LIVER X RECEPTOR POLYMORPHISM IN GENETIC SUSCEPTIBILITY TO VITILIGO

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JAYPEE UNIVERSITY OF INFORMATION AND TECHNOLOGY

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CERTIFICATE

This is to certify that the work titled "**Liver X Receptor Polymorphism In Genetic Susceptibility To Vitiligo**", submitted by "**Silky Agarwal**" in partial fulfillment for the award of degree of **Master of Technology in Biotechnology** of Jaypee University of Information Technology, Waknaghat has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

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Date :

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Dreams really come true. This thesis represents a part of my dream which would not be possible without the contribution of many others, who directly or indirectly contributed in the development of this work and who influenced my thinking, behavior and acts during the course of project. I, hereby, received the opportunity to express my gratitude to all of them for their constant guidance and inspiration to me.

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Date:
Place: Waknaghat

SUMMARY

Vitiligo, a common depigmentation disorder, caused by loss of melanocyte from the lesion site. Multiple gene and environmental triggers are thought to play a role in inducing vitiligo. Upregulated expression of LXR-alpha in the melanocytes from perileisional skin as compared to normal skin of vitiligo patient speculates its role in vitiligo pathogenesis. Genetic variation in gene may contribute to disease pathogenesis. In this study, we investigated the association of LXR-α gene polymorphism (rs2279238 (+1257 C>T) and rs11039155 (-6 G>A)) with vitiligo susceptibility among north India population.

39 patients diagnosed with vitiligo and 50 matched healthy control subjects who did not have any history of vitiligo or any other autoimmune disorder were recruited. Informed consent was obtained from all patients and controls prior to blood sample collection. DNA samples were isolated from the blood samples and genotyped by PCR-RFLP method.

The LXR-α (-6 G>A (rs11039155) and +1257 C>T (rs2279238)) genotype and allele frequencies of the vitiligo patient differ significantly from those of healthy control (p= 0.0358 and p= 0.0237 respectively). We found a significant association between the LXR-α gene polymorphism and vitiligo susceptibility among north India population. (rs11039155: odds ratio (OR) = 2.51; 95% confidence interval (CI) = 1.08 - 5.84; rs2279238: OR= 1.94; 95% CI = 1.06 - 3.54)**.** Our results provide evidence that the LXR-α -6 A and +1257 T allele contribute to risk of vitiligo in north India population and underscores the importance of a genetic factor in the vitiligo pathogenesis. Though, a larger patient and control pool needed to confirm such a conclusion.

Signature of Student	Signature of Supervisor
Name	Name
Date	Date

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ABBREVIATION

ABBREVIATION	
bp	Base Pair
^{0}C	Degree Celsius
CI	Confidence Interval
DNA	Deoxyribonucleic acid
dNTP	Deoxyribose nucleotide Triphosphate
EtBr	Ethidium Bromide
EDTA	Ethylene diamine tetra acetic acid
g	Gram
l	Litre
LXR	Liver X Receptor
LXR-α	Liver X Receptor- alpha
MITF	Microphthalmia-associated transcription
MMP	Matrix Metalloprotinases
µl	Microlitre
mins	Minutes
mg	Milligram
mM	Millimolar
M	Molar
NSV	Non-Segmental Vitiligo
OR	Odd Ratio
PCR	Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
ROS	Reactive Oxygen Species
Secs	Seconds
SNP	Single Nucleotide Polymorphism
TAE	Tris –Acetate EDTA
TE buffer	Tris-EDTA Buffer
Tris	Tris [Hydroxymethyl aminomethane]
U	Unit
UVR	Ultraviolet Rays
V	Volts

CHAPTER 1

INTRODUCTION

INTRODUCTION

Vitiligo is an acquired, idiopathic skin depigmentation disorder affecting approximately 1% of the world population characterized by milky white patches on the skin. It is caused by destruction of pigment-forming cells known as melanocytes at the leisional site. Melanocytes are the cell solely responsible for the melanin production which imparts color to the skin. Loss of melanocyte resulting in devoid of melanin and thus, milky white patches appear at the leision site.

The exact cause of the loss of melanocyte is not known. But various theories based on autoimmunity, oxidative stress, neural factors, genetic defects explain the loss of melanocyte that has been suggested to be the key event in the vitiligo pathogenesis. Consequences of vitiligo are not life threatening but it can have profound psychological consequences which may range from mild embarrassment to a severe loss of self-confidence and social anxiety. Basically, vitiligo affects the quality of patient's life.

Various research groups are focusing their efforts to elucidate the regulation and the mechanism of skin pigmentation with a goal of developing a new treatment for vitiligo. Various biological and chemical agents have been developed that target the vitiligo susceptibility marker for therapeutic intervention, but most of them have adverse effects in the skin and are not effective in many cases. Moreover, similar treatment cannot be given to all patients because of different clinical types of vitiligo. Furthermore, there are various reports that demonstrated varying treatment outcomes to a treatment in different patients. Therefore, there is a need to develop new therapeutic interventions as well as to identify new markers that could help to monitor and predict treatment outcome of vitiligo therapy.

The goal of my work was to identify potential vitiligo susceptibility genes with the hope that this knowledge may lead to better understanding of disease pathogenesis and more targeted disease treatment.

Recently, role of Liver X Receptor (LXR) in skin physiology and pathology has evolved rapidly because of their role in epidermal proliferation, carcinogenesis, differentiation

and permeability barrier function, which identifies them as promising drug targets for the treatment of skin diseases(Jamroz-Wisniewska, Wojcicka et al. 2007; Kumar, Parsad et al. 2012).

Liver X receptors (LXR-α / NR1H3 and LXRβ / NR1H2) are ligand (such as oxysterols, high concentrations of D-glucose and phytosterols,) activated nuclear receptors that have important roles in the regulation of genes involved in lipid biosynthesis, cholesterol homeostasis, immunity and inflammation. LXR exists in two isoforms: LXR-alpha and LXR-beta. LXR-alpha is highly expressed in several metabolically active tissues, such as liver, intestine, adipose tissue, and macrophages, whereas LXR-beta is ubiquitously expressed in most tissues (Steffensen and Gustafsson 2004; Lee, Park et al. 2013). Both isoforms are activated by endogenous oxysterol. LXR are also found to be expressed in the skin tissues such as sebaceous glands, hair follicle, epidermal keratinocyte and fibroblast(Billoni, Buan et al. 2000; Russell, Harrison et al. 2007). The function and characteristics of LXRs in skin have been recently widely studied but their expression and function in relation to melanocyte have not yet been investigated so much. Recently, Kumar *et.al.(Kumar, Parsad et al. 2010)* demonstrated that LXRs are also expressed in melanocytes. They demonstrated that Liver X receptors (LXRs) are upregulated in the melanocytes from perilesional skin as compared to the normal skin of vitiligo patient. Further, they have shown that LXR-α agonist 22(R)-hydroxycholesterol treatment significantly downregulate the cell adhesion molecule, which leads to the detachment of melanocytes from the basement membrane in perilesional vitiligo skin resulting in melanocytorrhagy(Kumar and Parsad 2012). Study by Lee *et.al*.(Lee, Park et al. 2013) reported that the activated LXR inhibit the melanogenesis through the activation of extracellular signal–regulated kinase (ERK) mediated Microphthalmia-associated transcription factor (MITF) degradation. Study by Lei *et.al*.(Lei, Vieira et al. 2002) demonstrated that MMP 2 plays an important role in melanoblast migration from the border of vitiligo lesions into clinically depigmented epidermis which is crucial for the repigmentation of vitiliginous skin. Additionally, study by Kumar *et.al.(Kumar, Parsad et al. 2011)* reported that MMP-2 and MMP-9 are downregulated in vitiligo patient and further, it was shown that LXR-α gene knock-down significantly increases the expression of MMPs. Thus, Higher expression of LXR-α in perilesional skin melanocytes

significantly decreases the adhesion, proliferation and matrix metalloproteinases and increases apoptosis. Based on all these studies LXRs appear to have important role in vitiligo pathogenesis and considered as a potential therapeutic target for vitiligo.

The aim of my study was to identify polymorphic marker in the Liver X Receptor-alpha gene which is involved in vitiligo susceptibility so that in the future, it could be used as prognostic marker. In this study, we would explore the association of LXR-alpha gene polymorphisms (rs2279238 and rs11039155) with vitiligo susceptibility**.** Single Nucleotide Polymorphisms in LXR-α gene (rs2279238 and rs11039155) has been found to be strongly associated with various disease e.g. metabolic syndrome, coronary atherosclerosis, obesity(Legry, Cottel et al. 2008; Dahlman, Nilsson et al. 2009).

Significance of the Study:

In India, incidence rate of vitiligo has been reported highest, approximately 8.8%(Sehgal and Srivastava 2007) and treatment provided to patients are not effective and costly and are associated with side effects. Individual respond differently to the treatment may be because of different genetic make up. Moreover, vitiligo affects the quality of patient's life and presence of depigmented spot is the only diagnosis of vitiligo. Change in single nucleotide in the DNA sequence may affect how human develops disease, respond to drug etc. Identification of SNP in a gene associated with the vitiligo can act as a susceptibility marker. Associated SNP can be used as a prognostic marker to track the inheritance of disease genes within the families. Associated SNP can also be used to develop new therapeutic intervention and can be used as a marker for prediction of a treatment outcome in an individual undergoing a vitiligo therapy. Since, LXR-alpha has been associated with the vitiligo and is a promising drug target in skin. Association of any LXRs SNP with vitiligo will help us to predict the treatment outcome where LXRs act as a therapeutic target and SNP can also provide us the genetic fingerprint for use in vitiligo identity testing.

CHAPTER 2

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Vitiligo, a common skin depigmentation disorder, characterized by milky white patches on the skin caused by melanocyte destruction resulting in devoid of melanin at the lesion site.

The following section explains the structure and function of the skin, giving more emphasis on melanocyte and melanogenesis which provide the background to the vitiligo which is the main focus of the project.

2.1 SKIN - MELANOCYTE AND MELANOGENESIS

Human skin, largest organ, consists of two main layers: the **Epidermis**, a stratified squamous epithelium mainly consisting of keratinocyte along with melanocyte, dendrocytes (Langerhans cells and Granstein cells), and the **Dermis,** an underlying layer of vascularized connective tissue. Skin act as a physical and chemical barrier and provide a protection from the environmental stress such as DNA damaging UV rays etc. (Costin and Hearing 2007) Melanocytes are one of the cells present in the skin which helps it in fulfilling its role. (Lin and Fisher 2007)

Melanocytes, located at the basal layer of epidermis, functionally connected to underlying fibroblasts in the dermis and to keratinocyte in the overlying epidermis, are solely responsible for the pigmentation of skin and hair, and thereby, contribute to the skin appearance and provide protection from damage by ultraviolet radiation. (Lin and Fisher 2007) Melanocytes are derived from the precursor cells called melanoblast which resides at the outer root sheath (OSR) of hair follicle. Melanoblasts originate from the neural crests of the embryonic ectoderm (Rawles 1947). Studies related to human embryogenesis show that undifferentiated melanoblast migrate into the developing epidermis at approximately the seventh week of gestation, and subsequently reside in the basal epidermal layer. (Holbrook, Underwood et al. 1989; Suder and Bruzewicz 2004)

2.1.1 Melanogenesis:

Melanocyte cells are solely responsible for the production of melanin (colored pigment) by the process named melanogenesis which is a highly complex pathway involving many

enzymes and co-factor. Melanogenesis occur in the membrane bound organelle named as **melanosomes**. (Simon, Peles *et al*. 2009) The different steps of melanin production are shown in figure 1.

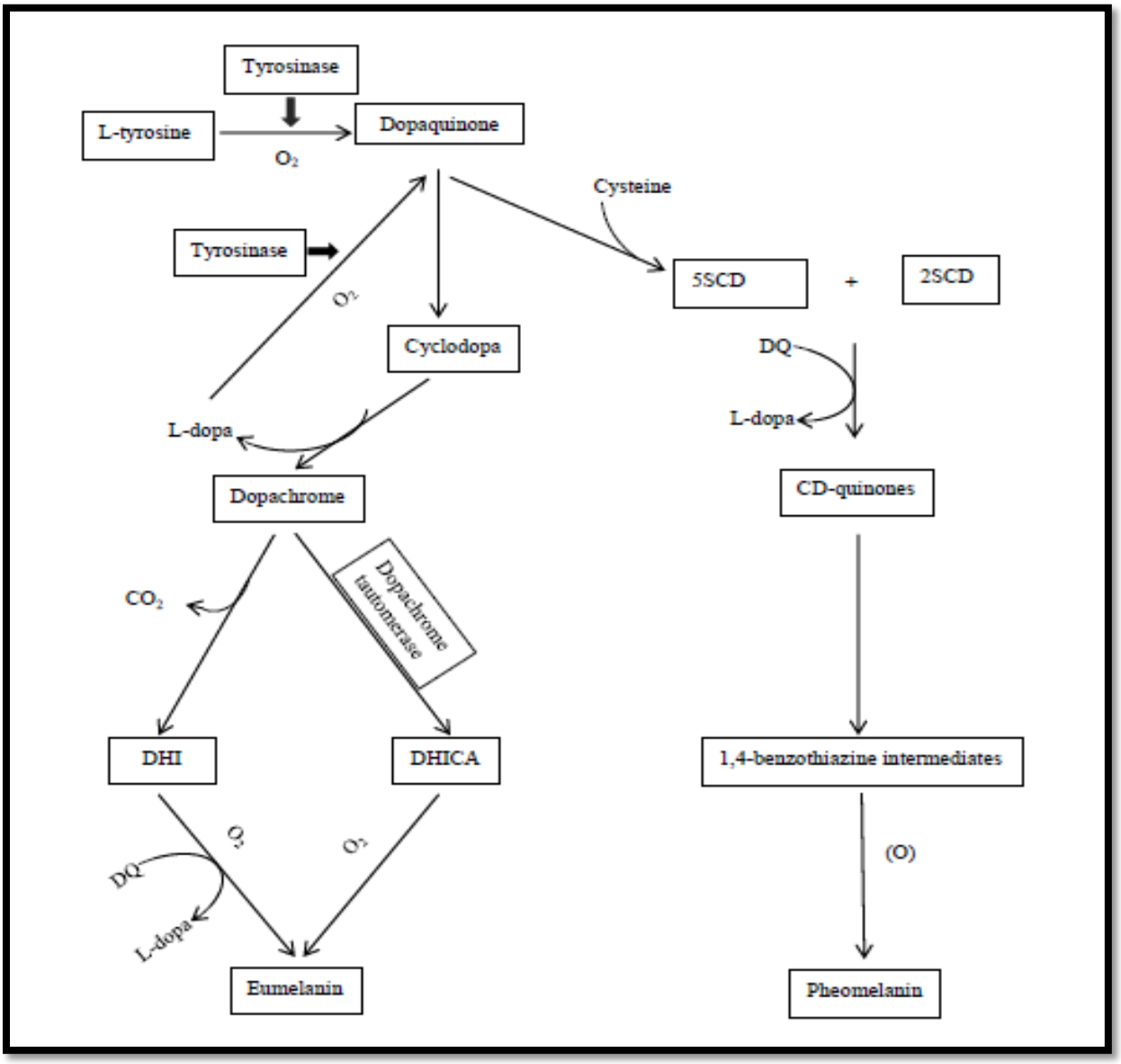


Figure 1: A diagrammatic overview of the melanogenesis pathway in human melanocyte *(Denat, Kadekaro et al. 2014)*

Two distinct kinds of melanins can be formed depending on the environment: **Eumelanin**, which are the predominant pigments found in dark skin and black hair, and **Pheomelanin**, which is associated with the red hair/freckled skin phenotype. The first

and rate-limiting step in melanogenesis is the conversion of L-tyrosine to DOPA (Dihydroxyphenylalanine) catalysed by tyrosinase. Tyrosinase is regulated by the transcription factor named **MITF**. DOPA undergoes oxidation to form dopaquinone which is immediately converted into dopachrome and then to DHI(5, 6 dihydroxy indole). Also tyrosinanse related protein 2 (TRP 2) converts dopachrome to dihydroxy indole carboxylic acid (DHICA). DHI and DHICA polymerize to form eumelanin. Cysteine/glutathione reacts with the dopaquinone to produce cysteinyldopas that may undergo further cyclization to benzothiazines and higher condensates giving rise to pheomelanins. (Taieb 2000; Simon, Peles et al. 2009; Denat, Kadekaro et al. 2014)

Melanin production is triggered by ultraviolet rays present in sunlight. UVR fall on the epidermal layer consisting of keratinocytes which activates the p53 tumor suppressor protein. Activation of p53 in keratinocytes increases the expression of POMC peptides(Cui, Widlund et al. 2007). POMC is a precursor of melanotrophic peptides α-melanocyte-stimulating hormone (α-MSH) and adrenocorticotrophic hormone (ACTH). α-MSH are secreted by keratinocytes and then competitively bind to melanocortin 1 receptor (MC1R) on the melanocyte which in turn activate the cell-signalling pathways, including the cyclic adenosine monophosphate (cAMP)-dependent protein kinase pathway and the mitogen-activated protein (MAP) kinase pathway. Induction of cyclic AMP production leads to phosphorylation of cAMP responsive-element- binding protein (CREB) transcription factor family members.(Videira, Moura et al. 2013)

CREB, in turn, transcriptionally activates various genes, including that encoding microphthalmia transcription factor (*MITF*), master regulator of melanogenesis which is a transcription factor that is pivotal to the expression of numerous pigment enzymes such as tyrosinase (TYR, enzyme involved in rate limiting step of melanogenesis) and differentiation factors. MITF transcriptionally activates the pigmentory enzymes which is then transported into melanosomes, site for melanin production(Levy, Khaled et al. 2006; Park, Kosmadaki et al. 2009).

Melanocytes are highly dendritic and are in contact with the neighboring keratinocytes. Each melanocyte is in contact with approximately 36 keratinocytes. Melanosomes containing the melanin migrate from the center of the melanocyte cell body to the end of the dendrites and are deposited into keratinocytes.(Shajil, Chatterjee et al. 2006) The

melanosomes accumulate in the keratinocytes and form a shield of melanin. Melanin is the most important photoprotective factor considered as a broadband UV absorbent which provides protection to the skin against ultraviolet radiation from sunlight, along with it has antioxidant and radical scavenging properties(Swalwell, Latimer et al. 2012).

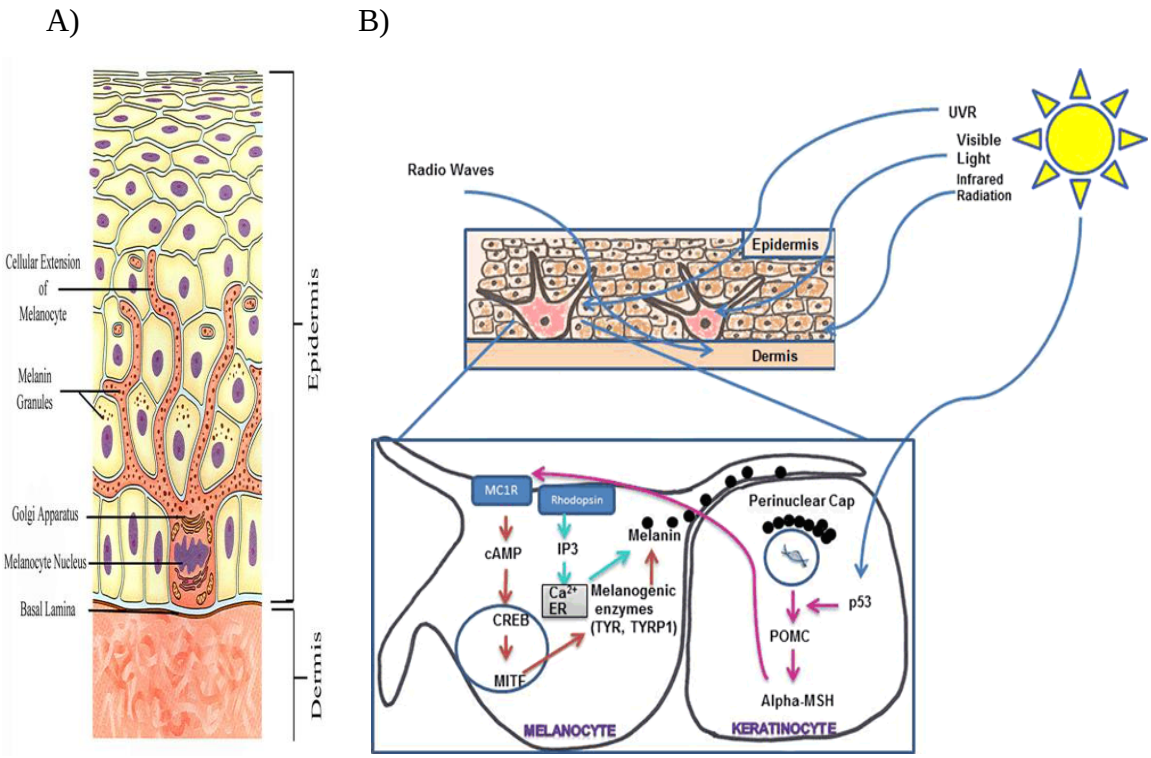


Fig 2: A) Melanocyte in the skin layer B) Overview of the melanogenesis process from the melanin production trigger to transfer of melanosome to keratinocyte.

2.1.2 Melanocyte Distribution and Melanin Content:

Melanocyte density in the skin of different individual of different ethnic background is found to be in similar range but melanocyte density in different sites of the skin may differ to an extent. Melanocyte density in the Asian, Black and white individual's skin was found to be in the range of 12.2 to 12.8 melanocytes/mm. Although, differences in melanocytes in different sites of the body in Asians have been reported. The density of melanocytes in palmoplantar skin is 2.5± 0.3 melanocytes/mm and in nonpalmoplantar skin is 13.3 ± 1.7 melanocytes/mm(Tadokoro, Yamaguchi et al. 2005).

Melanin content account for skin appearance. The total amount of melanin in unirradiated skin from Asian and from White subjects is very similar, whereas the amount in Black skin is about four-fold higher. Difference in melanin content in different ethnic groups may be due to differences in the rate of melanin synthesis, the type of pigment produced, and the way melanin is distributed within keratinocytes. Iozumi *et.al.*(Iozumi, Hoganson et al. 1993) reported higher melanin content in melanocytes cultured cell from Black skin compared to White skin and high melanin content is due to 10-fold higher catalytic activity in darker skin types. Similar study by Fuller *et.al.*(Fuller, Spaulding et al. 2001) reported activity of tyrosinase, the rate-limiting enzyme for melanin synthesis, is higher in black skin melanocytes than in melanocytes derived from caucasian skin but there is no difference in the amount of enzyme.

Fig: 3. Human pigmentation—the main skin types: African-American, Asian, Caucasian, and Hispanic (left to right).(Costin and Hearing 2007)

A large number of genes (approximately 125) are known to be involved in melanogenesis, mutations in any of them account to developmental pigmentary disorder(Bennett and Lamoreux 2003). In addition to genes expressed by melanocytes, signaling factors originating from adjacent tissues play critical roles in guiding melanogenesis. Alteration in any of the factor involved in melanin production or in regulation of melanocyte will lead to the pigmentary disorder such as albinism, vitiligo etc.(Videira, Moura et al. 2013)

Vitiligo is one of the example of pigmentary disorder in which melanocyte cells are destroyed because of alteration in both genetic and non genetic factors resulting in hypopigmented lesions that look like milky white patches on the skin because they are devoid of melanin. Keratinocytes still migrate to the surface of the epithelium without carrying the pigment.

2.2 VITILIGO: DEPIGMENTATION DISORDER

Vitiligo is an acquired, idiopathic, hypomelanotic dermatological disorder characterized by white patches of different shape and size on the skin that may gradually enlarge and may appear anywhere on the body.(Shajil, Chatterjee et al. 2006; Taieb and Picardo 2009) It affects both the gender equally at any age belonging to any ethnic group. Generally, the average age of onset is considered to be 22 ± 16 year.(Halder and Chappell 2009) Approximately, half the patients affected from vitiligo are under the age of 20 years and nearly 70-80% before the age of 30 years. (Jaigirdar, Alam et al. 2002; Tarle, Nascimento et al. 2014)

2.2.1 Prevalence And Incidence:

The overall prevalence of vitiligo worldwide is approximately 1%. However, its incidence ranges from 0.1 to > 8.8% across the country and in different countries of the globe(Alkhateeb, Fain et al. 2003). The highest incidence of the condition has been recorded in Indians from the Indian subcontinent, followed by Mexico and Japan. This may be because in India, there is a stigma associated with vitiligo and affected person and their families particularly girls are socially ostracized for marital purpose, so people out here go for early consultation(Cho, Kang et al. 2000; Handa and Dogra 2003; Shajil, Chatterjee et al. 2006). That's why maximum number of incidence was reported from India. In India, Rajasthan and Gujarat have reported high incidence rate i.e. around 8.8% (Shajil, Chatterjee et al. 2006). The different ethnic backgrounds of the population residing in different geographic regions with different environmental conditions may contribute to the wide variation in the prevalence of vitiligo.(Halder and Chappell 2009)

2.2.2 Consequences:

Vitiligo, considered as a minor disease, is not a life threatening disorder but it can have profound psychological consequences which may range from mild embarrassment to a severe loss of self-confidence and social anxiety.(Ongenae, Beelaert et al. 2006) These symptoms are mostly being observed in the racially darked skinned people where these

white patches become very prominent. During the social gathering, vitiligo patient may experience feelings of stress, embarrassment or self-consciousness. In India, mostly, vitiligo patients are isolated from the society. Psychological consequences are more pronounced in female as they may face more social difficulties such as marriage.(Parsad, Dogra et al. 2003; Dolatshahi, Ghazi et al. 2008) Individual with vitiligo undertake diet restriction such as avoidance of food which is white in color such as milk, avoidance of non vegetarian food, citrus food etc because of the local belief concerning the aetiology of the vitiligo.

2.2.3 Clinical Classification of Vitiligo:

There is no uniform classification of vitiligo, several reports on classification are there at present.

Milky white patches on the body of vitiligo patient are of different shape and size and in number and may enlarge and spread throughout the body. Mostly, vitiligo is classified according to the distribution, pattern and extent of depigmentation.

Majorly, the disease is best classified into either segmental vitiligo (SV) or non-segmental vitiligo (NSV)(Shajil, Chatterjee et al. 2006).

SEGMENTAL VITILIGO	**NON- SEGMENTAL VITILIGO**
Begins often in the childhood	Later onset
Autoimmunity rare	Autoimmunity associated
Frequently facial	Trauma prone site and koebnerisation
Stable results after autologous grafting	Unstable results after autologous grafting
Dermatomal, Unilateral distribution	Non-dermatomal bilateral distribution
No family history	Mostly, Family history is present
Most common Affected area: Neck and trunk	Most common affected area: Extensor surfaces of the elbow, knee and metatarsal/metacarpal interphalangeal joints

Table 1: Clinical subtypes of vitiligo .(Shajil, Chatterjee et al. 2006)

According to another classification proposed by Nordlund and Lerner (Nordlund and Lerner 1982):

Localized		Generalized			Universal
Focal	**Segmental**	**Acrofacial**	**Vulgaris**	**Mixed**	
One or more patches in one area but not in segmental patterm	One or more macules in dermatomal, unilateral distribution	Affects face and distal extremities	Symmetrical distribution of leisions in typical zones	Segmental along with vulgaris or acrofacial	Involves more than 80% of the body

Table 2: Clinical classification of vitiligo.(Shajil, Chatterjee et al. 2006)

Generally, segmental vitiligo is considered as localized vitiligo and non segmental vitiligo as generalized vitiligo.(Mohammed, Gomaa et al. 2015) According to another classification, vitiligo classified as "active" and "stable" vitiligo.(Chen, Yang et al. 2004; Lotti, Gori et al. 2008)

- **Active Vitiligo**: Lesions must have spread within the last three months.
- **Stable Vitiligo**: Lesions have not changed (depigmenting or repigmenting) within the past three-month to two-year period.

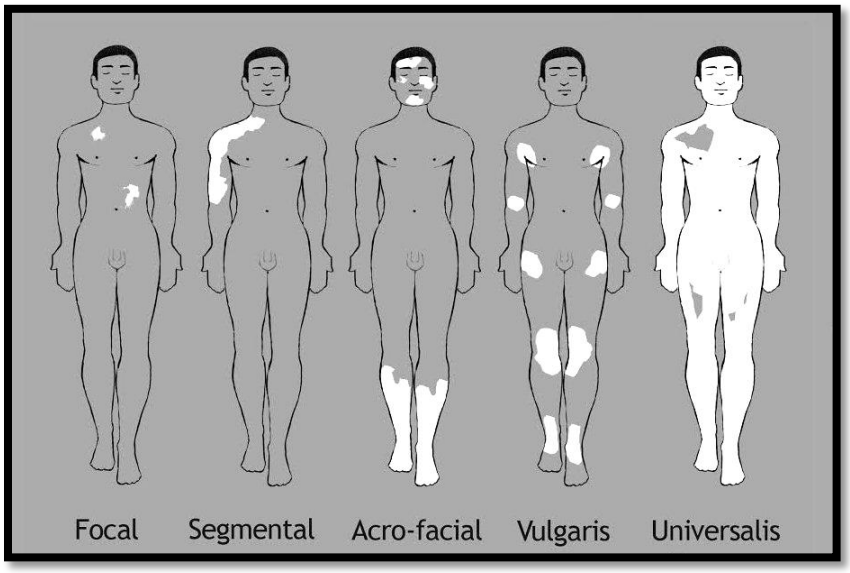


Fig 4: Different Clinical pattern of vitiligo

(Source: https://www.afvitiligo.com/le-vitiligo/les-types-de-vitiligo/)

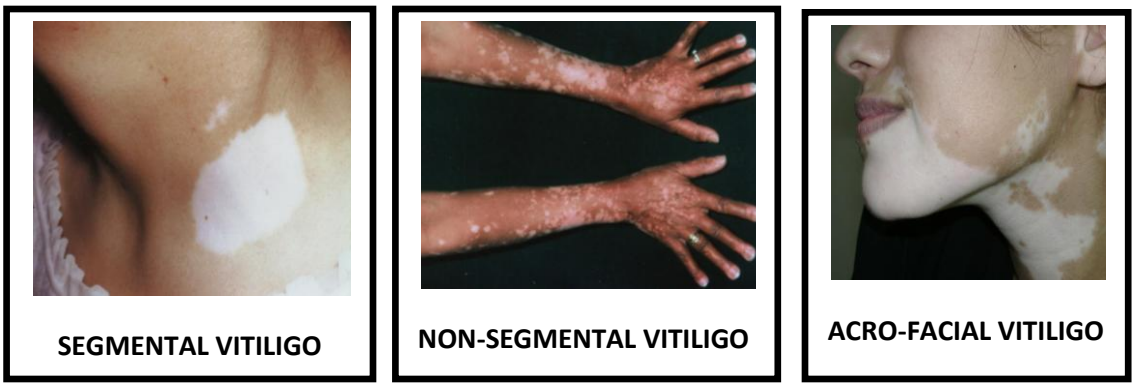


Fig 5: Clinical Pattern of Vitiligo

(Source: The photograph was kindly provided by Prof. David Gawkrodger, Department of Dermatology, Royal Hallamshire Hospital, Sheffield, UK.)

2.2.4 Vitiligo Pathogeneisis - Theories of Vitiligo:

Immunohistochemical and melanocyte culture studies suggest that depigmentation is caused by loss of melanocyte from the leisional area rather than by melanocyte inactivation or dormancy (Kumar, Parsad et al. 2012). The exact etiology of the vitiligo is still unknown but is thought of an intricate interaction of environmental trigger, genetics, biochemical and immunological factor that leads to melanocyte destruction and thus, leads to onset and development of the disease.(Toosi, Orlow et al. 2012) Stress, extreme exposure to pesticides, dyeing chemicals, phenolic compound etc, sunlight are considered to be some of the precipitating factors. Electron microscopic studies confirm the loss of melanocytes from the leisional sites of vitiligo patients.

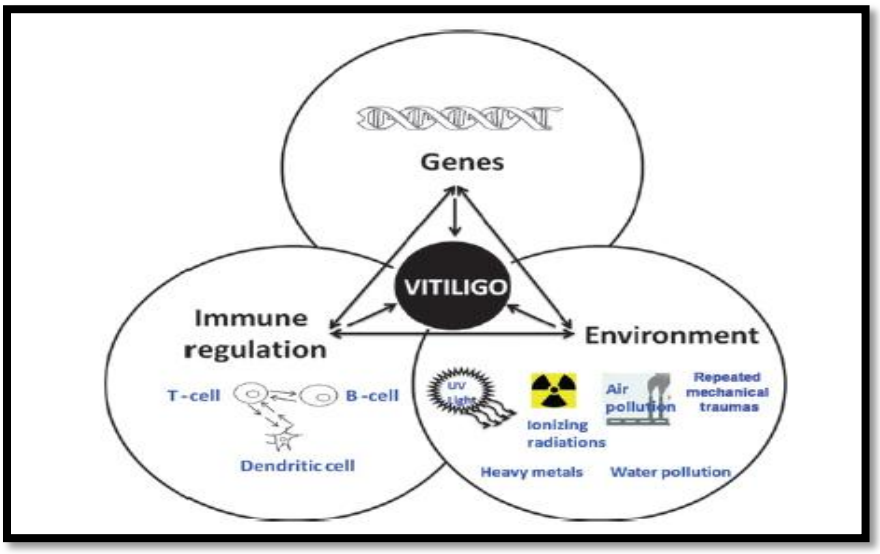


Figure 6: Interplay of genes, environmental trigger such as pollution, heavy metal, generation of ROS etc and immune response in precipitation of vitiligo.(Laddha, Dwivedi et al. 2013)

Various theories were postulated explaining the destruction of melanocyte at the leisional site. Lerner *et. al.* (Lerner 1959) in the 1950's firstly proposed the neural theory, and after that, model of reactive oxygen species (ROS), the autoimmune hypothesis and the melanocytorrhagy hypothesis have appeared. But none of these hypothesis can explain the entire spectrum of the disorder.

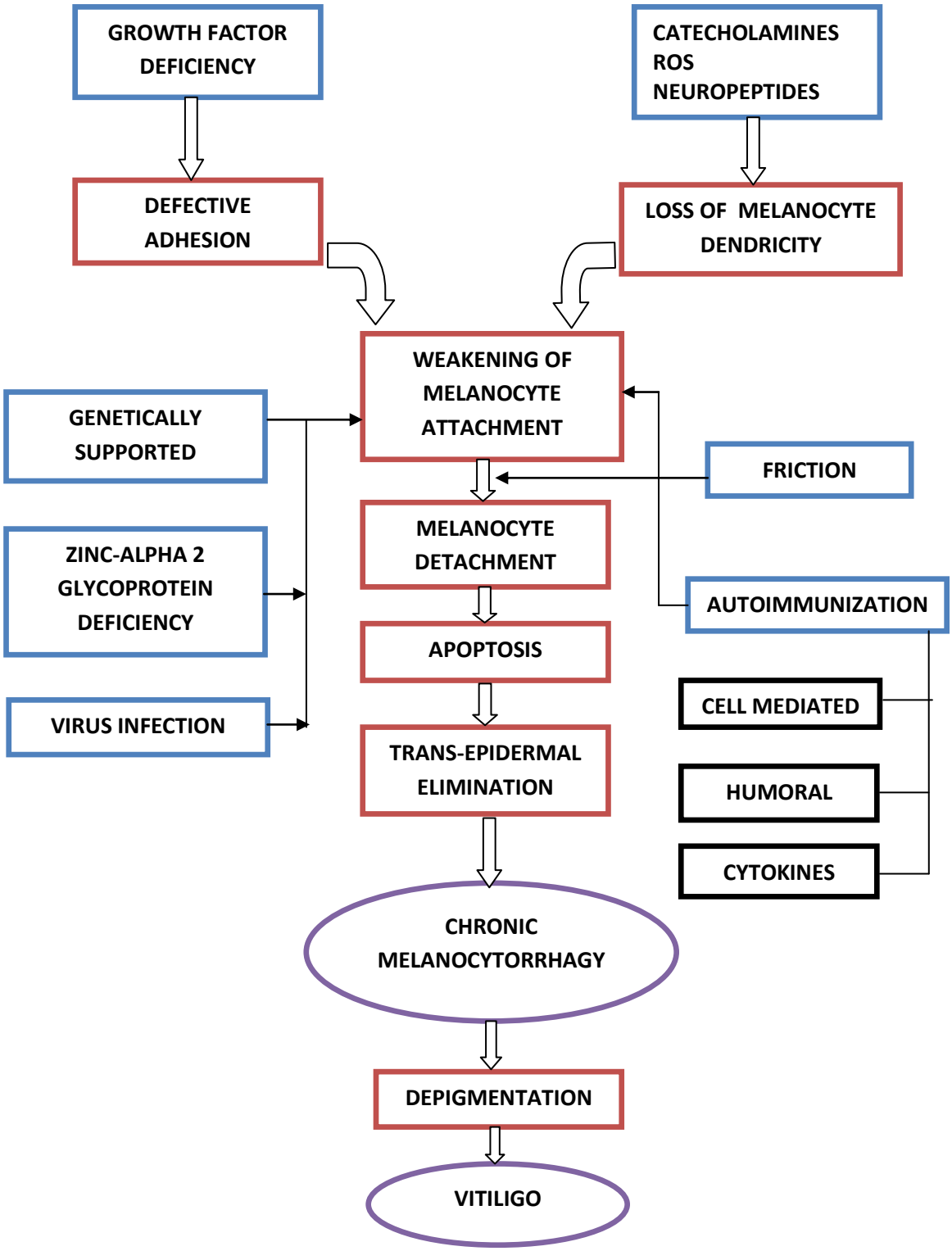


Fig 7: Pathogenesis of Vitiligo (Mohammed, Gomaa et al. 2015)

- **Neurochemical Theory:**

Neurochemical hypothesis suggests that release of neurochemical factor such as norepinephrine and acetylcholine from the peripheral nerve endings destroy the melanocyte or inhibits melanin production giving rise to depigmentaion spot and thus contribute to vitiligo pathogenesis.(Shajil, Chatterjee et al. 2006; Panja, Bhattacharya et al. 2013) High concentration of neurochemical may be due to both genetic and non-genetic factors. High concentration of norepinephrine and its metabolite in vitiligo patients may be due to a reduction in phenylethanolamine-N-methyl transferase(PNMT) activity and increase in tyrosine hydroxylase (TH) activity. These enzymes play a key role in production of L-DOPA from L-tyrosine. Increased activity of TH enzyme is due to decreased 4a-hydroxy-6BH4 dehydratase (DH) activity in vitiligo patients (Schallreuter, Wood et al. 1994). Increased level of norepinephrine also appear to induce another catecholamine degrading enzyme, monoamine oxidase (MAO)(Bindoli, Rigobello et al. 1992). Keratinocyte and melanocyte in the depigmented skin exhibit increased monoamine oxidase-A activity which further causes keratinocytes to produce 4-fold more norepinephrine and 6.5-fold less epinphrine that control keratinocytes(Schallreuter, Wood et al. 1996).

Basically, neural theory explains the occurance of segmental vitiligo which shows the dermatomal unilateral distribution. Taieb A *et.al.*(Taieb 2000) suggested that segmental vitiligo may be associated with the dysfunction of cholinergic sympathetic nerves as acetylcholine esterase activity was found to be lowered in vitiliginous skin during depigmentation, suggesting that acetylcholine may aggravate the process of depigmentation in vitiligo(Iyengar 1989).

Aberration in beta-endorphin and met-enkephalin secretion are reported in vitiligo patients. Met-enkephalin levels are found to be higher and this abnormality may be correlated with the emotional stress, which precipitates vitiligo in some patient. Abnormalities of neuropeptide are also observed in perilesional skin and blood of vitiligo patients. NPY released by either exogenous stimulus like trauma or by endogenous stimulus eg stress alters the balance of neuropeptides in vitiliginous skin. neuropeptides are also reported to have imunoregulatory effects (Shajil, Chatterjee et al. 2006).

- **Oxidative Stress Hypothesis:**

Oxidative stress is the result of overproduction or inadequate removal of reactive oxygen species (free radical). Oxidative stress considered to be the initial trigger in the onset of the disorder as H_2O_2 accumulation is observed in the epidermis of active vitiligo patients(Maresca, Roccella et al. 1997). Melanogenesis process itself a major source of generating reactive oxygen species (ROS) and removal of these ROS are controlled by several redundant antioxidant enzymes(Denat, Kadekaro et al. 2014).

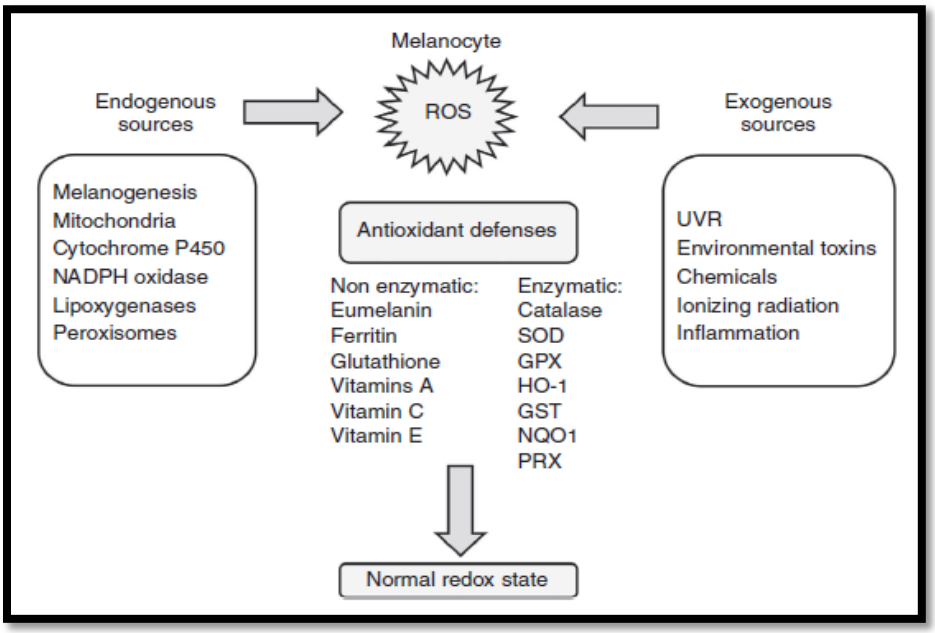


Fig 8: Induction of reactive oxygen species (ROS) by endogenous and exogenous sources and antioxidant defenses that restore normal redox state in melanocytes. *(Denat, Kadekaro et al. 2014).*

But defect in enzymatic and non enzymatic antioxidant system such as catalase, glutathione eroxidase, monoamine oxidase, xanthine oxidase etc may result in accumulation of ROS in the skin which is the basis of oxidative stress hypothesis(Passi, Grandinetti et al. 1998; Agrawal, Shajil et al. 2004; Koca, Armutcu et al. 2004).

It can be hypothesized that biochemical defects in the melanin biosynthesis pathway, as well as possible defects in patient's antioxidant enzymes, are responsible for the generation of ROS in the epidermis of patients with vitiligo.

Some of the defect leading to increment in intracellular production of ROS is:

- ✓ Defective recycling of tetrahydrobiopterin($6BH_4$) which leads to increased non enzymatic production of $7BH_4$, an isomer, concomitant with an increased production of H_2O_2(Schallreuter, Wood et al. 1994).
- ✓ Mitochondrial impairment.(Shajil, Chatterjee et al. 2006)
- ✓ Antioxidant imbalance. The low level of catalase may contribute for the generation of oxdidative stress in segmental vitiligo while the generation of oxidative stress in non –segmental vitiligo may be due to lower glutathione peroxidase and reduced glutathione.
- ✓ In response to electromagnetic radiation from the environment and acquired directly as oxidizing pollutants such as ozone and nitrogen dioxide (Betteridge 2000).

This accumulated oxidative stress causes DNA damage, lipid and protein peroxidation. Many proteins are altered and show partial or complete loss of functionality due to H_2O_2-mediated oxidation. H_2O_2 can also function as an inhibitor of tyrosinase, or in the presence of H_2O_2, DOPA (dihydroxyphenylalanine) substrate can generate a secondary complex that can bind and inhibit tyrosinase which ultimately, inhibit melanin production.(Laddha, Dwivedi et al. 2013)

Further, increased levels of ROS in melanocytes may cause defective apoptosis resulting in release of aberrated proteins, which can serve as autoantigens leading to autoimmunity. The intracellular levels of H_2O_2 and other ROS also increase in response to cytokines such as TNF-α (tumor necrosis factor a) and TGFb1 (transforming growth factor b1), which are potent inhibitors of melanogenesis.

Schallreuter *et. al.(Schallreuter, Moore et al. 1999)* reported massive accumulation of H_2O_2 in vitiligo skin which caused due to the impaired catalase and glutathione peroxidase activity. Inhibition of thioredoxin reductase, a free radical scavenger located in the membrane of melanocytes also contribute to oxidative stress generation in the epidermis(Schallreuter, Hordinsky et al. 1987).

- ➢ **Autoimmune Hypothesis:**

As neural theory explains the segmental vitiligo occurance, similarly, generalized vitiligo or non-segmental vitiligo occurance are better explained by autoimmune hypothesis. This is the most widely accepted hypothesis for vitiligo pathogenesis. Epidemological studies

have reported presence of autoantibodies against melanosomal protiens such as tyrosine, TRP1, TRP2 etc in the serum and autoreactive Cytotoxic T-cell Lymphocytes (CTLs) in peripheral blood and perileisional skin of vitiligo patients which supported the autoimmunity theory of vitiligo.(Kemp, Waterman et al. 2001)

Autoimmune hypothesis is also supported because of association of vitiligo with other autoimmune diseases such as diabetes, pernicious anemia, thyroid disease, Addison's disease etc(Laberge, Mailloux et al. 2005) . The autoimmunization mechanism are mediated by humoral, cell-mediated or by cytokines.

- **Humoral Immune Response in patient:**

Antibodies, mainly belonging to IgG class antibodies, against melanocyte antigens are found in the sera of vitiligo patients(Rocha, Oliveira et al. 2000; Farrokhi, Hojjat-Farsangi et al. 2005). The principle melanocyte antigens recognized by these antibodies are tyrosinase(Kemp, Emhemad et al. 2011), gp100/pmel 17(Kemp, Gawkrodger et al. 1998), tyrosinase related protein (TRP1 and TRP 2)(Kemp, Waterman et al. 1998) etc. Study by Shajil *et.al*.(Shajil, Chatterjee et al. 2006) reported that 84% of vitiligo patients at Baroda exhibit antimelanocyte antibodies in their circulation. Study by Ongenae *et.al.(Ongenae, Van Geel et al. 2003)* reported autoantibodies against HLA class I molecule in vitiligo patients. The disease activity has been positively correlated with the level of melanocyte antibodies(Harning, Cui et al. 1991). Exact etiology of antimelanocyte antibodies is still unknown but it has been showed through in vitro studies that antibodies derived from vitiligo patients are able to destroy melanocytes by complement mediated damage and antibody dependent cellular cytotoxicity(Gilhar, Zelickson et al. 1995).

Generation of autoantibodies against the melanocyte may be the result of:

- ✓ Genetic predisposition to immune dysregulation at T cell level. One of the best example exploring the given fact is Protein tyrosine phosphatase non receptor 22 (PTPN 22) 1858 C > T polymorphism which is considered to be the negative regulator of T-cell, found to be associated with vitiligo pathogenesis(Song, Kim et al. 2013).
- ✓ Presence of cross reacting antigen expressed on target cells or infecting microorganisms.

- ✓ Damage to melanocyte which exposes the melanocyte antigen.

- **Cellular Immune Response in patient**:

Histopathological investigation of the perileisional skin of vitiligo have confirmed the presence of CD4+ and CD8+ cells in the infiltrate(Le Poole, van den Wijngaard et al. 1996), thus suggesting the involvement of lymphocytes in depigmentation process. High frequency of Melanocyte Reactive-CTLs has been characterized in the peripheral blood of vitiligo(Middelkamp-Hup, Bos et al. 2007). Circulating melanocyte-specific CD8+ cytotoxic T lymphocytes that target melanocyte-specific antigens, including MelanA (MART-1), PMEL and tyrosinase, have been detected in vitiligo patients

- **Autoimmunization mediated by Cytokines:**

Beyond the humoral and cellular immune response, various studies demonstrated the potential role of cytokines in the vitiligo etiology(Grimes, Morris et al. 2004). In-vitro studies by Yu *et.al(Yu, Chang et al. 1997)*have demonstrated an increased production of proinflammatory cytokine IL-6 and IL-8 which play a important role in effector cell migration, effector target attachment and activation of B-cell. Grimes *et.al.(Grimes, Morris et al. 2004)* reported significant increase in the expression of tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and IL-10 which mediates the Th1 helpher response. Increased expression of TNF-alpha contributes to inhibition of melanocyte stem cell differentiation, melanogenesis process and initiates the apoptotic pathway in melanocyte, thus act as an intrinsic factor in melanocyte loss(Laddha, Dwivedi et al. 2012).

- **Melanocytorrhagy:**

Melanocytorrhagy is upcoming new hypothesis , first explained by Gauthier *et.al.(Gauthier, Cario-Andre et al. 2003; Gauthier, Cario Andre et al. 2003)* in 2003 which proposes that non-segemntal vitiligo is a primary cause of "melanocytorrhagic disorder".

Melanoctytorrhagy explains that melanocyte with defective adhesion system along with with altered melanocyte responses to friction and possibly other types of stress, inducing their detachment and subsequent transepidermal loss. Detachment and transepidermal

elimination of melanocytes following minor trauma such as stress, accumulation of ROS etc are probably the cause of depigmentation occurring in the isomorphic response known as Koebner phenomenon.(Kumar and Parsad 2012)

Adhesion system of melanocytes is far weaker than the system which firmly holds epidermal keratinocytes to the basement membrane. Dendrites are critically important for melanosome transfer, because one melanocyte contacts several keratinocytes in the epidermis through dendritic cell processes.

In NSV melanocytes, loss of dendricity induced either by oxyradicals (impaired redox status hypothesis) or by increased release of catecholamines (neural biochemical hypothesis) increase melanocytes transepidermal loss. This loss of dendricity could also affect melanosome transfer and contribute to depigmentation. Besides defective adhesion and dendritic loss, other abnormalities may lead to a decrease in the frictional resistance of melanocytes in NSV and eventually to their detachment. After their detachment, melanocytes undergo transepidermal elimination and thus lead to melanocyte loss which result in loss of pigmentation from the lesional site(Kumar and Parsad 2012).

Cario-Andre *et.al.(Cario-Andre, Pain et al. 2007)* in an *in vitro* study reported that NSV melanocytes have an intrinsic defect, which limits their adhesion in a reconstructed epidermis, with an enhancer effect of the vitiligo keratinocyte milieu. Namazi *et.al.(Namazi 2007)* hypothesized that a combination of pathogenic mechanisms (neurogenic dysregulation, oxidative stress, autoimmunity, and melanocytorrhagy) that act in concert can lead to NSV. Study by Kumar *et.al.*(Kumar, Parsad et al. 2011) demonstrated that melanocytes from unstable vitiligo/NSV showed significantly low adhesion to collagen type IV compared with control and stable vitiligo melanocytes. Results showed that caspase 3 and annexin V staining was significantly greater in melanocytes cultured from unstable vitiligo compared with the control which conclude morphological and adhesion findings support the theory of melanocytorrhagy as the primary defect underlying melanocyte loss in unstable vitiligo.(Kumar, Parsad et al. 2011)

There are various other theory such as viral infection, biochemical defect in pathway etc which may also be contributing to the vitiligo pathogenesis which is not explained above.

In conclusion, single hypothesis cannot explain all the various clinical type of vitiligo but they do work in combination such as onset of oxidative stress act as a trigger to cause the autoimmunization which leads to melanocyte loss. It has been speculated that local systemic factors affect the homeostasis of the epidermal melanin unit in segmental vitiligo whereas an impaired redox status of the epidermal melanin unit acts as the trigger further leading to inappropriate immune response in non-segmental vitiligo. The neural theory is more related to segmental vitiligo whereas the autoimmune theory is involved in non-segmental vitiligo(Anbar Tel, Abdel-Raouf et al. 2011).

2.2.5 Genetics of Vitiligo:

Genetic factors are playing a major role in the etiology of the disease. Vitiligo is considered to be a complex polygenic hereditary disorder. Various studies demonstrated that a family history for vitiligo exists in 6.25-38% of patients(Njoo and Westerhof 2001). However, the exact mode of inheritance remains unclear. Hereditary basis of the disease is governed by a set of recessive alleles situated at several unlinked autosomal loci which may be involved in the generation of oxidative stress, melanin biosynthesis, autoimmunity, that could collectively confer the vitiligo phenotype(Nath, Majumder et al. 1994). The inheritance pattern of vitiligo does not follow the simple mendelian pattern. Genetic linkage and candidate gene association studies have implicated several potentially contributory loci, though few have been consistently supported by the data. Few genes that are reported to vitiligo susceptibility are discussed below:

Gene	Function	Polymorphism Associated With Vitiligo	Reference
Lymphoid Protein Tyrosine Phosphatase(PTPN 22)	PTPN 22 Encodes For LYP Protein Which Act As A Negative Regulator Of T-Cell Activation	+1858C>T (Missense R620W)	Song et.al(Song, Kim et al. 2013)., 2013
Estrogen Receptor gene (ESR-I)	ESr-I play a role in the Pigmentation. High concentration of estrogen associated with increase pigmentation and successful treatment.	Intron 1 C/T	Jin et.al(Jin, Park et al. 2004), 2004
Angiotensin Converting Enzyme gene (ACE)	Capable of inactivating bradykinin, modulating cutaneous neurogenic inflammation and degrading substance and other neuropeptides	ACE gene Insertion/ Deletion(I/D)poly morphism	Badran et.al(Badran, Nada et al. 2015).,2015
Melanocortin 1Receptor (MC1R)	MC1R gene code for melanocyte stimulating hormone receptor(MSHR) to which alpha-MSH or ASIP bind. Binding of these initiate the melanogenesis process. MC1R act as major determinant of sun sensitivity	+274 G>A(val92Met)	Na et.al.(Na, Lee et al. 2003), 2003
Agouti Signalling Protein (ASIP)	ASIP binds to alpha-MSH which blocks the production of cAMP, leading to downregulation of eumelanogenesis	+488 A>G(arg 163Gln)	Na et.al.(Na, Lee et al. 2003), 2003
Catalase (CAT)	Anti-oxidant enzyme. Removal of hydrogen peroxide from the skin	389 C>T	Lu et.al.(Lu, Liu et al. 2014), 2014
Tumor necrosis factor-alpha(TNF-α)	Inhibit melanocyte stem cell differentiation, melanogenesis, initiate apoptosis	-308(G>A)	Laddha et.al.(Laddha , Dwivedi et al. 2012),2012
Cytotoxic T-lymphocyte antigen 4 (CTLA-4)	CTLA-4 is a T-cell surface molecule involved in T cell apoptosis and regulation of T- cell activation	CT60 A>G	Song et.al(Song, Kim et al. 2013)., 2013

Table 3: Genes that contribute to vitiligo susceptibility

Recently, role of Liver X Receptor (LXR) in skin physiology and pathology has evolved rapidly because of their role in epidermal proliferation, carcinogenesis, differentiation and permeability barrier function, which identifies them as promising drug targets for the treatment of skin diseases(Kumar, Parsad et al. 2012).

2.3 Liver X Receptor Gene:

Liver X Receptor (LXR-alpha/ NR1H3 and LXR-beta/ NR1H2) are ligand (such as oxysterols, high concentrations of D-glucose and phytosterols) activated nuclear transcription factors which regulate the expression of target genes involved in lipid biosynthesis, cholesterol homeostasis, immunity and inflammation(Jamroz-Wisniewska, Wojcicka et al. 2007). Oxysterol (eg 22(R)-hydroxy cholesterol (22 (R)-HC), 24(S), 25-epoxycholesterol (24(S), 25-EC)etc) are the natural ligand of LXR enzyme which are either intermediates in cholesterol biosynthesis or originate by the oxidation of cholesterol by various cytochrome P450 (CYP) isoforms.

LXRs contain a central DNA-binding domain consisting of a zinc-finger module and a large ligand-binding domain with a lipophilic core that binds specific small-lipid molecules such as oxysterol. After ligand binds, nuclear receptors undergo a conformational change that promotes interaction with retinoid X receptor. This heterodimer bind to the LXR response element (LXRE) which usually consists of direct repeats of the hexamer AGGTCA, separated by four nucleotides (DR4). Thus, LXRs regulate gene expression through binding to DNA as heterodimers with the retinoid X-receptor (RXR) (NR2B1)(Chen, Li et al. 2008).

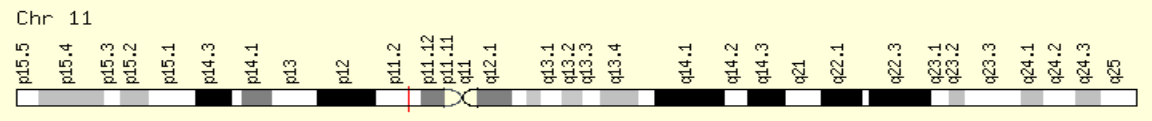


Figure 9: NR1H3 Gene in genomic location

(Source: http://www.genecards.org/cgi-bin/carddisp.pl?gene=NR1H3&snp=494&search=nr1h3#snp)

Originally, Liver X Receptor considered as “orphan” nuclear receptors, because their natural ligands were unknown following the discovery of oxysterols as there natural

ligand which bind to and activate these receptors at physiological concentrations(Willy, Umesono et al. 1995) , they have been removed from orphan category. Since there expression and function is high in liver, they been named as Liver X Receptor.

LXR exists in two isoforms: LXR-alpha and LXR-beta. LXR-alpha is highly expressed in several metabolically active tissues, such as liver, intestine, adipose tissue, and macrophages, whereas LXR-beta is ubiquitously expressed in most tissues(Steffensen and Gustafsson 2004; Lee, Park et al. 2013). Both isoforms are activated by endogenous oxysterol.

LXR are also found to be expressed in the skin tissues such as sebaceous glands, hair follicle, epidermal keratinocyte and fibroblast(Billoni, Buan et al. 2000; Russell, Harrison et al. 2007). They found to have role in skin physiology and pathology such as proliferation, carcinogenesis, differentiation and permeability barrier function(Kumar, Parsad et al. 2012). LXR activation induces keratinocyte differentiation and inhibits proliferation(Komuves, Schmuth et al. 2002). LXR induces lipid synthesis in sebocytes and inhibits the expression of cytokines and metalloprotinases in skin–photoaging models.(Kumar and Parsad 2012) Marked expression of LXRs in skin speculate that it might have an important role in pathogenesis of skin disorders such as psoriasis, acne vulgaris, vitiligo etc.

LXR activator exhibit potent anti-inflammatory activities in many inflammatory disorders including dermatitis, artherosclerosis and pulmonary inflammation through the suppression of several NF- kappa B target genes such as MMP 9, CCL2, CCL7, IL-1β and cyclooxygenase-2(Bensinger and Tontonoz 2008).

The function and characteristics of LXRs in skin have been recently widely studied but their expression and function in relation to melanocyte have not yet been investigated. Recently, Kumar *et.al.(Kumar, Parsad et al. 2010)* demonstrated that LXRs are also expressed in melanocytes. Kluger *et.al.*(Kluger, Cotten et al. 2008) reported a marked expression of LXR-alpha in cells adjacent to dermal papilla, speculating that it may correlate with the site of hair melanocytes. Important genes involved in regulation of melanocytes such as MITF, MMPs, certain inflammatory genes such as IL-6, IL-1beta are target genes of LXR which suggest that LXRs might be playing a role in regulation of melanocyte and melanogenesis(Lee, Park et al. 2013) .

Recently, Kumar *et.al.(Kumar, Parsad et al. 2010)* demonstrated that LXR-alpha are upregulated in the melanocytes from perilesional skin as compared to the normal skin of vitiligo patient suggesting its role in vitiligo pathogenesis.

2.3.1 Role of LXRs in Pathogenesis of Vitiligo:

LXRs function as nuclear cholesterol sensors that are activated in response to elevated intracellular cholesterol levels in multiple cell types. Bellei *et.al.*(Bellei, Pitisci et al. 2013) has reported high cholesterol content in the VHM (Vitiligo Human Melanocyte) culture isolated from the vitiligo patient compared to the NHM(Normal Human melanocyte) culture isolated from healthy individuals. Along with, higher amounts of oxysterols, in particular 7-beta-hydroxycholesterol and 7-ketocholesterol has been reported in vitiligo melanocytes. These cholesterol oxidation products have important pathophysiological role in oxysterols-induced cell death that can take part in degenerative pathologies(Gamba, Leonarduzzi et al. 2011). High content of oxysterol activates the LXR gene and marked expression of LXR might be involved in vitiligo pathogenesis.

Study by Kumar *et.al.*(Kumar, Parsad et al. 2010) demonstrated that expression of liver X receptor-alpha (LXR-α) at both mRNA and protein level was significantly higher in perilesional skin as compared to the normal skin of vitiligo patient. Further, they have demonstrated that on treating control melanocytes with LXR-α agonist 22-hydroxy cholesterol, the adhesion of melanocytes to type IV collagen and laminin 5 decreases significantly. Increase in LXR-α expression might decrease the cell adhesion molecule, which ultimately leads to the detachment of melanocytes from the basement membrane in perilesional vitiligo skin which can ultimately lead to Melanocytorrhagy (Kumar and Parsad 2012).

For repigmentation to occur at the leisional site of vitiliginous skin, migration of melanoblast (melanocyte precursor) from the outer root sheath of hair follicles into clinically depigmented epidermis is the major requirement naturally or in any of the photochemotherapy treatment (Norris, Horikawa et al. 1994; Taieb 2000). But for such migration, melanoblast require release from ORS and need to penetrate extracellular matrix tissue barrier in vivo to finally reach the basal layer of epidermis. These processes are directed by limited proteolysis of the extracellular matrix(ECM) by Matrix

metalloproteinases (MMPs). The MMPs are initially expressed as inactive pro-MMP zymogens which are then activated in the extracellular environment. Such activation occurs at the cell surface and an important consequence is that proteolysis is greatest in the immediate pericellular environment, where it can influence cell–cell and cell–ECM interactions(El Fahime, Torrente et al. 2000). Study by Giannelli G. *et.al.(Giannelli, Falk-Marzillier et al. 1997)* reported that MMP2 induces the migration of breast epithelial cells by specifically cleaving laminin-5. Study by Lei *et.al.*(Lei, Vieira et al. 2002) demonstrated that MMP 2 plays an important role in melanoblast migration from the border of vitiligo lesions into clinically depigmented epidermis which is crucial for the repigmentation of vitiliginous skin. Additionally, study by Kumar *et.al.(Kumar, Parsad et al. 2011)* reported that MMP-2 and MMP-9 are downregulated in vitiligo patient and further, it was shown that LXR-α gene knock-down significantly increases the expression of MMPs. Thus, high expression of LXR-alpha might be contributing to the inhibition of MMPs because of which melanoblast are not able to migrate at the basal layer of the epidermis. No repigmentation is observed as a phenomenon.

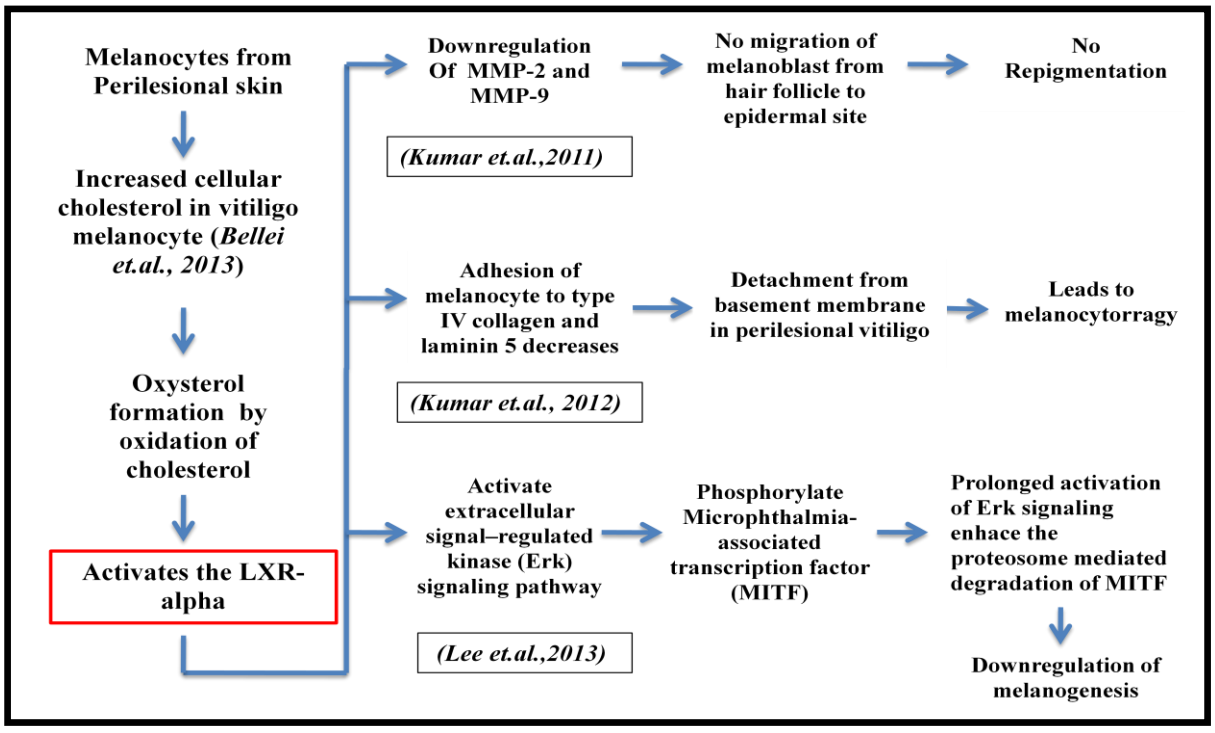


Fig10: Role of LXR-alpha in vitiligo pathogenesis

Study by Lee *et.al*.(Lee, Park et al. 2013) reported that the activated LXR inhibit the melanogenesis through the activation of extracellular signal–regulated kinase (ERK) mediated MITF degradation supporting that LXRs might be crucial intrinsic factor for the regulation of melanogenesis. LXRs ctivate the MEK/ERK.RSK-1 signaling pathway which phosphorylate Ser409 of MITF prolonged activation of Erk signaling pathway result in proteosome dependent degradation and thus down regulate melanogenesis.

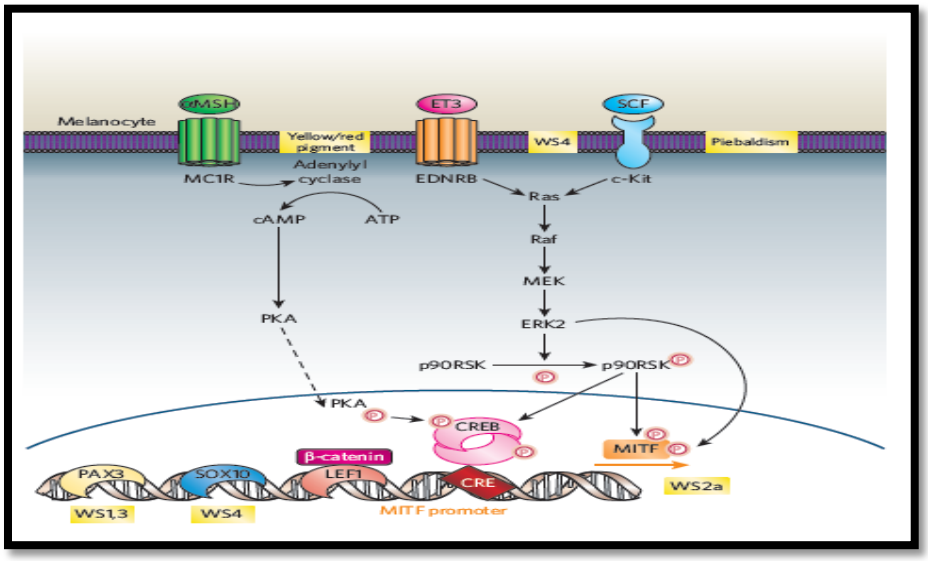


Fig11: Regulation of MITF promoter.*(Lin and Fisher 2007)*

Study by Oh *et.al.(Oh, Katz et al. 1997)* demonstrated that LXR suppress the expression of IL-6, which inhibits the growth of melanocyte.

Thus, Higher expression of LXR-α in perilesional skin melanocytes significantly decreases the adhesion, proliferation and matrix metalloproteinases and increases apoptosis. Based on all these studies LXRs appear to have important role in vitiligo pathogenesis and considered as a potential therapeutic target for vitiligo.

Change in expression of gene which is considered as an intrinsic factor in a disease may be correlated to both genetic and non genetic factors. In case of LXRs:

- ✓ Non genetic factor such as level of agonists eg oxysterol, drugs such as statins, fibrates, thazolidinedione derivatives etc, level of antagonists eg oxysterol sulfonates, LPS injection etc regulate the level of LXRs gene.

- Genetic factor involves mutational changes, single nucleotide polymorphism, epigenetic change.

Focus of my project is to genotype the SNP in the LXR-alpha gene in study population (Vitiligo and cases) which is further used to assess the association between the LXR-alpha SNP with vitiligo susceptibility.

2.3.2 Single Nucleotide polymorphism:

Single Nucleotide Polymorphism is a single nucleotide change in the DNA sequence that is present at a frequency of greater than 1% in a population. By SNP genotyping information, researchers can investigate disease-associated genes and analyze the genetic structure of a population. SNPs may change the encoded amino acids (nonsynonymous) or can be silent (synonymous) or simply occur in the noncoding regions. They may influence promoter activity (gene expression), messenger RNA(mRNA) conformation (stability), and subcellular localization of mRNAs and/or proteins and hence may produce disease. Therefore, identification of numerous variations in genes and analysis of their effects may lead to a better understanding of their impact on gene function and health of an individual.

Approximately 494 SNPs have been identified in NR1H3 gene. Out of which, 2 of the SNP(rs11039155 and rs2279238), I have picked for the genotypying by PCR-RFLP and to asses the association of these with vitiligo.

- **rs11039155 (-6 G>A):**
    - G to A transition at position - 6 (is located six base pairs upstream from the ATG site). Ancesteral allele: G
    - Location: Chromosome 11:47259211 (forward strand)
    - Minor Allele frequency : 0.08 (A)

Functional characteristic of rs11039155:

-6 G>A polymorphism is located six base pairs upstream from the ATG site, a position shown by Kozak to affect recognition of the AUG codon. The fact that the sequence

flanking the AUG start codon in NR1H3 mRNA (GAAGA GATGT) does not closely match the Kozak consensus sequence (GACACCATGG) makes it more likely that the polymorphism might affect translational efficiency; that is a mutation in position -6 is more likely to have an effect in the absence of A in position -3 and G in position +4(Kozak 1987). It would be therefore interesting to compare the level of NR1H3 protein produced in various tissues for example, liver, macrophages, adipose tissue between individuals with contrasted genotypes for the NR1H3 SNP rs11039155. Thus, it can be speculated that presence of mutant allele A at position -6 might increase the expression of LXR-alpha gene in the vitiligo patient along with high amount of oxysterol which can act as a trigger to activate LXR-alpha.

Association of rs11039155 with other disease:

Legry *et al.*(Legry, Cottel et al. 2008) documented that rs11039155 AA carriers of LXR-alpha had higher HDL cholesterol and a 30% decrease in risk of having the metabolic syndrome in two cohorts of French subjects. Study by Zhao *et.al.*(Zhao, Hu et al. 2014)reported rs11039155 is associated with risk of polycystic ovary syndrome in a Chinese Han population.

- **rs2279238 (+1257 C>T) :**
    - C to T transition in the exon region of LXR-alpha. Ancestral allele : C
    - Synonymous SNP
    - Location: Chromosome 11:47260473 (forward strand
    - Minor Allele Frequency (MAF): 0.35 (T)

Function Characteristics of rs2279238:

The LXR-alpha SNP rs2279238 is located at an exon splicing enhancer (ESE) where splicing factor SRp55 binds but the nucleotide variation is synonymous, resulting in a protein sequence identical to that of the wild type.(Price, Pacanowski et al. 2011) Studies have demonstrated that synonymous polymorphisms can affect messenger RNA splicing, stability, and structure as well as protein folding. These changes can have a significant effect on the function of proteins, change cellular response to therapeutic targets, and often explain the different responses of individual patients to a certain medication. It can

be speculated due to change in nucleotide sequence SRp55 binding efficiency will be affected which in turn have a affect in LXR-alpha expression.

Association of rs2279238 with other disease:

Dahlman *et al.*(Dahlman, Nilsson et al. 2006; Dahlman, Nilsson et al. 2009) reported that LXRA SNP rs2279238 CC carriers had lower body mass index and rs2279238 CT carriers were associated with obesity phenotypes. Study by Han *et.al.*(Han, Liang et al. 2014) reported individual carrying T allele are associated with the protective against tuberculosis patient. Study by Price *et.al.*(Price, Pacanowski et al. 2011) reported that individual carrying T allele are associated with cardiovascular outcomes.

2.3.3 SNP Genotyping: PCR-RFLP

SNP genotyping is performed by many methods, including hybridization, allele-specific 10 polymerase chain reaction (PCR), primer extension, oligonucleotide ligation, direct DNA sequencing, and endonuclease cleavage. Each of these methods has its specific advantages and disadvantages.

If the SNP to be studied involves a restriction enzyme site, Polymerase Chain Reaction - Restriction Fragment length Polymorphism (PCR-RFLP) can be a genotyping procedure that is easy to set up in any molecular biology laboratory. The PCR-RFLP method allows very rapid, simple, and inexpensive detection of point mutations within the sequences of PCR products. The mutation is discriminated by the specific restriction endonuclease and is identified by gel electrophoresis followed by staining with ethidium bromide. This convenient and simple method is useful in a small basic research study.

PCR-RFLP involves two step:

1. PCR: Biochemical reactions to form allele-specific products and
2. RFLP: detection procedures to identify the products.

Polymerase Chain Reaction:

The polymerase chain reaction (PCR) is a biochemical technology in molecular biology to amplify a single or a few copies of a piece of DNA into thousands to millions of copies of a particular DNA sequence. The method relies on thermal cycling which

consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA.

There are three major steps in a PCR, which are repeated for 30 or 40 cycles. This is done on an automated thermocycler, which can heat and cool the tubes with the reaction mixture in a very short time.

- **Denaturation:**

  In this step, reaction is heated to 94–98 °C for 20–30 seconds. It causes DNA melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules.

- **Annealing:**

  As the temperature cools down to its annealing temperature, primers anneals with single strand DNA i.e. short oligonucleotide primer sequence bind with the complementary region of DNA and forms a stable bond.

- **Extension:**

  $72^{o}C$ is the ideal working temperature for the Taq polymerase enzyme. The polymerase adds dNTP's from 5' to 3'and reading the template from 3' to 5' side. The initiation of DNA synthesis occurs at 3'-hydroxly end of each primer. The primers are extended by joining the bases complementary to DNA strands using Taq polymerase enzyme.

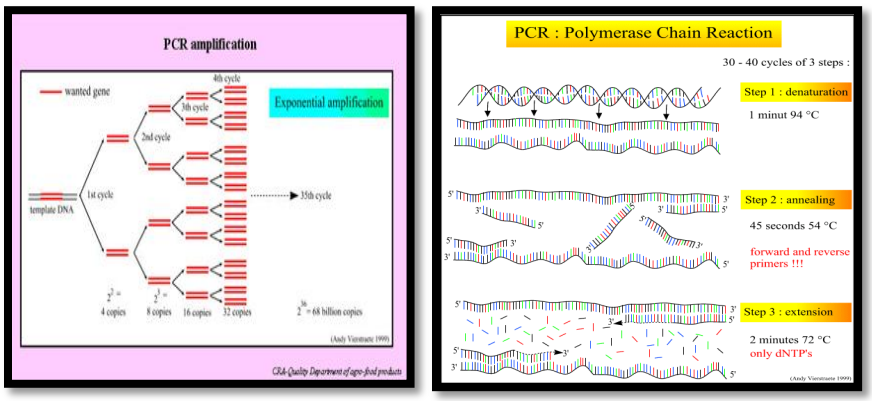


Fig 12: PCR Amplification cycle and major steps

Restriction Fragment length Polymorphism (RFLP):

RFLP arise because single base pair change can create or destroy the sites recognized by specific restriction enzymes, leading to variation between individual in the length of restriction fragment produced from identical regions of genome. A single base pair change i.e SNP is readily detectable genetic marker by RFLP because a mutated site is no longer cleaved by the enzyme in question. Two chromosome differ by such a mutation are then distinguishable on the basis of RFLP, which arises because a particular cleavage site is present in only one of the DNA molecule.

CHAPTER 3

OBJECTIVE

OBJECTIVE

In our study, we would explore the association of LXR-alpha gene polymorphisms (rs11039155 and rs 2279238) with vitiligo susceptibility which could be used as prognostic marker for Vitiligo susceptibility.

Objective of our study are:

• Optimization and Standardization of PCR for the SNPs

a) rs11039155
b) rs2279238

• To genotype SNPs rs11039155 and rs2279238 of LXR- alpha gene by PCR-RFLP in vitiligo patient and control individual belonging to North India.

• To look for the genetic association, if any, between polymorphism in LXR-alpha and susceptibility to vitiligo by statistical analysis.

CHAPTER 4

MATERIALS AND METHODS

Study Population:

The study group included 39 vitiligo patients (18 female/21 male) who referred to Dr. Rajendra Prasad Government Medical College (RPGMC), Kangra, Himachal Pradesh and Dr. Bansal's Skin laser centre, Manimajra , Chandigarh during 2015 (January-May 2015). The diagnosis of vitiligo was clinically based on the presence of depigmented patches on the skin. A total of 50 healthy individual with no history of vitiligo or apparent autoimmune disease were included as control; they matched to patients with regard to age, sex and geographical distribution. Conductance of the study was approved by the ethics Institutional Review Board of the Dr. Rajendra Prasad Government Medical College and Jaypee University of Information Technology. The importance of the study was explained to all participants and written consent was obtained from all patients and controls.

Sampling:

2 ml venous blood was collected from the patients and healthy subjects in Na_2EDTA coated tubes. Blood sample are stored at -20^{o}C till further use.

Isolation of Genomic DNA from Whole Blood sample (Miller *et.al.*, 1988):

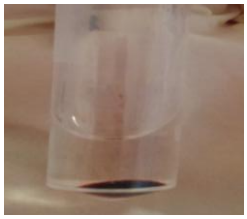
- 400µl blood sample was pippetted in a 2ml eppendorf. To this RBC lysis buffer was added (three times the volume of blood sample) and was kept for incubation on a rocker at room temperature until RBCs completely lysed. (Appendix 2.6)
- The solution was centrifuged at 13,000 rpm for 1 min to obtain a creamish white WBC pellet.
- The supernatant was discarded and the WBC pellet was thoroughly suspended in 400 µl TE buffer(pH 8.0) using a vortexing machine.(Appendix 2.7)
- 22 µl of 10% SDS solution was added to the suspended pellet solution and the mixture was incubated at 56^{o} C for 30 min on a dry bath. (Appendix 2.5)
- Subsequently, 160 µl of 7.5 M ammonium acetate was added to the solution and was mixed vigorously for about 1 min per sample on vortexer. The mixture was

centrifuged at 13,000 rpm at RT for 15 mins, thereby resulting in separation of the precipitated proteins as pellet.

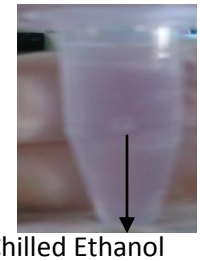
- The clear supernatant was transferred to a fresh sterile micro-centrifuge. To this chilled absolute ethyl alcohol was added (twice the volume of clear supernatant). the tube was gently rocked a couple of times to allow the precipitation of genomic DNA.
- The genomic DNA precipitate was centrifuged at 13,000 rpm for 10 min to pellet at the bottom of the tube. The latter were subsequently washed in 150 µl of 70% ethanol and air dried at RT for about 10-15 mins.
- The dried pellet was dissolved in 40 µl TE buffer (pH 7.3) by incubating at 65^{o}C for 10 mins. The dissolved DNA was finally stored at 20^{o}C till further use.(Appendix 2.8)
- The DNA quantification was done using Nano Drop plus Spectrophotometer (GE Healthcare, US). The concentration of DNA was read by measuring the absorbance of a sample at A_{260} on a spectrophotometer.

Blood+ RBC lysis buffer mixture on rocker

Creamish WBC pellet



Precipitated protein as pellet



Chilled Ethanol Precipitated DNA

OBJECTIVE 1 & 2 : SNP GENOTYPING : PCR-RFLP

PCR: Biochemical reaction to form allele specific product

PCR technology was used for the amplification of the desired LXR-alpha gene segment. PCR amplification of the genomic DNA isolated from blood sample was carried out using primers (Table 4) specific for the LXR-Alpha gene sequence which were made using software PRIMER3.

Polymerase chain reaction for the LXR-alpha gene amplification was performed using the following protocol:

- 25µl reaction mixture was prepared as per the composition given in the Table 5. Samples mixture was kept into the Thermocycler (Thermo scientific thermocycler) for amplification and parameters as given in Table 6 were set.

Qualitative Analysis of PCR product:

Agarose gel electrophoresis was conducted to see the desired band size of 366 bp and 377 bp. PCR amplification reactions were analyzed on 1.2% w/v agarose gel containing Ethidium Bromide (0.5mg/ml). A 50 bp (NEB,) marker was used as ladder. 10 µl of amplified product mixed with 1 µl of gel loading dye was loaded into the wells along with ladder into the separate well. The gel was then run for 30-45 min at 100Volts in 1X TAE buffer. Bands were visualized using U.V. transilluminator.

Table 4 : Nucleotide Sequence of the Primer used in PCR for *LXR*-alpha gene

	Primer Sequence	
	rs11039155	**rs2279238**
Forward Primer	5’ GTGAGAGGATCACTTGAGC 3’	5’ CTTTCTGAGCCTCACTTTCC 3’
Reverse Primer	5’ CAGACCGCAGGCTCCACGC 3’	5’ CGCAGCTCAGAACATTGTAG 3’
Amplicon Size	366 bp	377 bp

Table 5: Reagent Mixture Composition used in the PCR for DNA Amplification

Reaction Component	Per Reaction Volume(µl)
10X Buffer	**2**
dNTPs	**0.5**
Primer(Forward)	**0.5**
Primer(Reverse)	**0.5**
Taq polymerase	**0.25**
DNA template (40 ng/ µl)	**1**
MQ water	**20.25**
Total	**25**

Table 6 : PCR Cycling Conditions for the Amplification of Genomic DNA

STEP	TEMP (^{O}C)	TIME	CYCLES
Initial Denaturation	94	2 min	
Denaturation	94	30 sec	35 cycles
Annealing	62 (rs11039155) 60 (rs2279238)	30 sec 45 sec	
Extension	72	30 sec	
Final Extension	72	5 min	
Final Hold	4	Infinite	

Restriction Fragment Length Polymorphism (RFLP): Detection Procedure to identify the allele specific product

Amplified product was digested with the restriction enzyme to know about the which genotype is present in the particular individual. PCR product of rs11039155 and rs2279238 were digested with Hpy188III and Fnu4HI respectively. Reaction condition of RFLP for both the SNP are mention in Table 7.

Table 7 : Reaction Condition of RFLP Genotyping Method

Reaction Component	Reaction Volume (µl)	
	rs2279238	**rs11039155**
	Fnu4HI **5' GC|NGC 3'** **3' CGN|CG 5'**	**Hpy188III** **5' TC|NNGA 3'** **3' AGNN|CT 5'**
Enzyme	0.1	0.2
Buffer	1.5	1.5
Water	3.4	3.3
DNA	10	10
Total	15	15

Analysis of Digested Product:

Agarose gel electrophoresis was done to visualize the digested band pattern. Digested Band pattern for different genotype will be as shown in fig 13 and Table 8 . RFLP digested product were analyzed on 3% w/v agarose gel containing Ethidium Bromide (0.5mg/ml). A 50 bp (NEB,) marker was used as ladder. 15 µl of amplified product mixed with 2 µl of gel loading dye was loaded into the wells along with ladder into the separate well. The gel was then run for 30-45 min at 100Volts in 1X- TAE buffer. Bands were visualized using U.V. transilluminator.

Table 8: Digested band pattern of SNP rs11039155 and rs2279238

rs11039155 (-6 G>A)		rs2279238 (C>T)	
Genotype	**Band Size (bp)**	**Genotype**	**Band Size (bp)**
GG	204 + 162	**CC**	325 + 48
GA	204 +162 + 103 + 59	**CT**	325 + 48 + 377
AA	204 + 103 + 59	**TT**	377

A) rs11039155 **B) rs2279238**

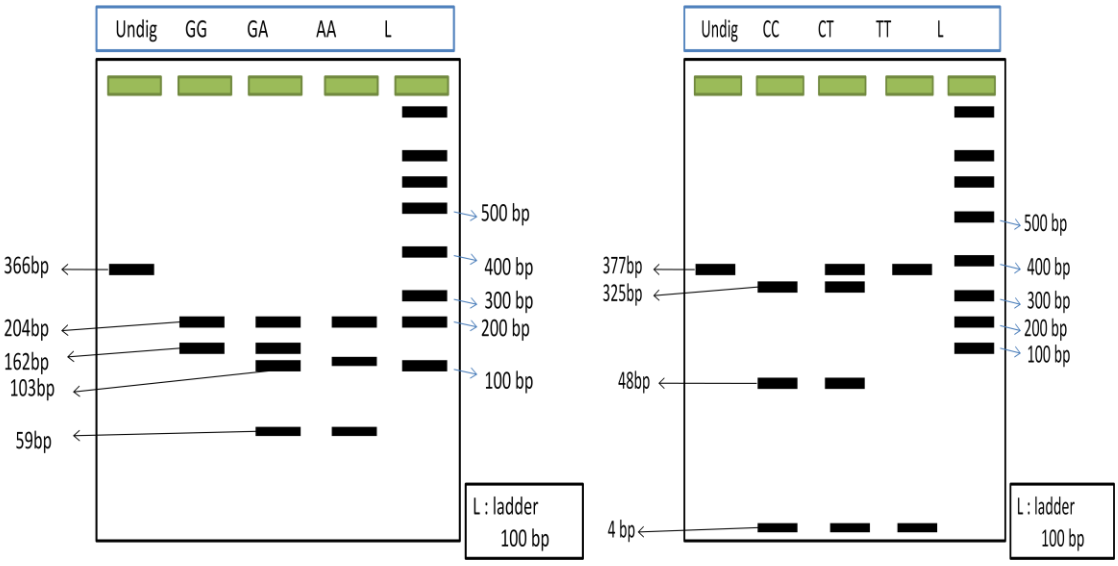


Fig13: Diagrammatic representation of Digested Band pattern for SNP A)rs11039155 B) rs2279238

OBJECTIVE 3: Statistical Analysis:

Statistical tool were applied to assess the association of LXR-alpha gene polymorphism with vitiligo susceptibility.

The significant differences in genotype and allele frequencies between patients and controls were analyzed by the Fisher exact test (http://www.quantpsy.org/fisher/fisher.htm). A chi-squared test was used to determine whether observed control genotype frequencies conformed to Hardy-Weinberg equilibrium (HWE) expectation using online HWE calculator (http://www.had2know.com/academics/hardy-weinberg-equilibrium-calculator-2 alleles.html). Observed frequency considered to be in disequilibrium if $p < 0.05$. Odds ratios and 95% confidence intervals were calculated to assess the risk associated with variant allele. Odd Ratio was calculated using software Review Manager v5.3. The significance of the odd ratio was determined by Z test ($p<0.05$ was considered statisticallysignificant)

CHAPTER 5

OBSERVATIONS & RESULT

OBSERVATIONS & RESULTS

Results:

Clinical and demographic characteristics of vitiligo patients are shown in Table 9. The sex ratio was almost equal (46% female (18 out of 39)). The average age of patient and control were 25.38 ±13.26 yr and 21.66 ± 6.08 yr respectively. The average age at onset of disease was calculated to be 16.63 ±11.44 yr. The majority of patients (82%) had less than 25% body coverage of the depigmented patches. Few patient (31% (12 out of 39)) reported occurrence of white spot at the injury site. 7% reported the family history of vitiligo. The genotype and allele frequencies of LXR-alpha polymorphisms (rs11039155 and rs2279238) in 39 vitiligo patients and 50 controls are summarized in Table 10.

39 vitiligo patient and 50 control subject were genotyped for -6 G>A and +1257 C>T in LXR-α gene. The genotype distribution for both the SNP showed no deviation from Hardy-Weinberg equilibrium in control population ($p>0.05$). Genotyping of the SNPs in the LXR-α gene revealed that the variant A allele of rs11039155 was found in 10% of controls and 22% of cases; the variant T allele of rs2279238 was found in 40% of controls and 56% of cases. There was a significant differences in the allelic frequencies of these two SNPs between vitiligo and control (rs11039155: $p=0.0358$; rs2279238: $p=0.0237$) which suggests the significant association of minor allele of both the SNP with the vitiligo. - 6 A and +1257 T alleles were found to increase the risk of vitiligo by 2.51 and 2.08 fold respectively. (**rs11039155**: odds ratio (OR) =2.51; 95% confidence interval (CI) = 1.08-5.84; **rs2279238:** OR= 1.94; 95% CI = 1.06-3.54) implicating that these particular SNP are associated with the vitiligo susceptibility and can be a prognostic marker for the disease.

Dominant and Co-dominant model of rs11039155 (Table 11) demonstrate that presence of single A in the individual genotype; predispose that individual to the vitiligo.

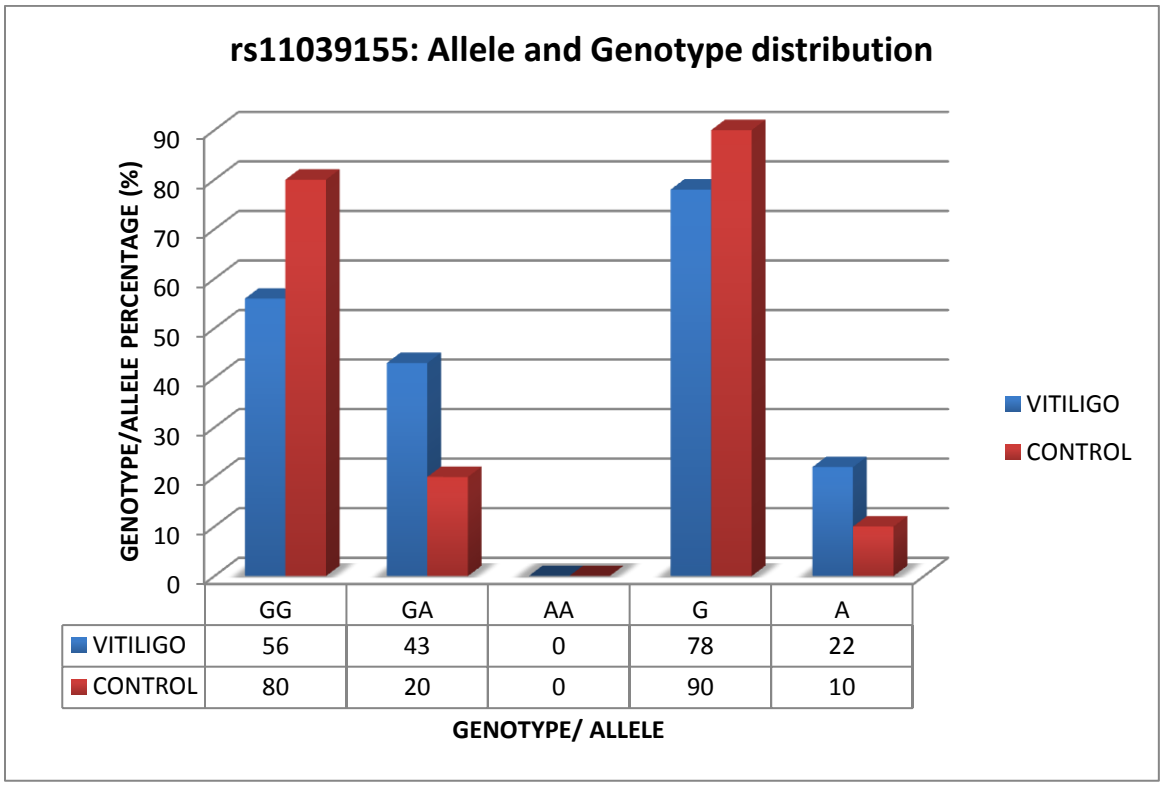
Table 9: Clinical and Demographic Characteristics of Vitiligo cases and Control group

Characteristics	Vitiligo (N= 39)	Controls (N= 50)
Sex (n/N [%])		
Female	18/39 [46]	14/50 [28]
Male	21/39 [54]	36/50 [72]
Age	25.38±13.26	21.66 ± 6.08
Age at the onset	16.63±11.44	
Changing size of depigmented patches (n/N[%])	24/38 [63]	
Body Coverage of depigmented patches (%)		
1-25	82	
26-50	5	
51-75	8	
76-100	5	
Reported Family history of vitiligo (n/N [%])	3/39 [7]	
White patches at Injury site (n/N [%])	12/39 [31]	

Table 10: Allele and Genotype distribution of the Liver X Receptor-alpha polymorphism in Vitiligo cases (N=39) and control (N=50)

Genotype/ Allele	Vitiligo (n/N [%])	Control (n/N [%])	p-value	Odd Ratio [95% CI]	HWE p-value
rs11039155 (-6 G>A)					
GG	22/39 [56]	40/50 [80]			0.43
GA	17/39 [44]	10/50 [20]			
AA	0	0			
G	61/78 [78]	90/100[90]	0.033	2.51[1.08-5.84]	
A	17/78 [22]	10/100 [10]			
rs2279238 (C>T)					
CC	6/39 [15]	16/50 [34]			0.24
CT	22/39 [56]	28/50 [54]			
TT	11/39 [28]	6/50 [12]			
C	34/78 [44]	60/100 [60]	0.03	1.94 [1.06-3.54]	
T	44/78 [56]	40/100 [40]			

Figure 14: Graphical view of Allelic and Genotypic distribution percent of the LXR-α polymorphism in vitiligo cases and control



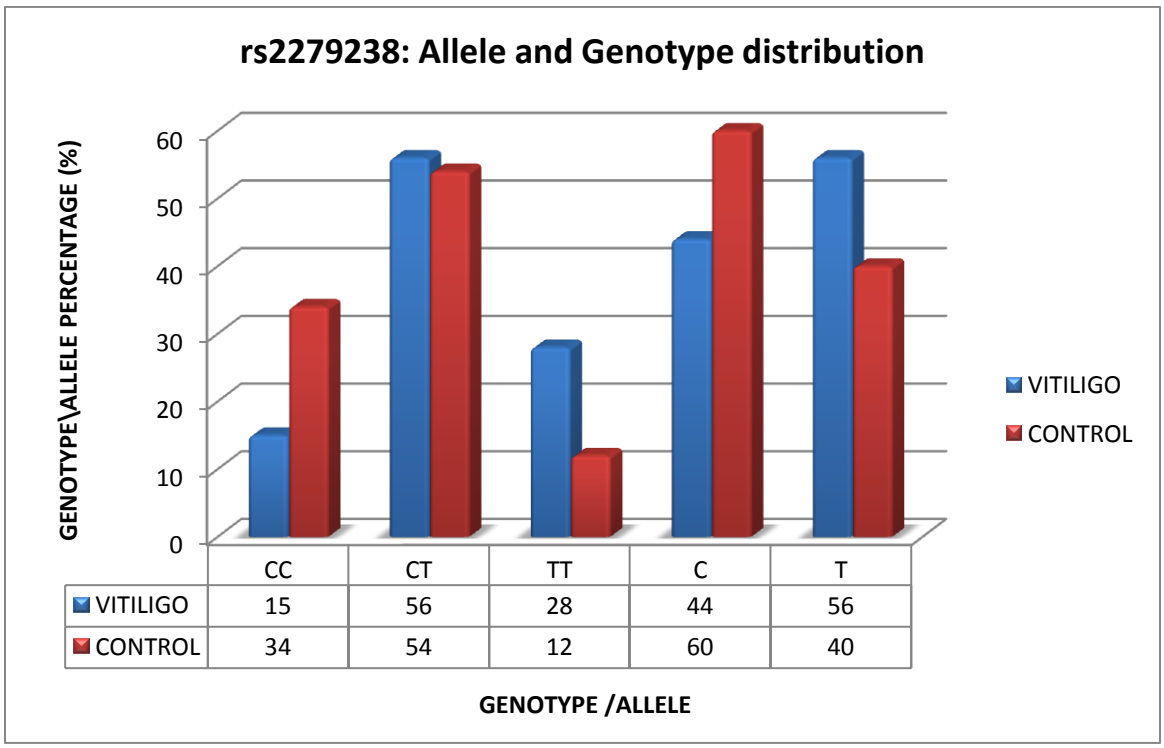
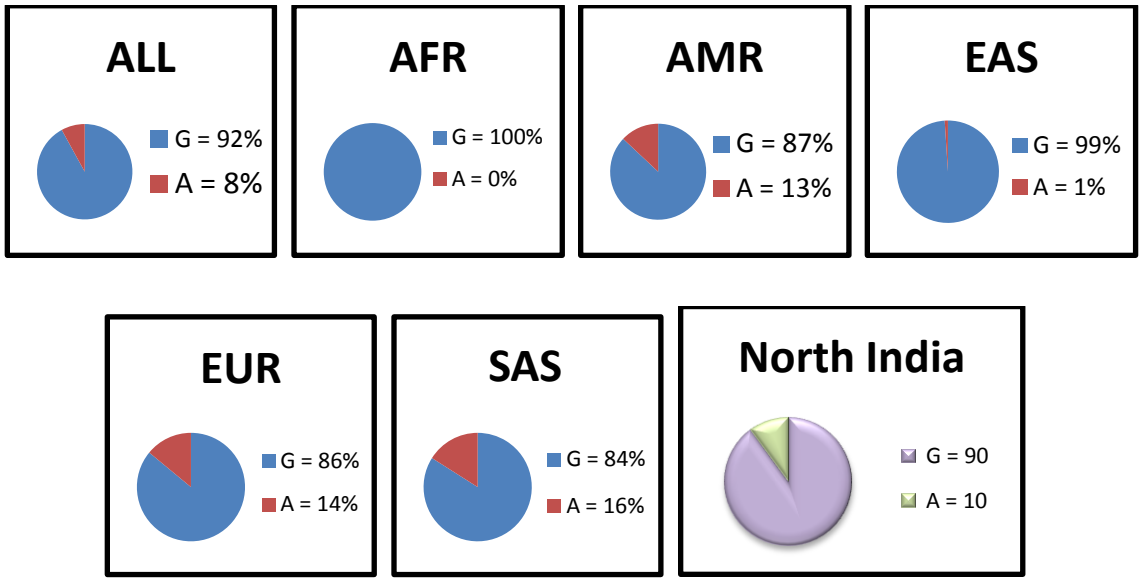


Table 11: Association between LXR-alpha polymorphism and Vitiligo Susceptibility

Polymorphism Model	Odd Ratio	95% CI	p-value
rs11039155			
Dominant Model (AA+GA vs GG)	3.09	1.209-7.901	0.0184
Recessive Model (AA vs GA+GG)		Not estimable	
Homozygous Model (AA vs GG)		Not estimable	
Co-Dominant Model (GA vs GG)	3.09	1.209-7.901	0.0184
rs2279238			
Dominant Model (CT + TT vs CC)	2.59	0.920 – 7.42	0.07
Recessive Model (TT vs CT + CC)	2.88	0.96 - 8.67	0.06
Homozygous Model (TT vs CC)	4.88	1.24 – 19.19	0.02
Co-Dominant Model (CT vs CC)	2.09	0.703 - 6.24	0.18

rs11039155:



A) rs2279238

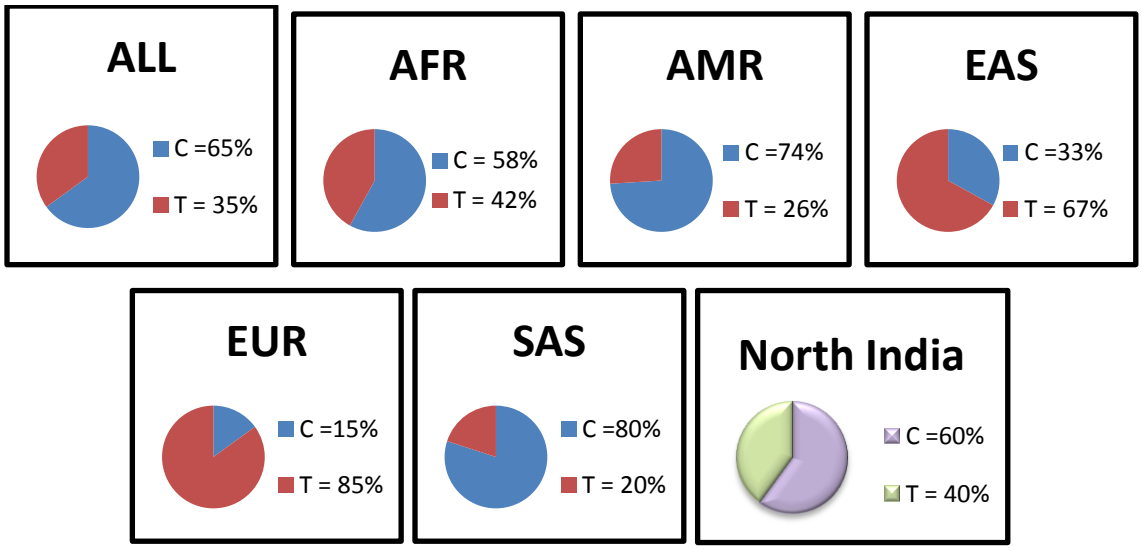
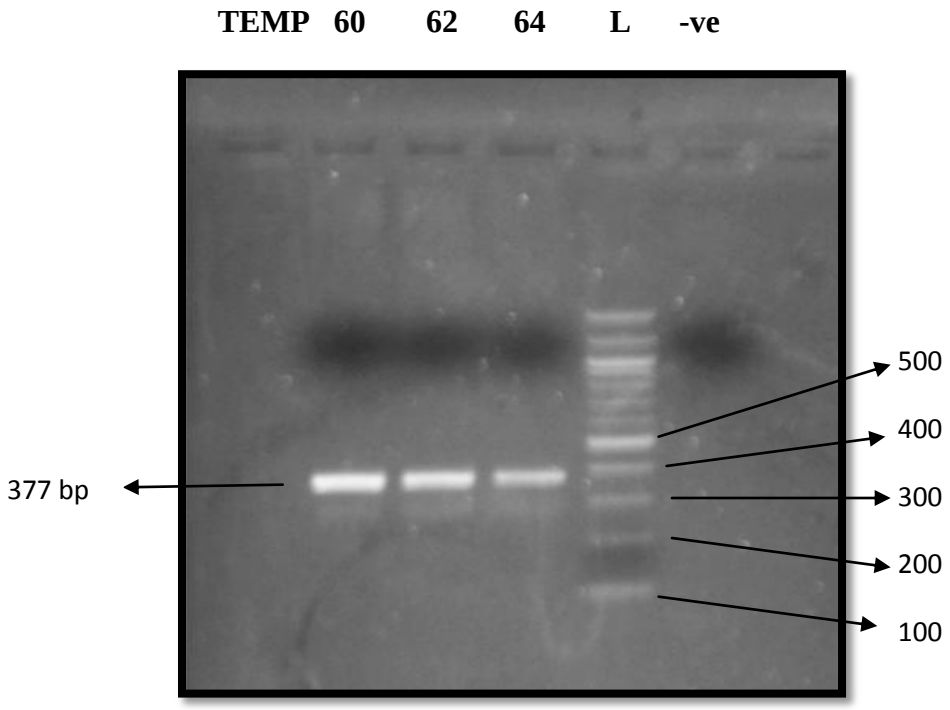


Figure 15: Allelic frequency of LXR-alpha gene polymorphism in different population compared to North India Population

Abbreviation: AFR: African; AMR: American; EAS: East Asian; EUR: European; SAS: South Asian

Fig 16 : PCR GRADIENT GEL IMAGE

A) rs2279238



B) rs11039155

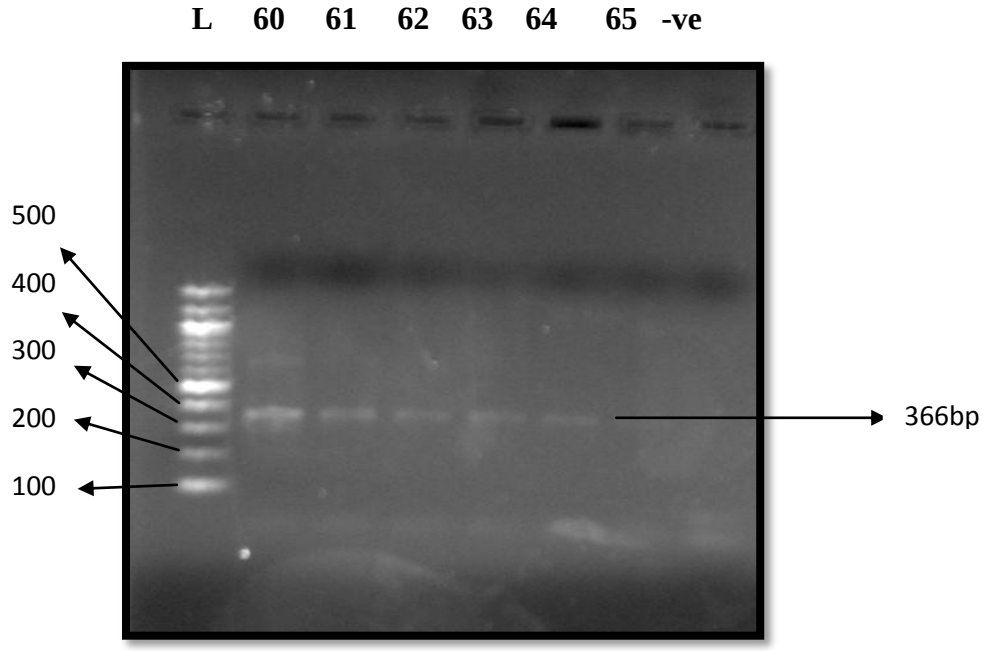
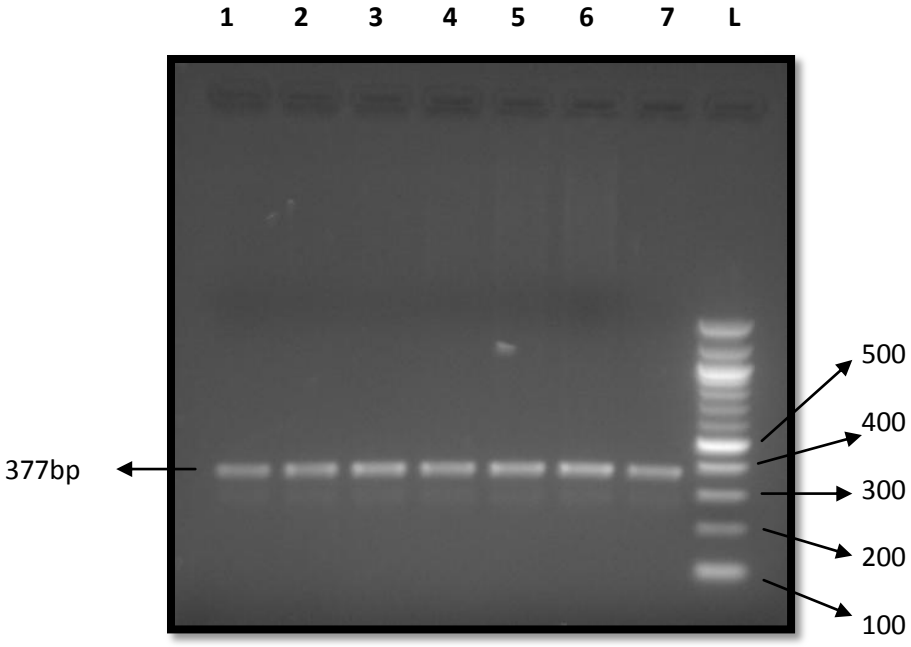
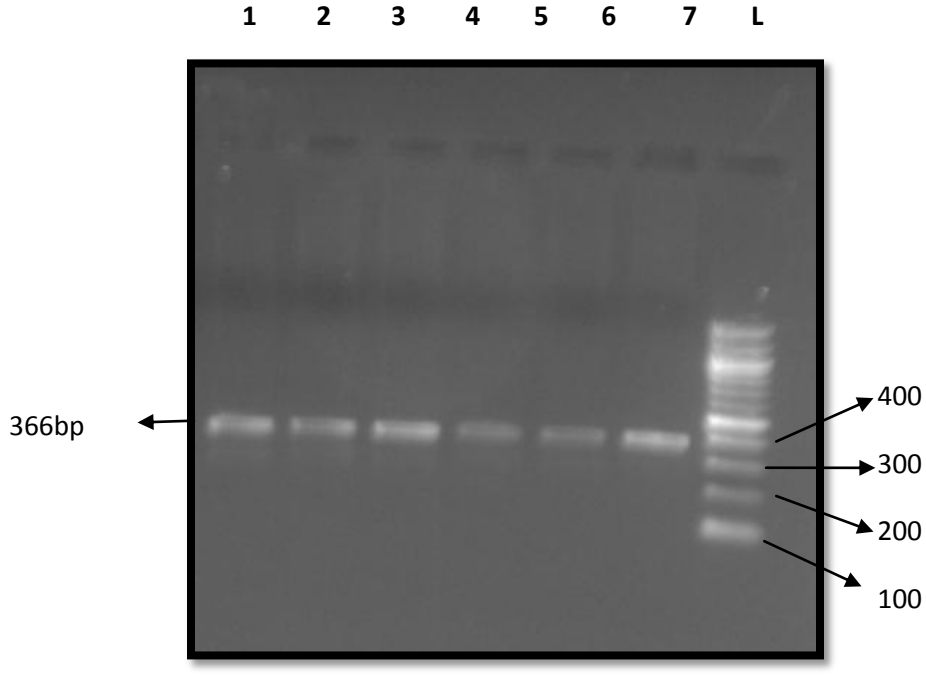


Fig 17 : AMPLIFIED PRODUCT

A) rs2279238



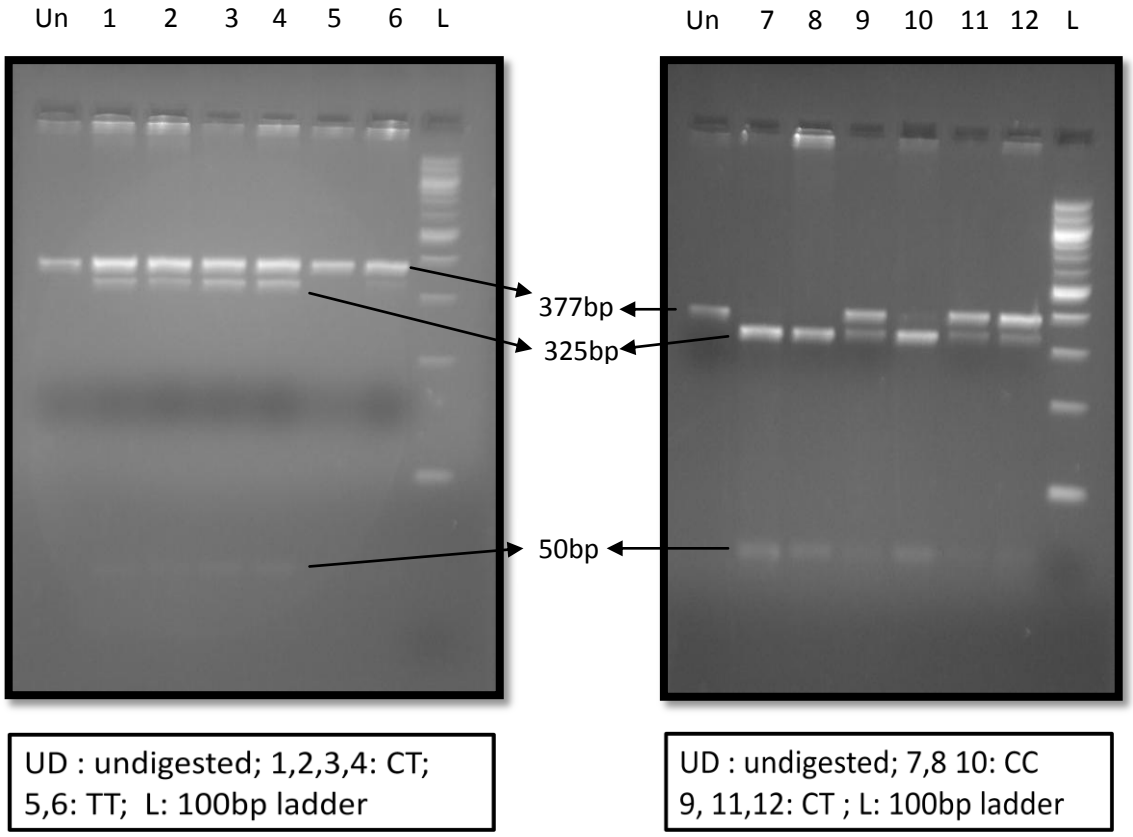
B) rs11039155



SAMPLE NO: 1-7; L: 100bp LADDER

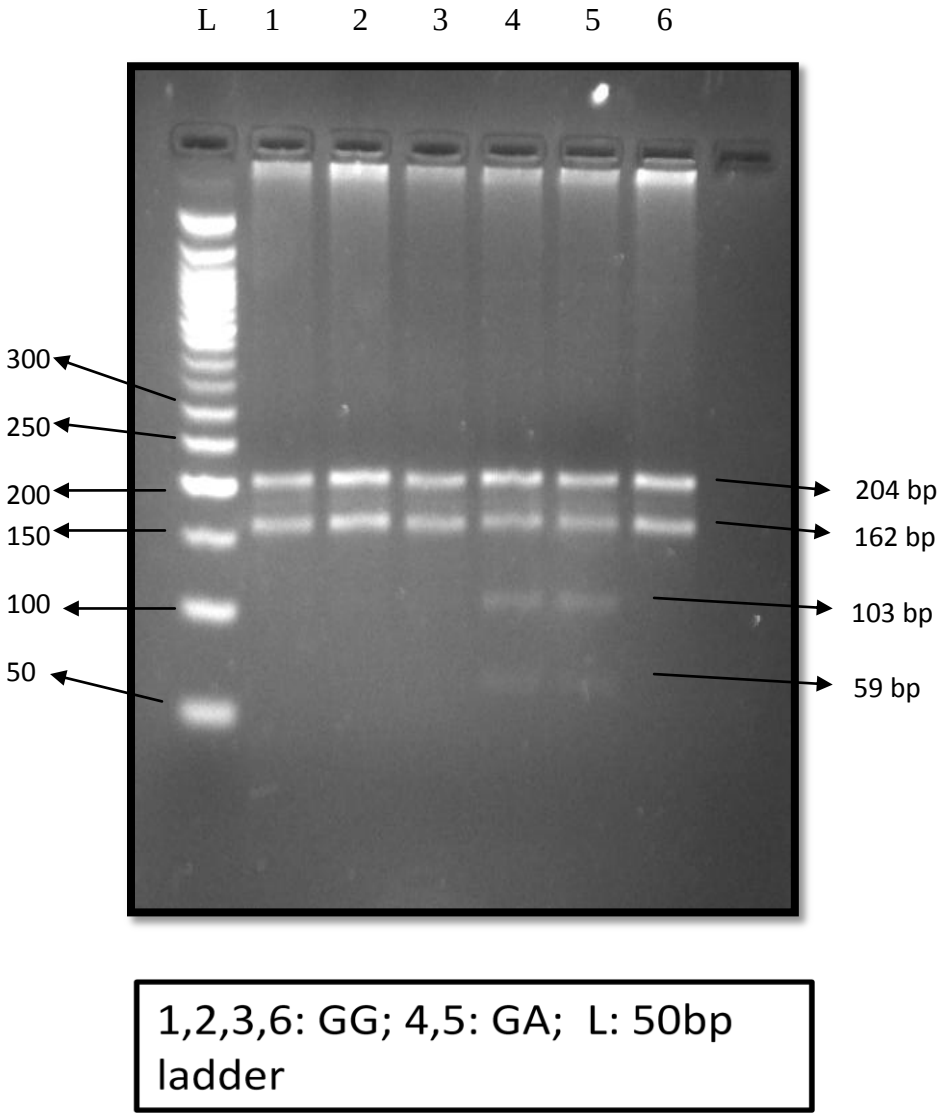
Fig 18 : RESTRICTION DIGESTION GEL IMAGE

A) rs2279238



Agarose Gel Electrophoresis image of digested DNA product by Fnu4HI on 3.5 % Gel

B)rs11039155



Agarose Gel Electrophoresis image of digested DNA product by Hpy188III on 3.5 % Gel

CHAPTER 6

DISCUSSION

DISCUSSION

Vitiligo is a complex genetic disorder with multiple genes and environmental triggers playing a role in inducing disease expression. Family based studies with vitiligo indicate that genetic factor is considered to be one of the determinants in vitiligo pathogenesis beside many other prevailing causes.(Sun, Xu et al. 2006) Gene playing a normal physiological role in healthy individual might have certain genetic variations in the genes that affect their stability, expression or regulation which might account for disease susceptibility in the patient.

Upregulated expression of LXR-alpha in the melanocytes from perileisional skin as compared to normal skin of vitiligo patient speculates its role in vitiligo pathogenesis.(Kumar, Parsad et al. 2010) In the present study, we investigated vitiligo patients for genetic variation in the LXR-alpha gene which may show association with vitiligo susceptibility. In our research, we focused on the association of SNP rs2279238 (+1257 C>T) and rs11039155 (-6 G>A) in LXR-α with the risk of vitiligo in north India population. The two SNPs studied here were chosen on the basis of previously identified genetic associations with other physiopathological conditions. We showed that the two genetic variants in LXR-alpha gene influence the susceptibility to vitiligo in an individual. The result showed that variant -6A and +1257 T allele were significantly associated with the risk of vitiligo. To the best of my knowledge, no previous report on association of LXR-alpha variant with vitiligo in any of population is present. This is the first report linking LXR-α SNP rs2279238 and rs11039155 with vitiligo in north India population.

−6 G>A polymorphism is located six base pairs upstream from the ATG site, according to Kozak it may affect the recognition of the AUG codon and translational efficiency.(Kozak 1987) Legry *et.al(Legry, Cottel et al. 2008),.* in their study, quantified LXR-alpha mRNA levels in human primary macrophages which showed that the rs11039155 SNP (−6G>A), located in the 5′ region of NR1H3, cannot modify LXR-alpha gene expression. Another possible explanation is that this SNP could be in linkage disequilibrium with another SNP affecting LXR-α function. Furthermore, according to Price *et.al.*(Price, Pacanowski et al. 2011), in-silico analyses suggested that rs2279238 is

located at an exon splicing enhancer (ESE) of NR1H3 where splicing factor SRp55 binds but the nucleotide variation is synonymous, resulting in a protein sequence identical to that of the wild type (Price et al., 2011). Various studies have demonstrated that synonymous polymorphisms can affect mRNA splicing, stability and translation efficiency (due to codon usage). However, the possibility of these gene variant on contribution to disease pathogenesis remains to be cleared.

LXR-alpha, ligand activated nuclear transcription factor, involved in the regulation of multiple physiological processes such as lipid biosynthesis, cholesterol homeostasis, immunity and inflammation. During the last decade, there have been several studies focusing on the association between LXR gene polymorphisms with many metabolic indicators and conditions, including circulating LDL and HDL-cholesterol concentrations(Robitaille, Houde et al. 2007; Legry, Cottel et al. 2008; Sabatti, Service et al. 2009; Legry, Bokor et al. 2011), type 2 diabetes mellitus(Dahlman, Nilsson et al. 2009; Ketterer, Mussig et al. 2011) and obesity(Dahlman, Nilsson et al. 2006). Legry *et.al.*(Legry, Cottel et al. 2008) reported that rs11039155 AA carriers had higher HDL cholesterol and a 30% decrease in risk of having the Metabolic Syndrome (major risk factor for type 2 diabetes mellitus and cardiovascular diseases) in two cohorts of French subjects. Similarly, further study by Legry *et.al.*(Legry, Bokor et al. 2011) reported that -6 A carrier had higher HDL-C in adolescent. In another population-based study, Dahlman *et.al.*(Dahlman, Nilsson et al. 2006) reported that rs2279238 CT carriers were associated with obesity phenotypes. Another study by Dahlman et.al.(Dahlman, Nilsson et al. 2009) reported genetic variation in *LXR*-alpha is unlikely to affect the risk of developing Type 2 Diabetes mellitus or quantitative phenotypes related to glucose homeostasis in French population.

Vitiligo is found to be associated with obesity (Mracek, Ding et al. 2010; Bagherani 2012), type 2 diabetes mellitus(Afkhami-Ardekani, Ghadiri-Anari et al. 2014) and several other diseases. High cholesterol and oxysterol content is one of the characteristics found in the vitiligo human melanocyte which further act as a trigger for LXR-alpha activation.(Bellei, Pitisci et al. 2013)

Looking at the allelic frequency of LXR-alpha -6G>A and +1257 C>T in different control population, we can see that variant -6A and +1257 T is highly frequent in French population and Chinese han population respectively (Table 12). On the other hand -6A and +1257T has a low frequency in North India and French population.

Table 12: Frequency of LXR-alpha polymorphism in different control population compared to North India population

Population	Allele frequency of mutated allele(%)
rs11039155	
North India(Present study)	10
Iran(Rooki, Ghayour-Mobarhan et al. 2013)	17.3
Germany(Knebel, Janssen et al. 2012)	23
French (Legry, Cottel et al. 2008; Legry, Bokor et al. 2011)	27.6 - 28.5
rs2279238	
North India(Present Study)	40
Swedish(Dahlman, Nilsson et al. 2009)	16
French(Dahlman, Nilsson et al. 2009)	12
Iran(Rooki, Ghayour-Mobarhan et al. 2013)	25.1
China(Han, Liang et al. 2014)	62.6
Germany(Ketterer, Mussig et al. 2011; Knebel, Janssen et al. 2012)	26 - 32.1

To confirm the association of -6A and +1257 T with vitiligo susceptibility requires the large cohort study which could improve the statistical significance of our finding. As vitiligo is a complex and multifactorial disease, both gene–gene and gene–environment interactions may occur, and a single genetic variant is unlikely to be sufficient to predict overall risk. Therefore, further research is needed to elucidate the role of other functional

SNPs of LXR-α and other related genes involved in similar biological pathways that may be involved in the aetiology of vitiligo.

The present study had a number of limitations. First, sample size was very small to come into any conclusion, which makes it necessary to confirm our findings in large scale studies. Small sample size will contribute to low statistical power. Secondly, Lack of knowledge about the functional influence of the identified polymorphisms with vitiligo which remains to be determined.

CHAPTER 7

CONCLUSION & FUTURE PROSPECTS

CONCLUSION & FUTURE PROSPECTS

The present study investigated the association of LXR-alpha gene variation with vitiligo susceptibility and our results showed that variant -6 A and +1257 T allele were significantly associated with the risk of vitiligo.

Identification of novel genes that are associated with vitiligo susceptibility would be a great benefit and can act as one of the prognostic marker that will help identifying individual at the risk of vitiligo at an earlier stage of disease. Furthermore, this would help to determine appropriate therapeutic and prophylactic approaches. Moreover, as this nuclear receptor is an inducible transcription factor, an understanding of its role in vitiligo could guide the development of new ligands for the treatment of vitiligo.

This piece of work provides support for additional prospective studies to confirm the contribution of LXR-alpha gene variants in vitiligo.

Larger population-based and in-depth molecular studies are required to validate our current findings and to elucidate the functional roles of the rs2279238 and rs11039155 polymorphism in the aetiology of vitiligo.

Moreover, as vitiligo is a complex and multifactorial disease, both gene–gene and gene–environment interactions may occur, and a single genetic variant is unlikely to be sufficient to predict overall risk. Therefore, further research is needed to elucidate the role of other functional SNPs of LXR-α and other related genes involved in similar biological pathways that may be involved in the aetiology of vitiligo.

APPENDIX

1. GLASSWARES AND INSTRUMENTS

Glasswares

- Beaker – 1000 ml, 500ml, 100ml
- Eppendorfs (autoclaved)- 2ml, 1.5ml, 0.5 ml and 0.2 ml
- Autoclaved Microtips (100-1000µl, 20-200µl, 0.1-10µl)
- PCR Tube stand
- Capped Bottles
- Measuring cylinder – 500 ml, 100ml, 10 ml
- Eppendorfs stand

Instruments

- PCR Tube stand
- Micro pipette
- Thermo-Cycler
- Laminar Air Flow
- Autoclave
- Incubator
- Hot air Oven
- pH meter
- MilliQ Water unit
- Microwave Oven
- Rocker
- Spinner
- Weighing balance
- Refrigerator (-80^{o} C, -20^{o} C, 4 ^{o} C)
- Nanodrop Spectrophotometer
- Vortex
- Agarose Gel Electrophoresis chamber
- UV transilluminator
- Centrifuge
- Water Bath

2. REAGENTS

2.1 Di-sodium ethylene diamine tetra acetate, Na_2EDTA (0.5 M, pH 8.0)

- Take 186.1g of Na_2 EDTA and add to it 800ml of MilliQ Water.
- Stir Vigorously on Magnetic stirrer.
- Adjust pH to 8.0 with 10M NaOH.
- Make up the volume to 1000ml.

2.2 Tris (hydroxymethyl) aminomethane-chloride, Tris-Cl (1M, pH 8.0)

- Take 121.2g Tris Base in 800ml of distilled water.
- Adjust the pH to 8 by adding 1N HCl.
- Make up the volume to 1000ml with MQ water.

Solution was filtered through Whatmann filter paper and stored in a sterile tight screw capped reagent bottle

2.3 Tris –Cl (1 M; pH 7.3)

- Take 121.2g Tris Base in 800ml of distilled water
- Adjust the pH to 7.3 by adding 1N HCl.
- Make up the volume to 100ml with MQ water.

Solution was filtered through Whatmann filter paper and stored in a sterile tight screw capped reagent bottle

2.4 Ammonium Chloride, NH_4Cl (1 M)

- 53.5 g of ammonium chloride dissolved in 800ml MQ water.
- Make up the volume to 1000ml with MQ water.

2.5 10% SDS

- Dissolve 10g SDS in 70ml of distilled water.
- Heat to 68^{o}C to mix the solution.
- Make up the volume to 100ml with MQ water.

2.6 Red Blood Cell Lysis Buffer

Composition: Tris 10mM, pH-8.0; EDTA 1mM; NH_4Cl 125 mM, pH 8.0

EDTA (0.5M)	2ml
Tris (1M , pH-8.0)	10ml
NH_4Cl (1M)	125 ml

Mixed the above reagent in MQ water to obtain the final volume of 1000 ml RBC lysis buffer.

2.7 Tris-EDTA (TE) buffer (pH 8.0)

Composition: Tris 10mM; EDTA 1mM, pH 8.0

EDTA (0.5M)	2ml
Tris (1M , pH-8.0)	10ml

Mixed the above reagent in MQ water to obtain the final volume of 1000 ml RBC lysis buffer.

2.8 Tris-EDTA (TE) buffer (pH 7.3)

Composition: Tris 10mM; EDTA 1mM, pH 7.3

EDTA (0.5 M)	2ml
Tris (1M, pH 7.3)	10ml

Mixed the above reagent in MQ water to obtain the final volume of 1000 ml RBC lysis buffer.

2.9 Ammonium Acetate (7.5 M)

- Dissolved 28.9g ammonium acetate salt in 20ml of MQ water
- Tthe final volume was adjusted to 50 ml.

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Brief Biodata of Student

Title of Thesis **:** LIVER X RECEPTOR POLYMORPHISM IN GENETIC SUSCEPTIBILITY TO VITILIGO

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Degree of Award **:** Master of Technology in Biotechnology

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