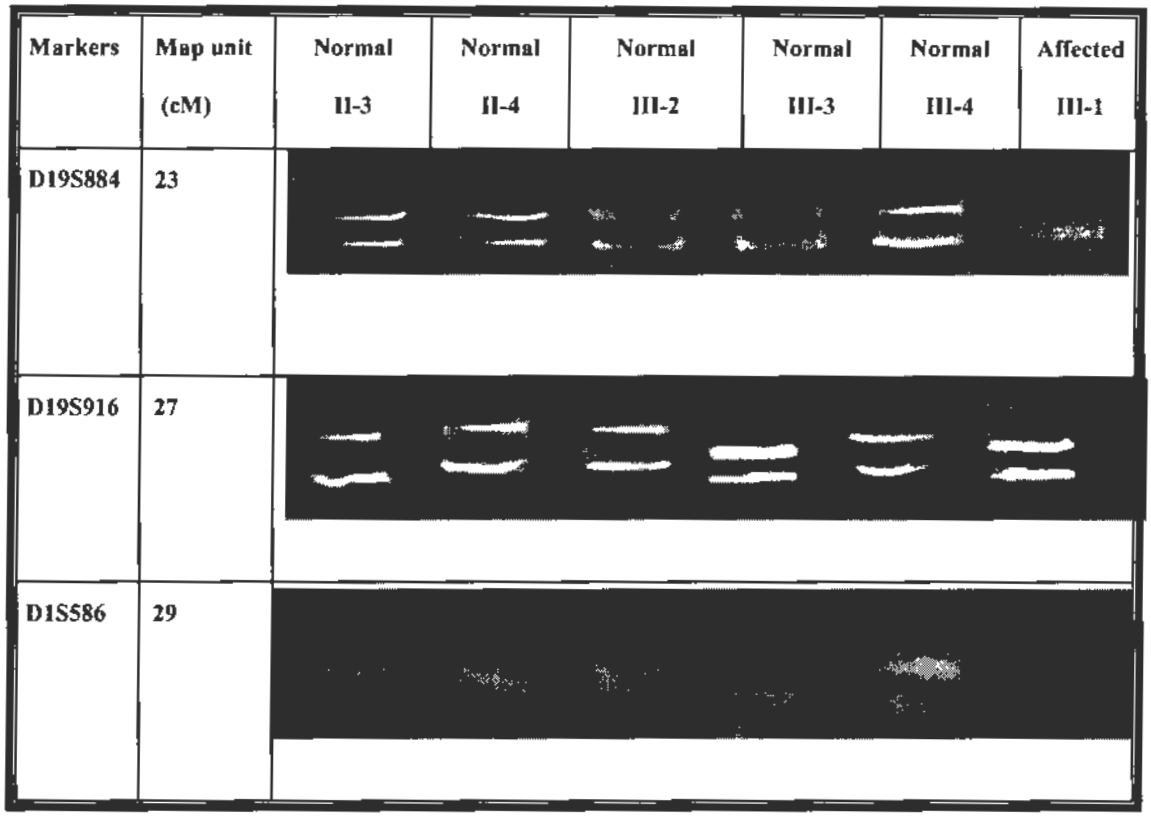


Figure 3.7 Gel images showing the banding pattern of chromosome 19p13 markers in six member of family A. These markers were used to test the *TYK2*gene. Markers with genetic location are mentioned on the left where as individual IDs are written above the respective lanes.

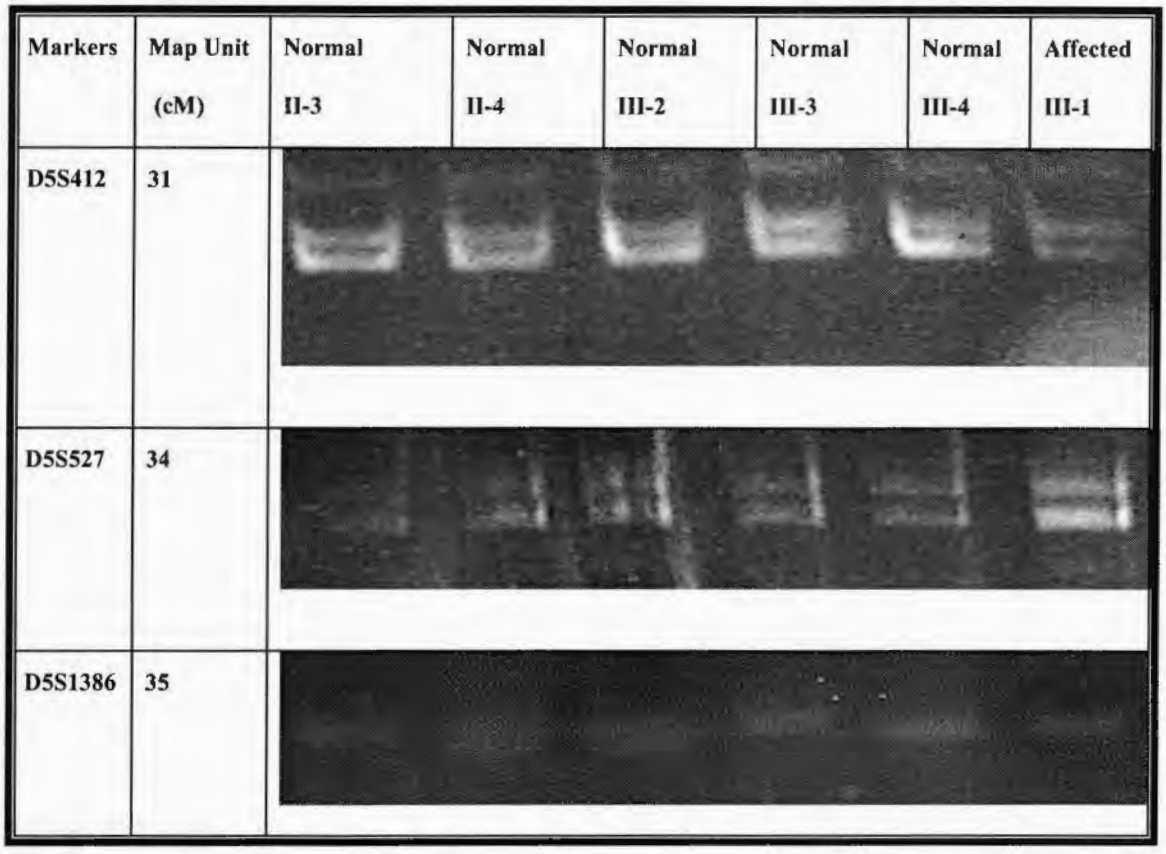


Figure 3.8 Gel images showing the banding pattern of chromosome 5q33.3markers in six members of family A. These markers were used to test the *IL12B*gene. Markers with genetic location are mentioned on the left where as individual IDs are written above the respective lanes.

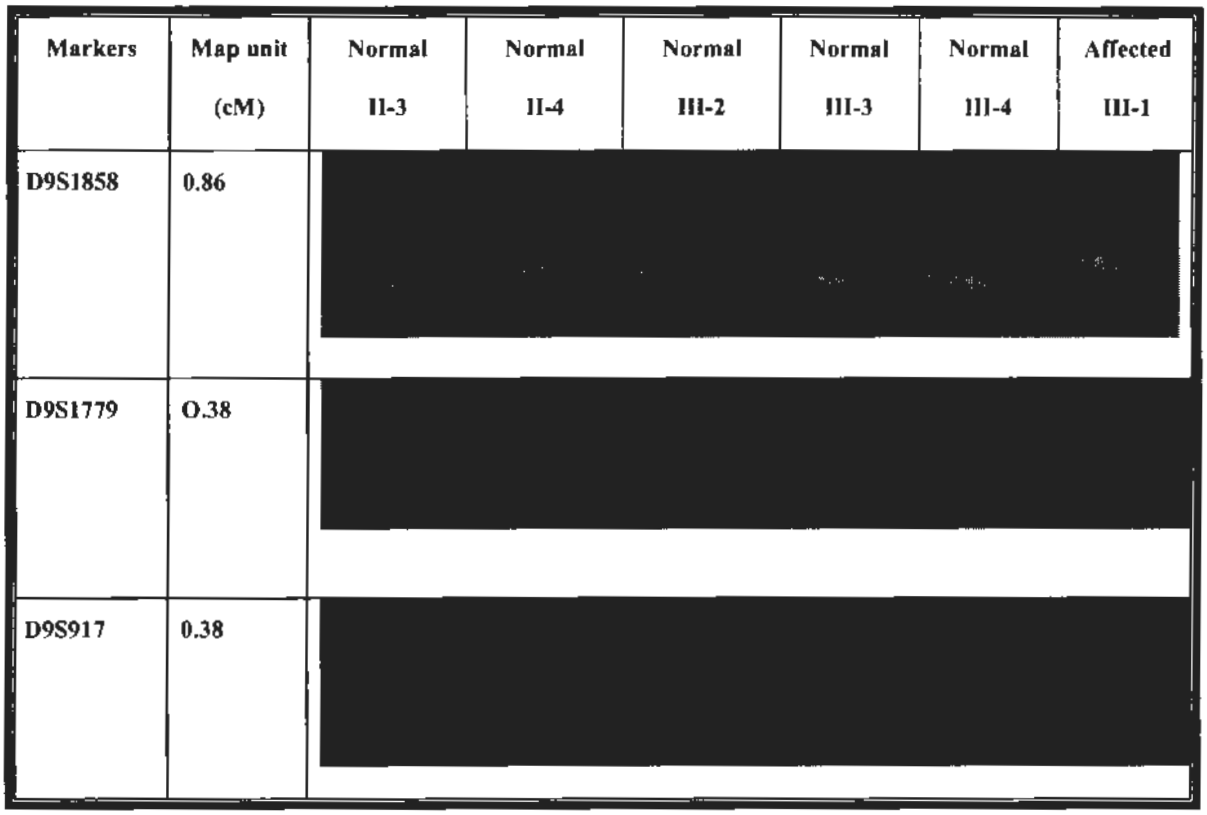


Figure 3.9 Gel images showing the banding pattern of chromosome 9p24.3markers in six member of family A. These markers were used to test the *DOCK8*gene. Markers with genetic location are mentioned on the left where as individual IDs are written above the respective lanes.

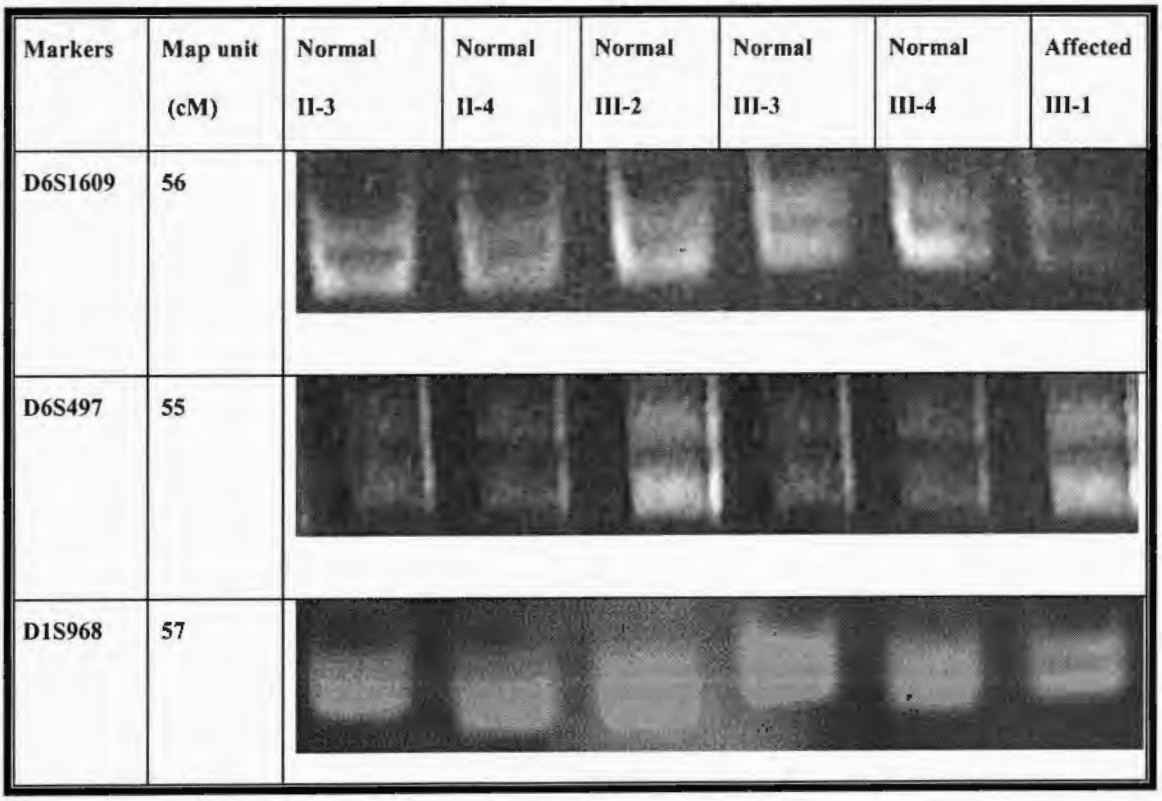


Figure 3.10 Gel images showing the banding pattern of chromosome 6q14.1markers in six member of family A. These markers were used to test the *PGM3*gene. Markers with genetic location are mentioned on the left where as individual IDs are written above the respective lanes.

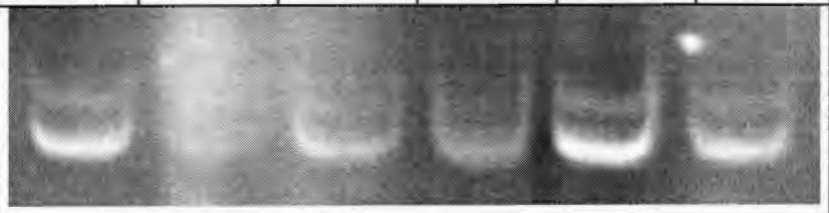
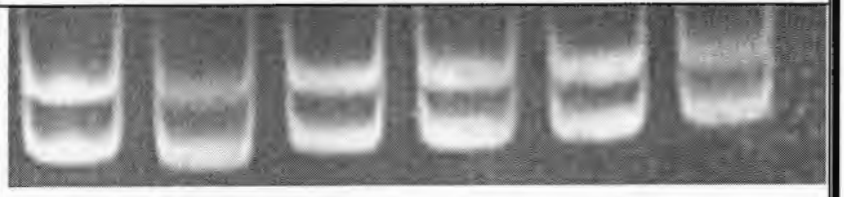
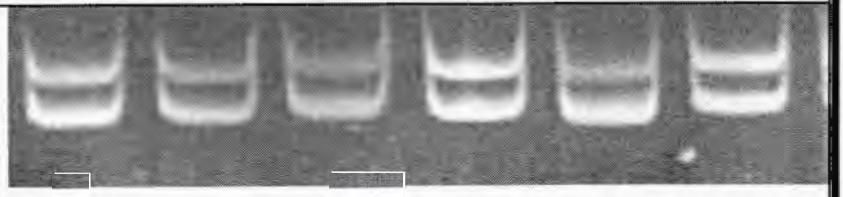
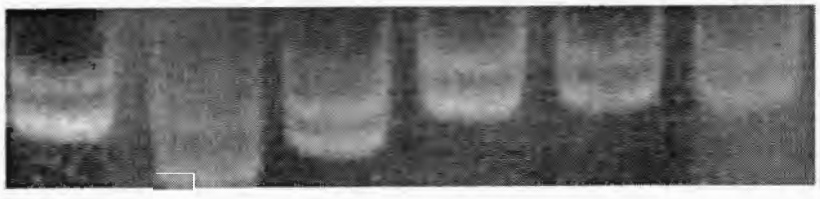
Markers	Map unit (cM)	Normal II-3	Normal II-4	Normal III-2	Normal III-3	Normal III-4	Affected III-1
D17S1294	52						
D17S1293	58						
D17S758	61						
D17S107	64						

Figure 3.11 Gel images showing the banding pattern of chromosome 17q12markers in six member of family A. These markers were used to test the *CCL11*gene. Markers with genetic location are mentioned on the left where as individual IDs are written above the respective lanes.

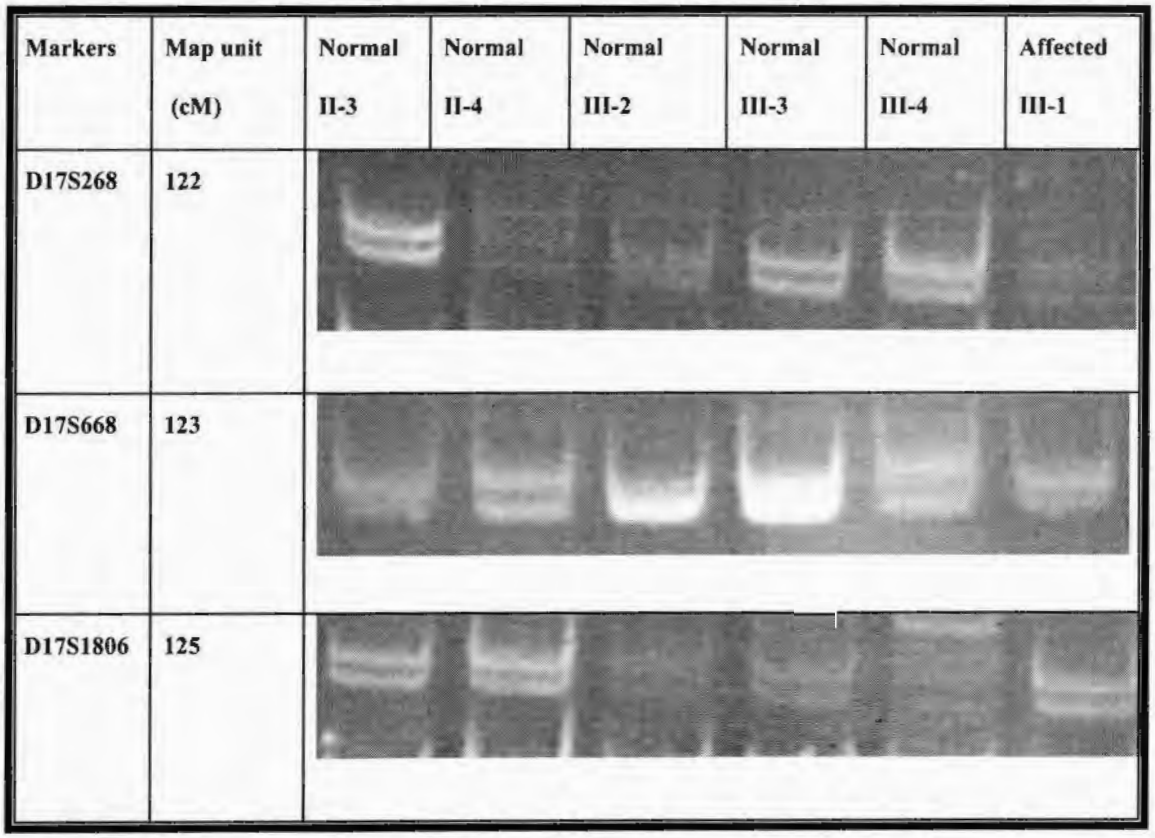


Figure 3.12 Gel images showing the banding pattern of chromosome 17q25.3 markers in six member of family A. These markers were used to test the *ZNF750*gene. Markers with genetic location are mentioned on the left where as individual IDs are written above the respective lanes.

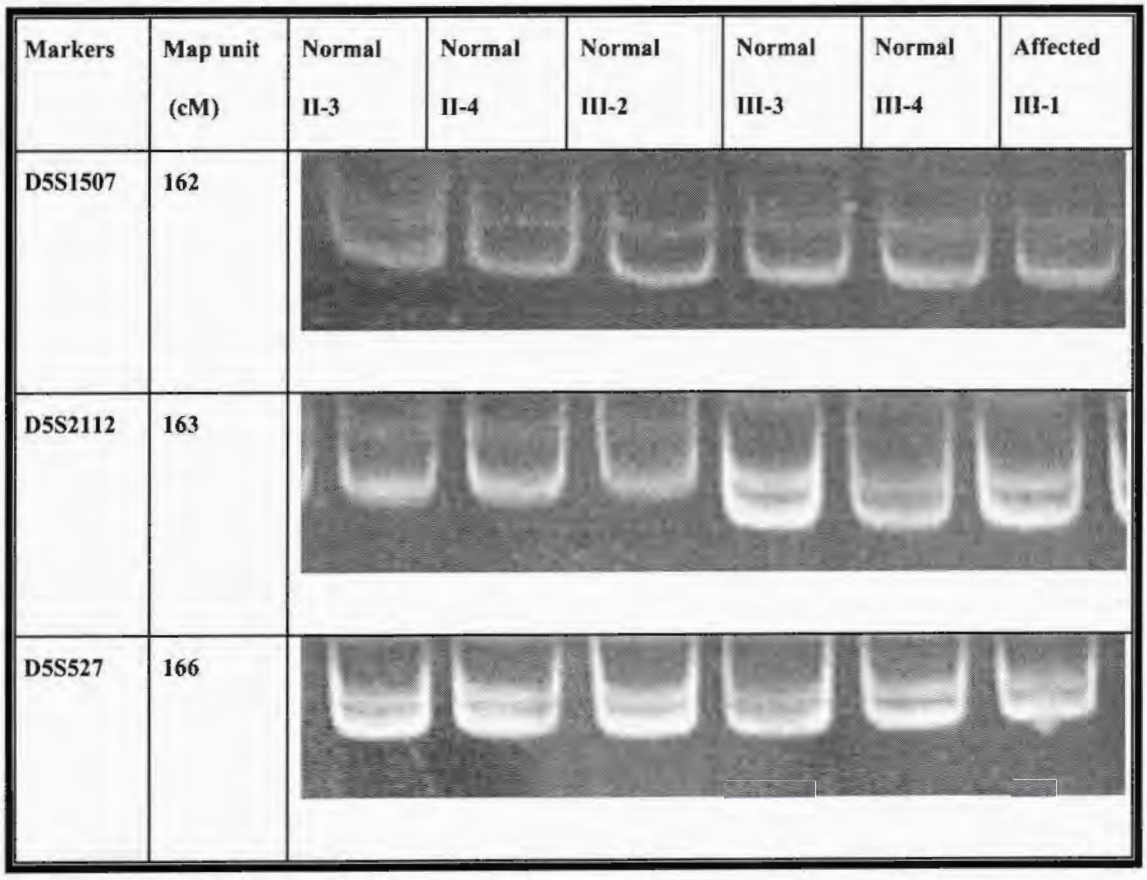


Figure 3.13 Gel images showing the banding pattern of chromosome 5q33.3 markers in six member of family A. These markers were used to test the *Havcr2*gene. Markers with genetic location are mentioned on the left where as individual IDs are written above the respective lanes.

Family B

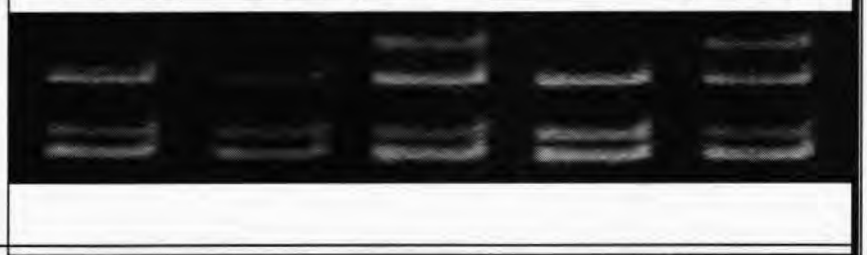
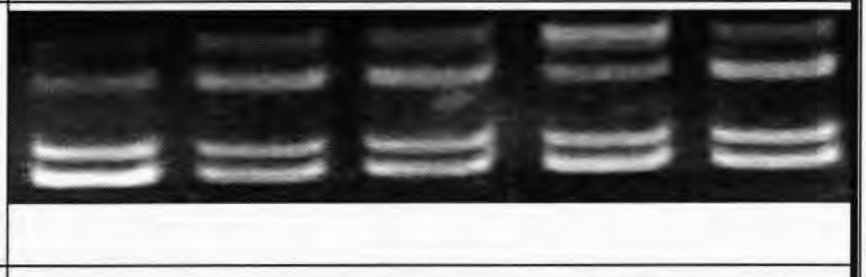
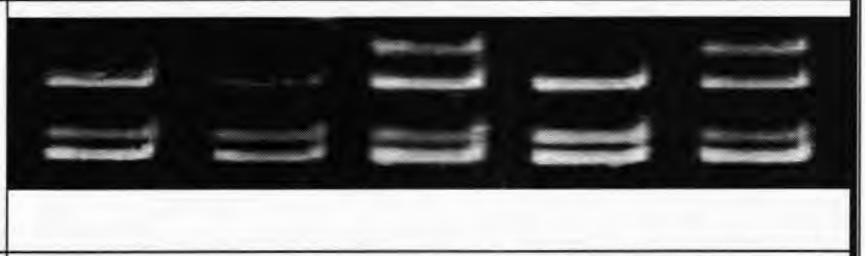
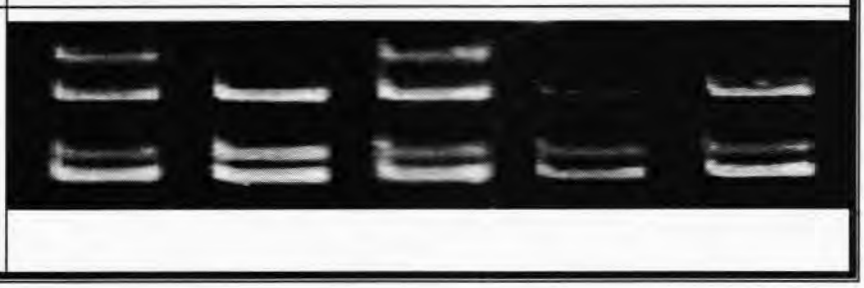
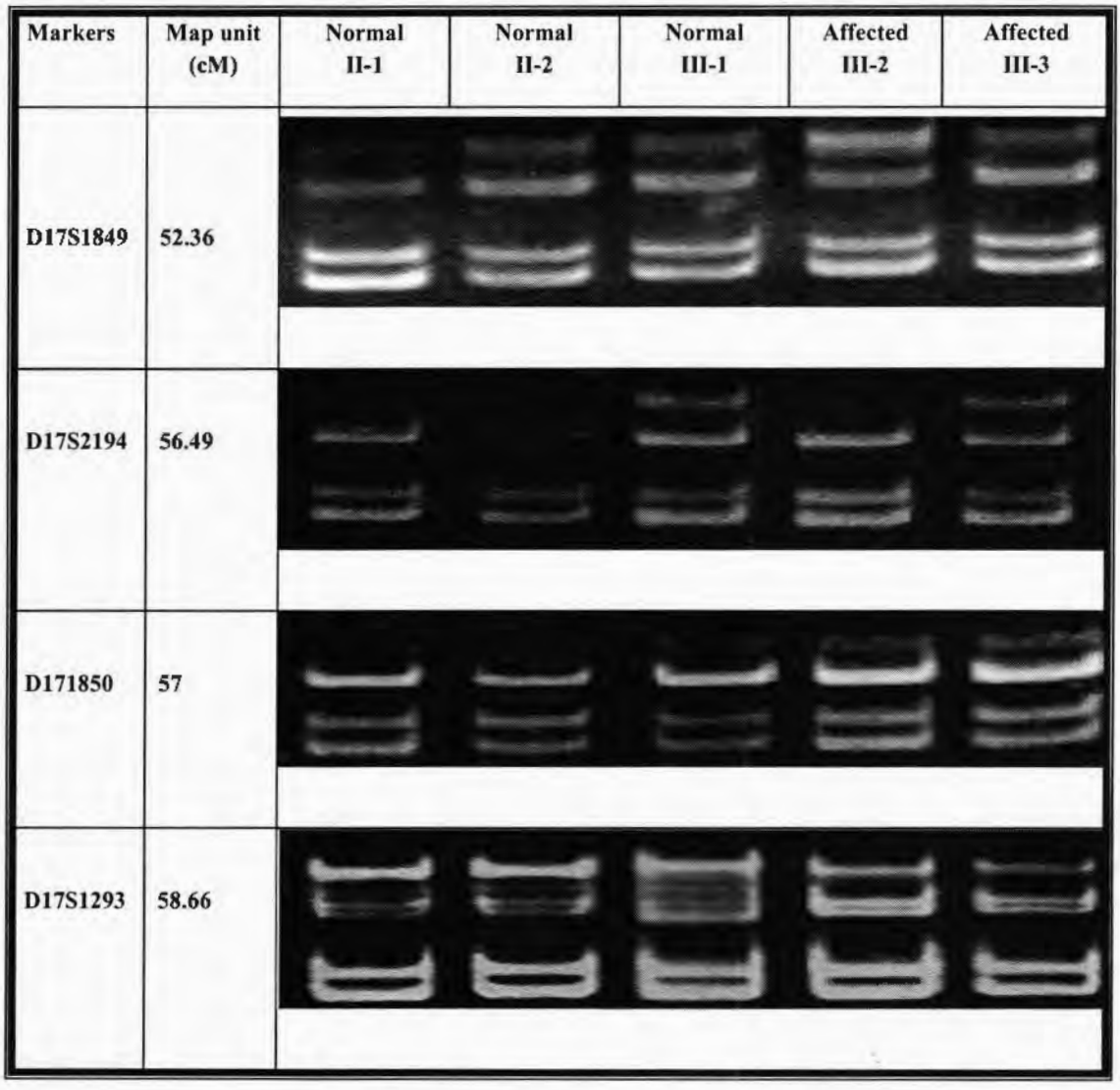
Markers	Map unit (cM)	Normal II-1	Normal II-2	Normal III-1	Affected III-2	Affected III-3
D1S1644	239					
D1S1595	243					
D1S292	244					
D1S2709	245					

Figure 3.14: Gel images showing the banding pattern of chromosome 1q21.3 markers in five members of familyB. These markers were used to test the *FLG*gene. Markers with genetic location are mentioned on the left where as individual IDs are written above the respective lanes.



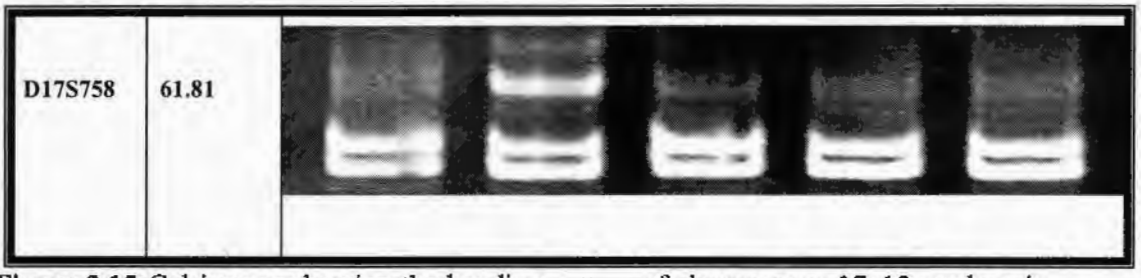
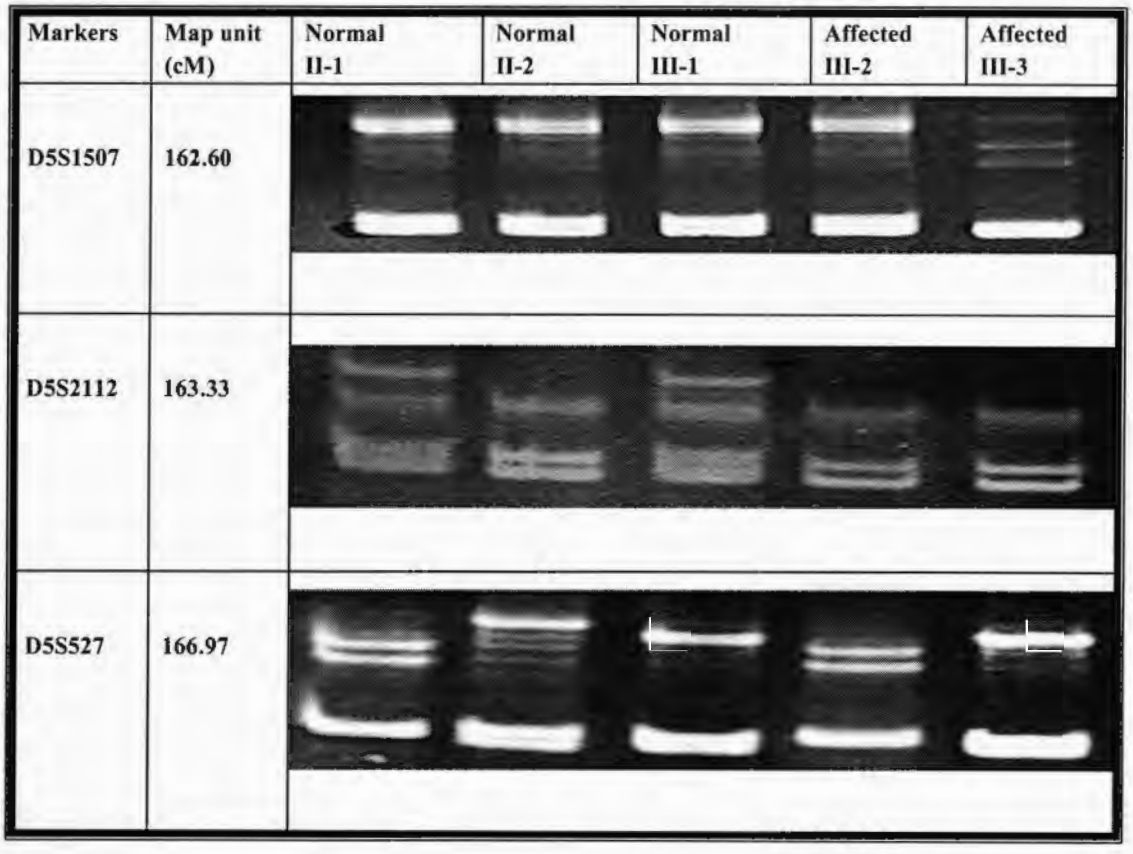


Figure 3.15: Gel images showing the banding pattern of chromosome 17q12 markers in five members of family C. These markers were used to test the *CCL11* gene. Markers with genetic location are mentioned on the left where as individual IDs are written above the respective lanes.



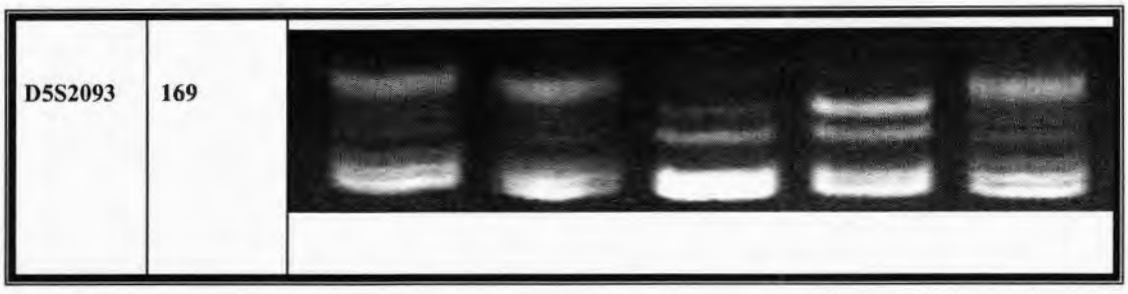


Figure 3.16: Gel images showing the banding pattern of chromosome 5q33.3 markers in five members of family C. These markers were used to test the *HAVCR2*gene. Markers with genetic location are mentioned on the left where as individual IDs are written above the respective lanes

Family C

Figure 3.17 Gel images showing the banding pattern of chromosome 1q21.markers in nine member of family C. These markers were used to test the *FLG* gene. Markers with genetic location are mentioned on the left where as individual IDs are written above the respective lanes

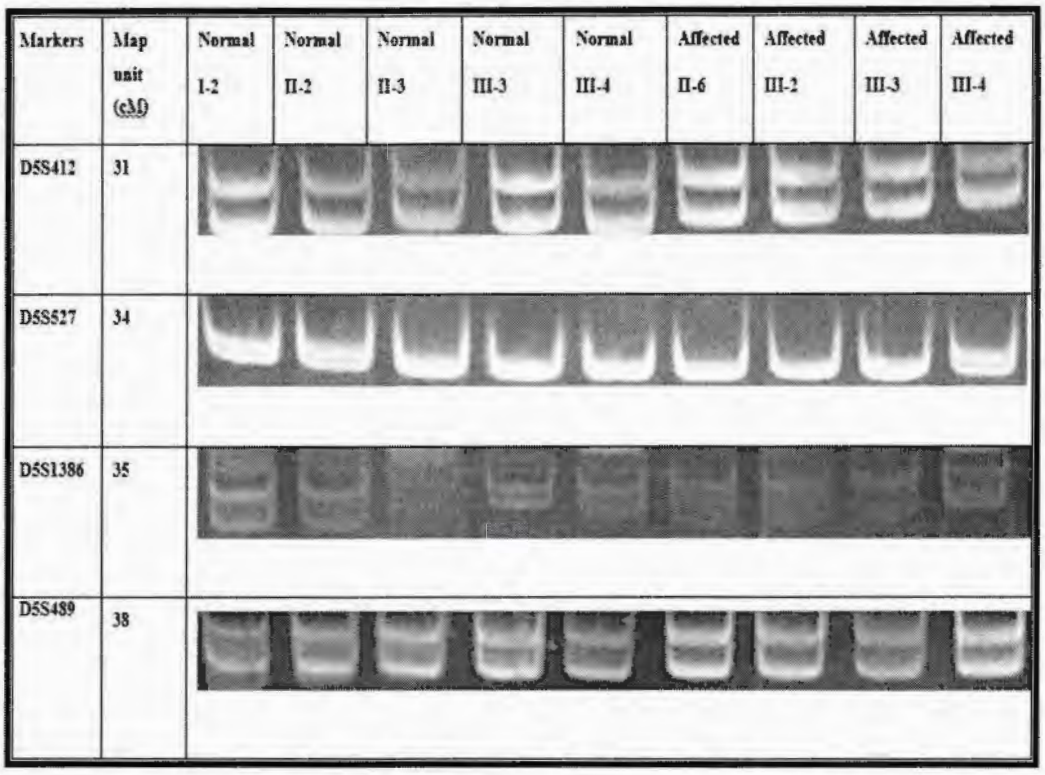


Figure 3.18 Gel images showing the banding pattern of chromosome 5q33.3markers in nine member of family C. These markers were used to test the *IL12B* gene. Markers with genetic location are mentioned on the left where as individual IDs are written above the respective lanes.

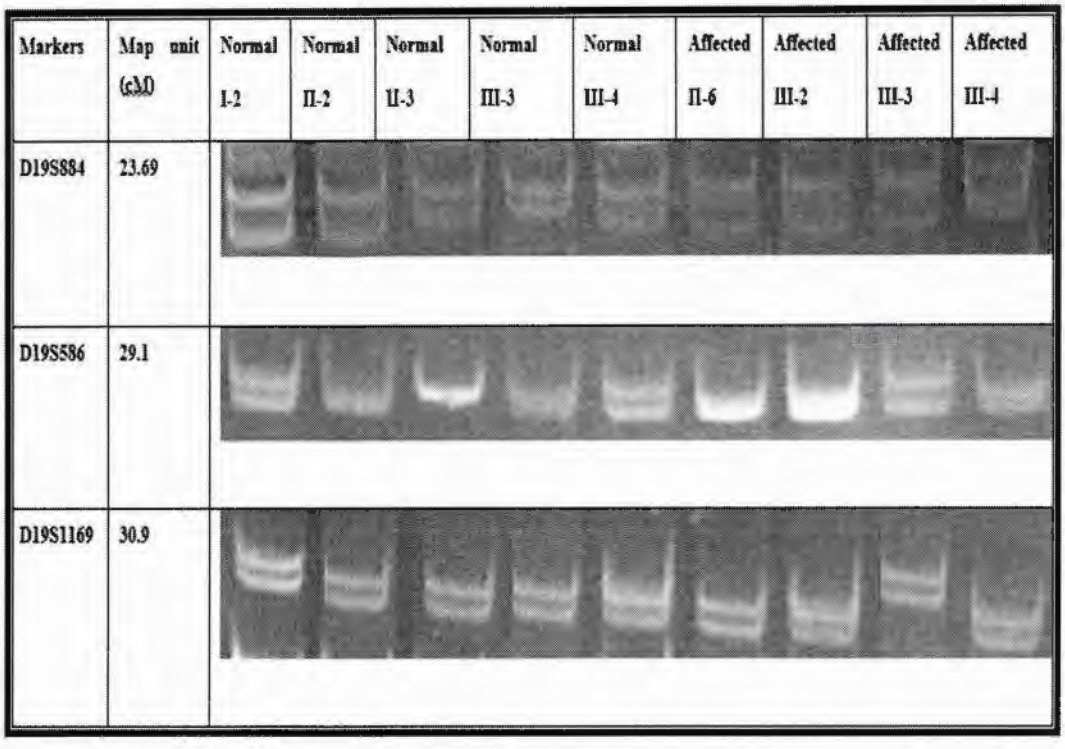


Figure 3.19 Gel images showing the banding pattern of chromosome 19p13 markers in nine member of family C. These markers were used to test the *TYK2* gene. Markers with genetic location are mentioned on the left where as individual IDs are written above the respective lanes.

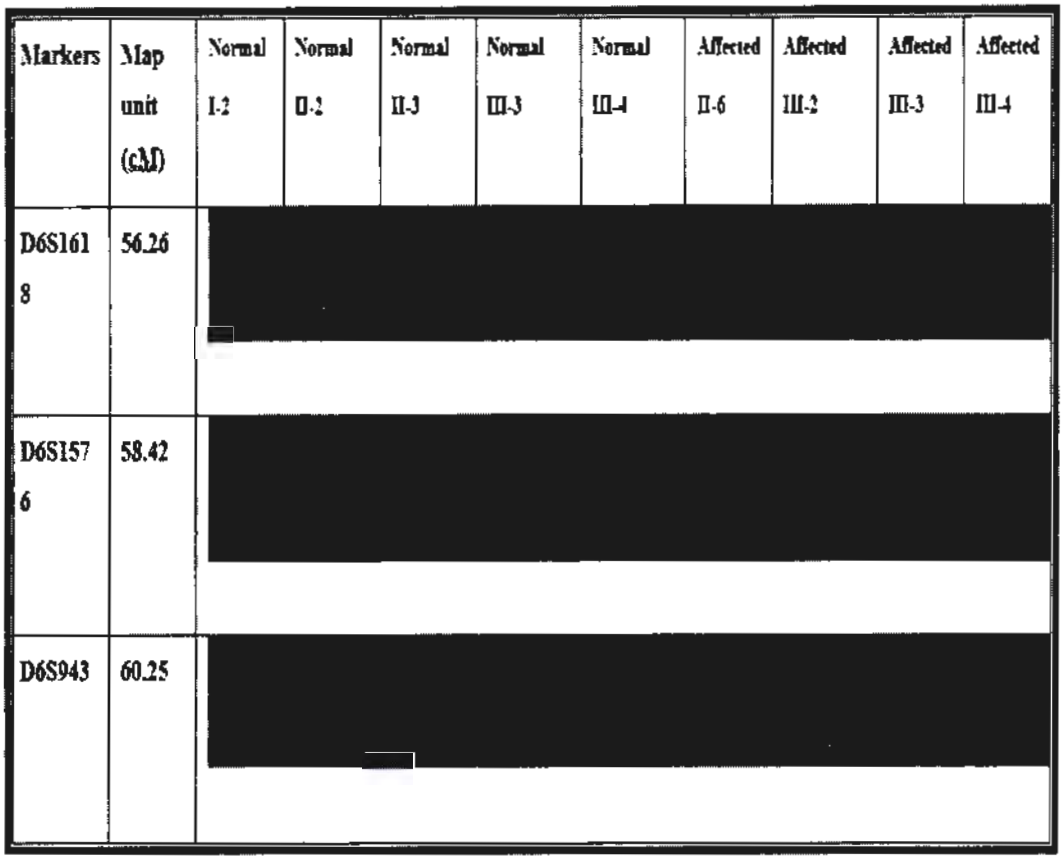


Figure 3.20 Gel images showing the banding pattern of chromosome 6q14.1.markers in nine member of family C. These markers were used to test the *PGM3* gene. Markers with genetic location are mentioned on the left where as individual IDs are written above the respective lanes.

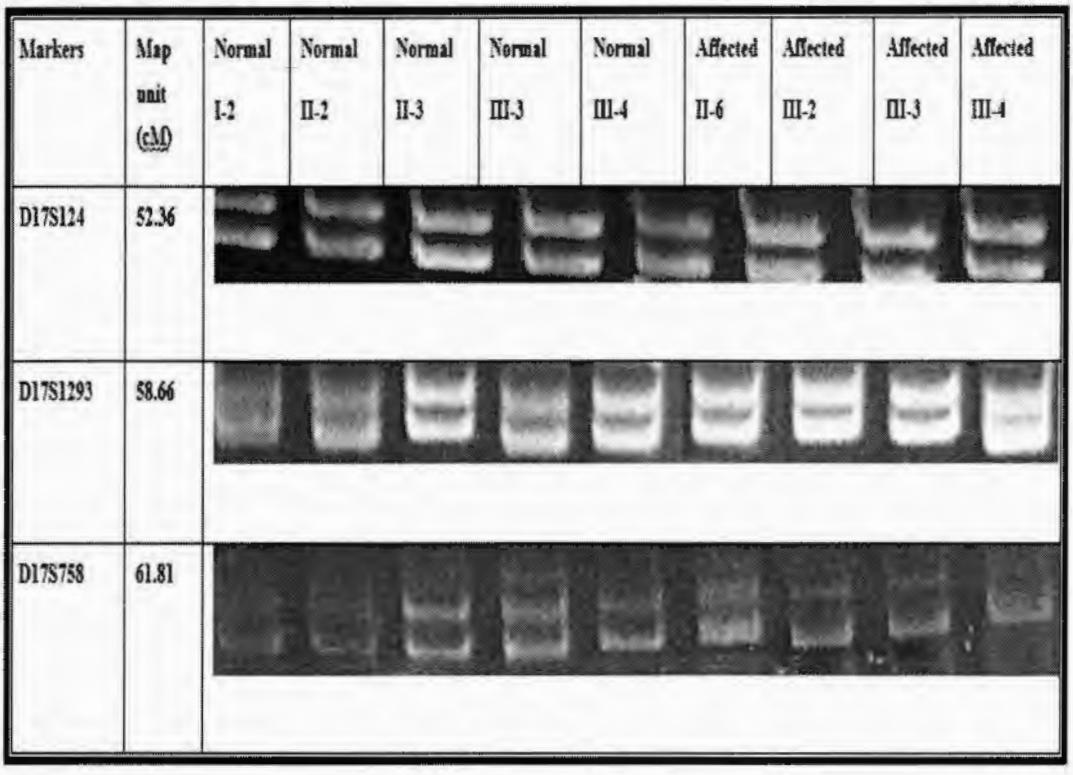


Figure 3.21 Gel images showing the banding pattern of chromosome 17q12markers in nine member of family C. These markers were used to test the *CCL11* gene. Markers with genetic location are mentioned on the left where as individual IDs are written above the respective lanes.

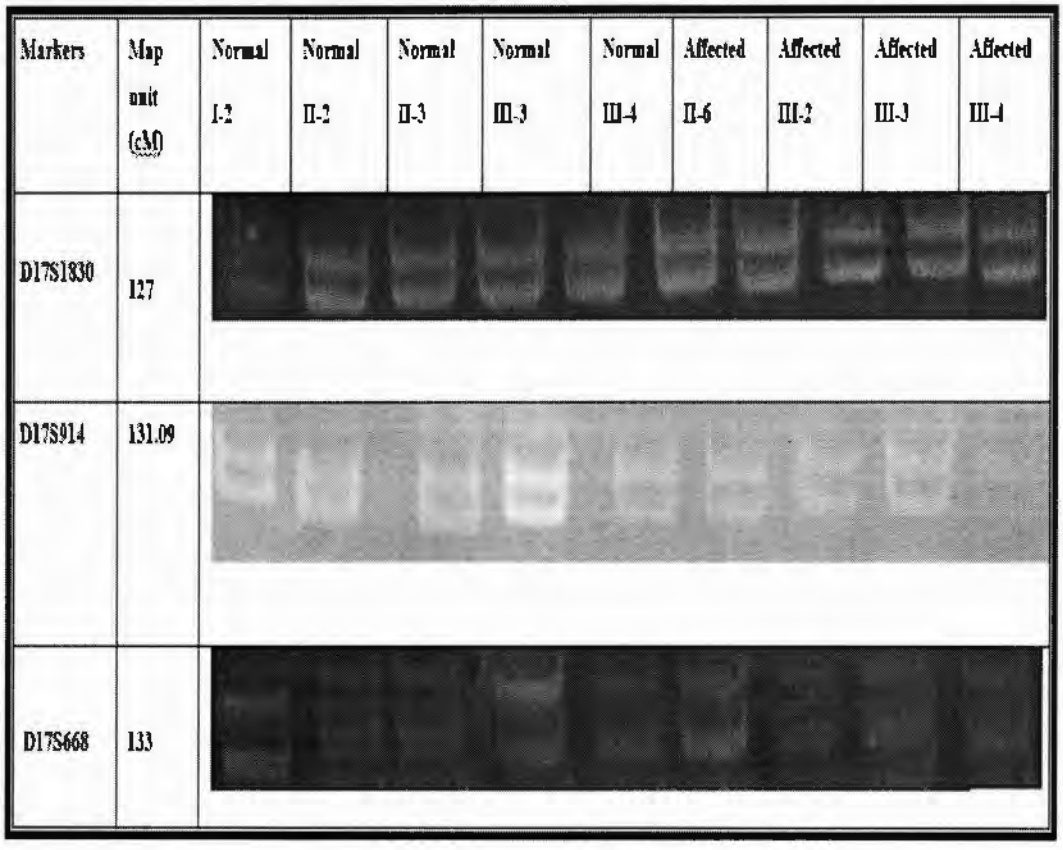


Figure 3.22 Gel images showing the banding pattern of chromosome 17q25.3.1markers in nine members of family C. These markers were used to test the *ZNF750SS* gene. Markers with genetic location are mentioned on the left where as individual IDs are written above the respective lanes.

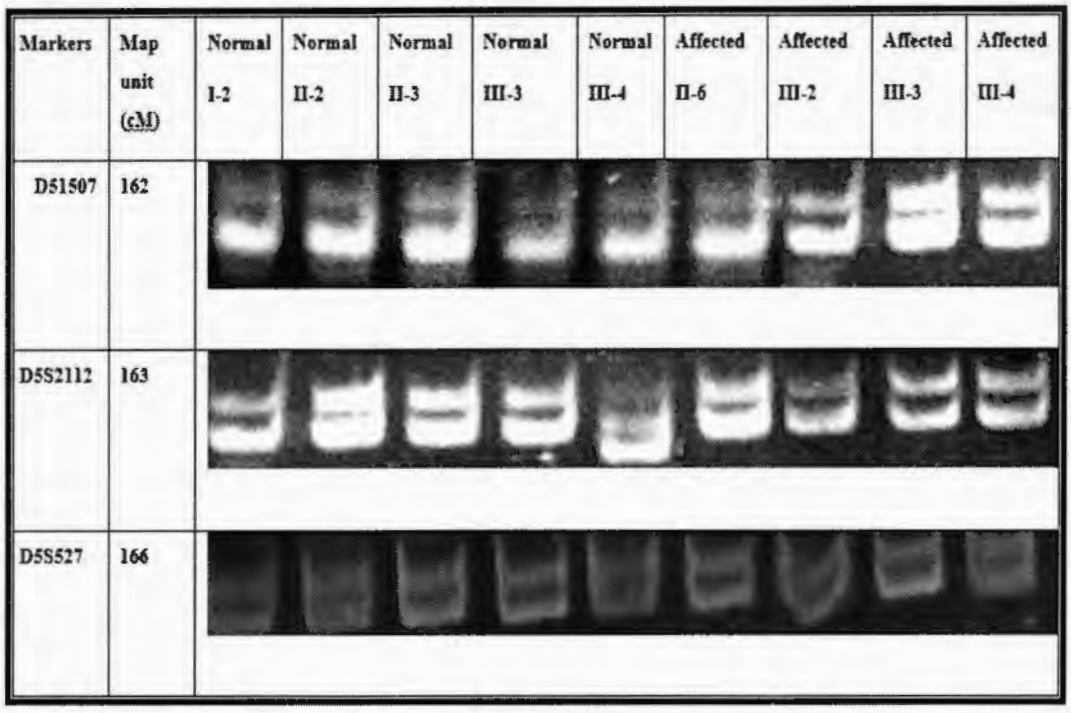


Figure 3.23 Gel images showing the banding pattern of chromosome 5q33.3 markers in nine member of family C. These markers were used to test the *Havcr2* gene. Markers with genetic location are mentioned on the left where as individual IDs are written above the respective lanes

Discussion

The epidermis generates various protective or defensive functions which are mediated by its distinctive differentiation end-product, the stratum corneum (Elias, 2005). Propensity to develop secondary infections (Baker, 2006) and defective epidermal permeability barrier (Patrick, 2010) are well-recognized features of AD, the barrier abnormalities in AD are not just epiphenomena, but to a certain extent the 'driver' of disease activity i.e., an 'outside-to-inside' view of disease pathogenesis. (Elias, 2014).

Tight junctions (TJs) in the skin have been shown to form a barrier in the granular cell layer of the epidermis. Inflammation of the skin is found after various external stimuli, e.g., UV radiation, allergen uptake, microbial challenge, or contact with irritants, as well as due to intrinsic, not always well-defined, stimuli, e.g., in autoimmune responses (Brander *et al., 2016).*

Atopic dermatitis (AD) is a chronically relapsing skin inflammatory disorder characterized by perivascular infiltration of immunoglobulin-E (IgE), T-lymphocytes and mast cells. The key pathophysiological factors causing this disease are immunological disorders and the compromised epidermal barrier integrity.(Shao *et al.*, 2016). Patients must have an itchy skin condition with three or more of the following symptoms: antiquity of flexural involvement, asthma/hay fever, dry skin, beginning of rash under the age of two years, visible flexural dermatitis (Williams *et al.*, 2006).

Depending on co-existence with allergic features; AD can be classified into either intrinsic or extrinsic AD. Penetration of foreign allergens of environment and food are strongly associated with aggravation of extrinsic AD barrier dysfunction. Allergen penetrates through skin barrier and bind to an epidermal dendritic cell (DC) to start cutaneous inflammation 13 and activate Th2 polarization damaged in case of an acute stage (Lee *et al.*, 2016). Disease is classified on the basis of pathogenesis. Primary defect in skin is known as intrinsic AD, whereas extrinsic AD is skin defect with allergy. In both conditions, genes involved in lipid metabolisim are upregulated, differences between the two types is due to differences in their cytokine pathways for example, decreased IL37 appearance in intrinsic AD (Yonsi, 2016).

Constant attempts by different authors to redefine and reclassify AD, reflects a recognition that the clinical syndrome encompasses more than one disease entity. To date, sub classification by algorithms based on phenotype have proved of limited use in clinical practice and do not define response to treatment or prognosis. A recent approach to address this issue in other complex diseases has been to define disease endotypes, which classify the disease based on pathogenesis through an analysis of both the clinical and molecular phenotype (Brandner and Basler, 2016).

Most experts currently agree that a defective skin barrier is central to the initiation of AD, but whether a putative distinction between intrinsic AD (primary defect in the skin) versus extrinsic AD (skin defect and allergy) is of clinical relevance, remains of interest.

Most experts currently agree that a defective skin barrier is central to the initiation of AD, but whether a putative distinction between intrinsic AD (primary defect in the skin) versus extrinsic AD (skin defect and allergy) is of clinical relevance, remains of interest.

The pathogenesis is multifactorial involving disruption of epidermal barrier, IgE dysregulation, defects in skin mediated immune responce and genetic factor. Desaturase activity is deficient in atopic dermatitis which leads to decreased linoleic and linolenic acid metabolites and results in AD (Piliang ,2009). It has been observed that astrogliosis in spinal dorsal horn affects chronic itch in AD-like model animals, and that the processing of the sensation of itch, that is characteristic in AD, occurs in the central nerves.It mostly occurs during childhood and early infancy, but it can persist or even start in adult hood. The life time prevalence of atopic dermatitis (AD) is 10 to 20 % in children and 1 to 3% in adults. In industrialized world its prevalence has increased two to three folds during past three decades (Leung *et al.,* 2004). AD has increased in frequency in recent decades (Palmer, 2006).Children having atopic dermatitis are at a strong risk of allergic asthma and allergic rhinitis. Those with AD during first two years of life. 50% will have asthma in future life (Hamid *et al.*, 2006).The disease is strongly heritable as shown by twin and family studies (Kwiatkowski *et al.*, 2006) Highest prevalence noticed in U.K and New Zealand. Moreover, its observed that AD risk is increasing in low income countries (Cookson *et al.*, 2008). The results indicate that AD is linked with genes with general effects on skin inflammation and immunity (Lawrence *et al.*, 2001). A genome wide association study of AD in Chinese population identifies previously undescribed susceptibility loci at 5q22.1,20q13.33, later also showing association for in German sample. Linkage to AD is found on chromosome 1, 3, 4, 5, 11, 13, 15, 17, 18, 19, 20 with only 3p24 locus showing significant replication. Genome wide linkage approach identifies few candidate genes linked with AD none has pinpointed a specific locus or gene (Barnese, 2010).

Evening primrose oil, a rich source of both linoleic acid and α-linolenic acid was prompted as early attempts to treat these AD patients with prominent barrier abnormality and inflammation (Senapati *et al.*, 2008) For the deployment of precise strategies to restore barrier function in AD, Lipid-Based Therapeutic treatments in Atopic Dermatitis together, describe a strong rationale (Bieber *et al.*, 2012]. Ultraviolet A1 (UVA1) is quite effective in healing flares, while narrow-band UVB has been suggested for chronic-moderate forms of the disease (Patrick et al., 2010).Many drugs are advised by experts to aid Atopic Dermatitis. Tacrolimus (FK 506) is an immunosuppressant drug for avoiding organ rejection after transplantation and is effective for treatment of atopic dermatitis too (Rubins *et al.*, 2014).

In the present study blood samples from three consanguineous families (A, B, C), having members affected with AD were collected from different regions of Pakistan. Onset of disease in all three families vary. Only one member affected in family A has onset of disease at the age of 18 years. In family B four affected individuals develop disease symptoms at different times. Onset of disease in in members of family B are 16 years,6 months, 2 years and 13 years of age respectively In family C two affected individuals have onset of disease at the age of 10 months and 1 year of age respectively. The inheritance pattern was found to be autosomal recessive and there were environmental influences as well.

These families were taken and verified for linkage by genotyping polymorphic micro satellite markers linked to different known Atopic dermatitis (AD) loci.Genotyping analysis in family A revealed that affected individuals were heterozygous for different combination of parental alleles, therefore linkage in this family was excluded to the

known Atopic dermatitis candidate regions. These results show that a novel gene is responsible for Atopic Dermatitis (AD) in this family.

Similarly genotyping analysis of family B and C showed similar results showing no linkage to the known atopic dermatitis candidate regions. Exclusion mapping analysis revealed that allelic pattern of the affected individuals were heterozygous with dissimilar combination of parental alleles. This reveals the probability that there might be novel unknown gene or locus responsible for disease pathogenesis.

Consanguineous marriages possess a major risk factor in causing autosomal recessive disorders including AD. Autosomal recessive conditions are enhanced in cousin marriages by expressing recessive default genes in off springs.(Romeo *et al.*, (2016) explained that one of the major reasons of increased autosomal recessive disorders is consanguinity. Consanguineous marriages are one of the most common cause of genetic disorders in Pakistan, therefore genetic counselling and parentel diagnosis is required, so that the frequency of affected children can be reduced as a precautionary measure.

The study will help to understand the fundamental molecular mechanisms of faulty genes, will provide insight to understand molecular characterization of human heredity disease AD. Precise genotype phenotype correlation can be established by improving genotyping ,along with complete structure of genetically homogeneous assemblies of patients.

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