Role of *Tyrosinase* (*TYR*) gene variants (rs28940876 and rs61754379) in genetic susceptibility to Vitiligo

Project report submitted in partial fulfillment of the requirement for the Degree of Bachelor of Technology

in

Biotechnology

Submitted by

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TABLE OF CONTENTS

CERTIFICATE

This is to certify that the work titled "**Role of *TYR* gene variants (rs28940876 and rs61754379) in genetic susceptibility to Vitiligo**", submitted by **Pallavi Raj Sharma** and **Tulika** in partial fulfilment for the award of degree of **Bachelor 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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ACKNOWLEDGEMENT

We express our sincere gratitude and indebtedness to our guide Dr. Harish Changotra for his exemplary guidance, valuable feedback and constant encouragement throughout the duration of the project. We are highly grateful to him for providing us the opportunity to perform research in his esteemed lab.

We are highly thankful to Mr.Rohit Randhawa, Ms.Avni Vij, Mrs.Ambika Sharma and Mr.Sanjay Singh for their constant support and motivation during our project work for their continuous guidance, inspiration, presence and blessings.

Special thanks to our dear friends Mr. Ankit Sharma and Mr. Sachin Sharma for their wonderful company and support. The moments spent with you both will always be cherished.

We are grateful to our parents for their faith in us and their constant support.

SUMMARY

Vitiligo is a pigmentary disorder characterized by areas of depigmented skin due to loss of epidermal melanocytes. It affects people from all races and gender equally and has a genetic component too which contributes to its inheritance. The pathogenesis of vitiligo is still not clear and it was concluded that multiple factors are involved in the occurrence of this disease. The present project is focused on one gene, *TYR*, and its role in susceptibility towards vitiligo.

TYR gene encodes the protein tyrosinase which first enzyme catalyzing the process of melanogenesis, and in the absence of tyrosinase enzyme, melanin will not be produced, hence resulting in vitiligo. Mutations in the *TYR* gene can contribute to the formation of an inactive enzyme. In this project, two SNPs – rs28940876 and rs28940876 were studied and investigated to find any association with disease susceptibility.

DNA was isolated from 108 diseased blood samples and 92 healthy control samples, and genotyping was performed using PCR-RFLP. The alleles were recognized by interpreting the band patterns by visualization on agarose gel electrophoresis. The allele frequencies of these two SNPs between vitiligo and control were, **rs28940876**: p=0.4398, Odds Ratio= 0.28, class interval= 0.02- 59.43 and **rs61754379**: p=0.9363, Odds Ratio= 1.17, class interval= 0.01- 6.97, which suggested no association of minor allele of both SNPs with vitiligo. Thus, no significant results were found through this study. Probably genotyping more number of samples would be required to validate the obtained results. Although insignificant with the casual risk of vitiligo, the study could be utilized further to understand the etiological factors of this disease in different population sets.

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LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

µl	Microliter
ml	Milliliter
g	Gram
mg	Microgram
l	Liter
M	Molar
min	Minute
s	Seconds
SDS	Sodium Dodecyl Sulphate
RFLP	Restriction Fragment Length Polymorphism
nsSNP	Non-Synonymous Single Nucleotide Polymorphism
dbSNP	Single Nucleotide Polymorphism Database
EtBr	Ethidium Bromide
EDTA	Ethylene Diamine Tetra Acetic acid
^{o}C	Degree Celsius
bp	Base Pair
CI	Confidence Interval
DNA	Deoxyribonucleic Acid
rpm	Rotations Per Minute
TAE	Tris Acetate EDTA buffer
TE	Tris EDTA buffer
UV	Ultra-Violet
V	Volts

CHAPTER 1: INTRODUCTION

An acquired disease, vitiligo is a disorder distinguished by depigmentation mostly of skin, skin and mucous membrane. Its etiology of is still not totally known. However, recent studies advocate that there is a systematic damage to melanocytes (cells responsible for producing melanin), especially in the mucous membranes, eyes and the membranes inside the ear. Other reports suggested an association between vitiligo and ocular manifestation, hearing loss and autoimmune diseases. Hearing loss is one of the most usual symptoms associated with vitiligo prevalent in 4% to 20% patients [1, 2].

The term vitiligo is derived from Latin and was first used by Celsus in his *De Medicane*[3]. In nineteenth century the clinical features of vitiligo were described Brocq and Kapose. Kaposi further depicted that in the basal layer cells of epidermis of the area of the skin affected by vitiligo showed absence of pigment granules.[4]

Vitiligo is a common pigmentation disorder with India showing the highest occurrence of 8.8% in the world[5]. Although its global incidence is 1%[6]. Both genders are influenced equally, irrespective of their age. However, in 70%-80% of the cases, the disease develops before the age of 30[7]. Additionally, it has also been observed that the onset of vitiligo before the age of 12 years is common.

Individuals suffering from vitiligo especially females face severe psychological and social problems. They feel distressed and stigmatized by their condition. Thus, the disease influences the quality of life of the patients quite gravely.

June 25 is celebrated as the World's Vitiligo Day. It is an initiative aimed to build global awareness about vitiligo.

Although the etiology of this condition is still elusive, a number of theories have been developed to explain it- autoimmune theory, genetic basis theory, autodestruct theory and neural theory. Autoimmunity theory is the leading one[8].

Researchers from all over the world have and are still working to find susceptibility genes involved in vitiligo and other autoimmune diseases and additional genes that may mediate the disease itself. Also several research institutions are intensively and extensively working to better understand the disease, improve its classification, epidemiology, diagnosis, prognosis and treatment.

Our project is an endeavor towards new findings regarding vitiligo pathogenesis. Focusing particularly on the genetics of the disease, we are looking into the presence of the non-synonymous single nucleotide polymorphism in *Tyrosinase (TYR)* gene as well as their association with vitiligo susceptibility.

The gene under study codes for the enzyme tyrosinase. It is the key enzyme, catalyzing the very first and the rate limiting step of melanogenesis – the melanin synthesis process taking place inside the melanocytes. Genetic studies so far have investigated association between gene polymorphism and vitiligo.

To study polymorphism in a bigger sample population, it is required to sort out the possible functional nsSNP first. dbSNP database along with other computational tools facilitate to carry out the profiling of nsSNPs in the *TYR* gene.

Significance of the study

Change in a single nucleotide in the DNA sequence may affect how human develops disease, respond to drugs etc. Identification of SNP in a gene associated with vitiligo can act as a susceptibility marker. Associated nsSNPs can be used as informative marker to trace the inheritance pattern of the genes involved within the families. It 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, *TYR* is involved in the process of melanogenesis, association of any *TYR* nsSNP with vitiligo will help us to better understand the disease and its cause.

The main objective of this study is to elucidate any association between nsSNPs (rs28940876 and rs61754379) in *TYR* gene and vitiligo susceptibility with the help of computational tools of analysis and later the experimental procedures to validate the above.

CHAPTER 2: REVIEW OF LITERATURE

2.1 STRUCTURE AND PHYSIOLOGY OF SKIN

Skin is the largest organ of or our body. An adult carries approximately 3.6 kgs of skin. The thickness of the skin varies across the body depending on the fact that how much we use the specified area of the skin.

Functions of Skin

Skin is a multifunctional organ. It acts as a protective layering functioning as a waterproof, insulating shield, guarding the body against extremes of temperature and harmful chemicals. It also secretes antibacterial substances that protect us from infection. It is also responsible for synthesis of Vitamin D for converting calcium into healthy bones. Besides this, it functions as a sensor to connect the brain with the outside world.

Skin is also involved in thermoregulation i.e. it helps us maintain the temperature of our body. When the body temperatures rises making us hot, there is vasodilation at the skin surface at the skin surface. This allows more heat to escape, thus allowing the body to cool down. On the other hand when we are cold, there is constriction of the blood vessels, so that now less heat can escape.

Structure of Skin

Skin is made up of three layers – epidermis, dermis and a fatty subcutaneous layer, the panniculus adiposus.

Epidermis

Epidermis, the outer most layer of the skin, comprises of no blood vessels. It relies on its underlying layer, dermis for acquiring nutrients and waste disposal through diffusion through the dermoepidermal junction.

Epidermis primarily is colonized by cells called keratinocytes, made from tough proteins-keratins. Epidermis also harbors other cells such as melanocytes responsible for producing

melanin which provide skin its distinctive colour and Langerhans cells which are antigen presenting cells.

There are five layers of skin namely-

(i) Stratum corneum: It is the outermost layer of epidermis. It consists of tightly packed cells which make skin tough, waterproof and prevent invasion of foreign matter.

(ii) Stratum lucidum: This layer consists of several dead cells. It is thick layer and is found in thickened skin, including soles of the feet.

(iii) Stratum granulosum: It is in this layer that keratin is formed which is important for providing strength to the skin.

(iv) Stratum spinosum: This layer contains cells that change shape from columnar to polygonal. Keratin is also produced here.

(v) Stratum basale: This layer is the deepest layer of the epidermis, in which many cells are active and dividing. The basement membrane, composed of collagen and proteins, segregates the stratum basale from the dermis.

Dermis

The dermis is a thick layer composed up of strong connective tissues. It is further classified into two levels –the *papillary region*, which is made of loose connective tissue and the lower layer is made of tissue that is more closely packed is called the *reticular layer*.

The dermis is made up of a matrix of collagen, elastin and network of capillaries and nerves. Collagen contributes to the strength, the elastin maintains its elasticity and the capillary network provides nutrients to the different layers of the skin. Numerous specialised cells and structures are additionally present in the dermis which includes hair follicles, sweat glands, sebaceous glands (produce sebum which helps lubricate skin & hair) and nails.

SKIN COLOUR

Melanin is responsible for the color of our skin. It is a pigment synthesized by melanocytes in the epidermis. Melanin plays a protective role, by shielding us from the harmful effects of ultraviolet (UV) rays present in the sunlight and to which are regularly exposed to. A higher amount of melanin particles is found in dark-skinned people.

2.2 MELANOGENESIS

Melanogenesis is the process of production of melanin and distribution. This process takes place inside epidermal units consisting of melanocytes surrounded by keratinocytes. Melanin is the pigment which primarily determines the colour of our skin, hair and eyes. Besides this, melanin is also involved in protecting us from the harmful effects of UV rays using its ability to absorb ultraviolet radiation [9]. UV absorbed by melanin is transformed into heat- a less toxic form of energy[10].

Melanocytes originate in neural crest as melanoblasts and then move towards different destinations including basal layer of epidermis and hair follicles[11, 12].

Melanocytes contain organelles called melanosomes- lysosome related organelles (LROs) are the site of synthesis, storage and transport of melanin. They are synthesized in mammalian skin melanocytes, in choroidal melanocytes and retinal pigment epithelial (RPE) cells in the eye, and in melanophores (a class of pigment-containing cells) in lower vertebrates. The function of melanosomes depend on the on the cell type. For example, epidermal melanocytes supply neighboring keratinocytes with melanosomes which result in the pigmentation of skin and hair where as in the case of RPE cells they are contained inside the cells[13, 14].

The key proteins involved in skin pigmentation and melanogenic enzymes are localized in melanosomes. After the completion of synthesis of melanin, melanosomes move towards the melanocyte dendrites in a movement controlled by microtubule proteins [15].

In the epidermis, each melanocyte interacts through dendrites with 30-40 keratinocytes, allowing transfer of melanosomes to the cytoplasm of keratinocytes positioned strategically over the nuclei.

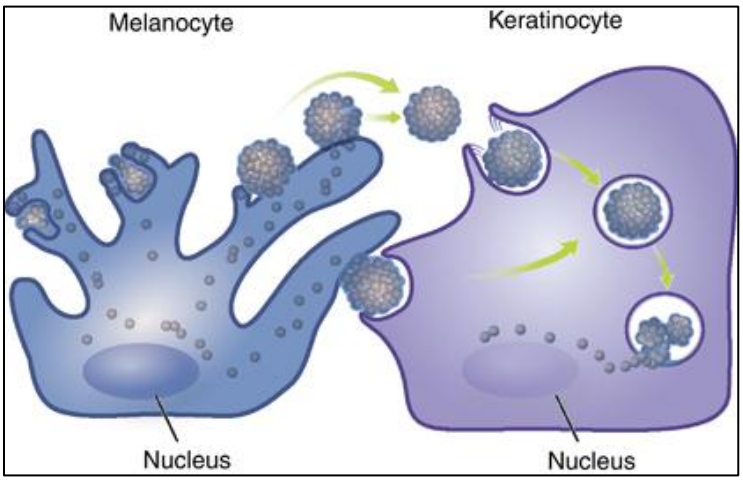

Fig 1: Diagram depicting transfer of melanosomes from a melanocyte through its dendrites to one its neighboring keritinocytes. Melanosomes are packed in globules, released into the extracellular space, phagocytized by keratinocytes and distributed around nucleus. [16]

The melanocyte number is relatively same in different ethnic groups. The phenotypic diversity of pigmentation is because of the size and number of melanosomes, the type and amount of melanin and melanin transfer and distribution in keratinocytes [17]. People with dark skin have longer, more numerous and elongated melanosomes, resulting in delayed degradation in keratinocytes and consequently in increased visible pigmentation. These physical differences in melanosomes are not affected by external factors such as UVR rather are present at birth of the individual.

There are two types of melanin- eumelanin and pheomelanin. Eumelanin is brown-black colour (dark insoluble polymer) and pheomelanin gives gives red-yellow colour (soluble polymer former by the conjugation of cysteine and glutathione)[18]. Eumelanin is the major type in individuals with dark skin and hair and is more efficient in photoprotection whereas pheomelanin is mostly found in individuals with red hair.

The process of melanogenesis is initiated by with oxidation of L-tyrosine to dopaquinone (DQ) by the enzyme tyrosinase (TYR). This step of formation of DQ is the rate limiting step because the rest of the steps of melanogenesis can proceed spontaneously as physiological pH[19]. DQ

serves as the substrate for the synthesis of eumelanin and pheomelanin[20]. DQ is highly reactive and in the absence of thiol compounds, it undergoes intramolecular cyclization to produce indole leukodopachrome. The redox exchange between leukodopachrome and DQ give rise to dopachrome and L-3,4 dihydroxyphenylalanine (L-DOPA), which is also a substrate for TYR and oxidized to DQ again by the enzyme. Dopachrome gradually decomposes to give dihydroxyindole (DHI) and dihydroxyindole-2-carboxylicacid (DHICA). The later process is catalyzed by TRP-2. Finally both DHI and DHICA are oxidized to eumelanin. TRP-1 is believed to catalyze the oxidation of DHICA to eumelanin.

At the same time, if a thio group containing compound is present – cysteine and glutathione DQ is converted to 5-S-cysteinyldopa or glutathione in the presence of cysteine and glutathione respectively. Subsequent oxidation gives benzothiazine intermediates and finally to produce pheomelanin.

The process of melanogenesis involves three enzymes namely TYR, TRP-1 and TRP-2. However it is TYR which is the most important of them all for melanogenesis [21].

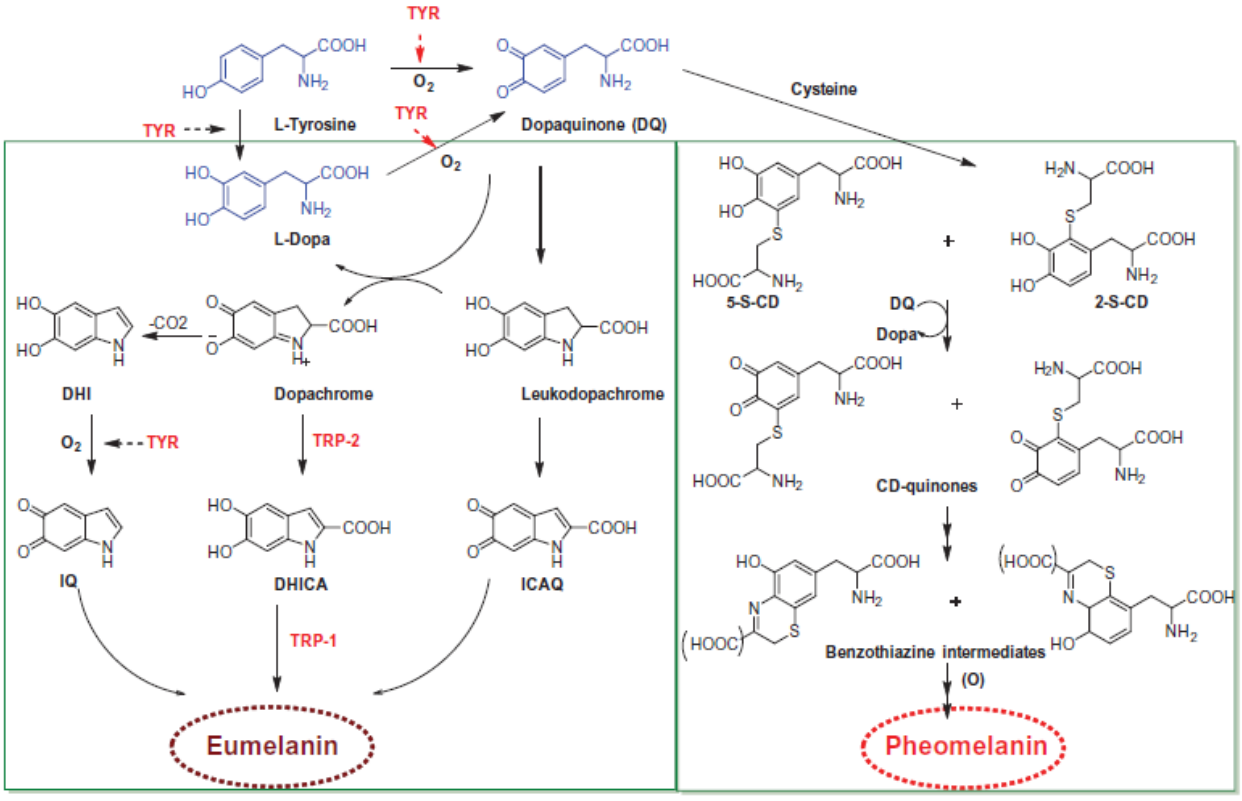

Fig 2: Melanaogenesis pathway- production of eumelanin and pheomelanin. Adapted from[21].

2.3 VITILIGO

Vitiligo is a pigmentation disorder which is characterized by the presence of white patches on skin due to the chronic and progressive loss of melanocytes from the cutaneous epidermis. With a worldwide incidence of 0.5–2%, the disease begins before the age of 20 in half the cases [22-24]. Vitiligo susceptibility is not dependent on race, gender, or age, but 6 – 38% of patients have family members with the disease which suggests it might be genetically inherited. However, the inheritance pattern of the disorder is not a simple Mendelian mechanism, it is consistent with that of a polygenic trait.

Even though it is not a life threatening condition, one should not be oblivious of its impact on an individual's psychological well-being and social interactions [25, 26]. Patients often have low self-esteem and confidence, which results in chronic depression in most cases.

Vitiligo is a multi-factorial complex disease and its etiology still remains elusive, however, there have speculations about it. Scientists have agreed on a "Convergence Theory", which states that there is no one cause for occurrence of this disorder and it is a resultant of a combination of various factors. These factors may include infections, stress, neural abnormalities, melatonin receptor dysfunction, impaired melanocyte migration and genetic susceptibility[27].

CLASSIFICATION

Depending on the distribution of white patches on the body vitiligo has been classified broadly as generalized, localized and universal type vitiligo. Generalized vitiligo is subdivided into acrofacial, vulgaris and mixed subtypes and localized is subdivided into focal, segmental and mucosal subtypes[28].

1. Generalized vitiligo

- Acrofacial - Patches observed on distal extremities and face
- Vulgaris – Patches are scattered that are widely distributed
- Mixed – Patches are of both kinds (acrofacial and vulgaris)

2. Localized vitiligo

- Focal – One or more patches in an area
- Segmental – One or more macules present in an unilateral segment of the body
- Mucosal – Mucous membranes are affected

3. Universal vitiligo – Complete or nearly complete depigmentation of the body

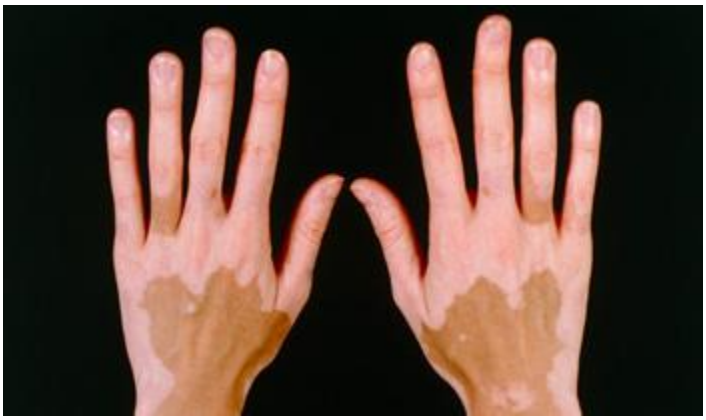

Fig 3: Non-segmental vitiligo

EPIDEMIOLOGY

The worldwide prevalence of vitiligo ranges from 0.4 to 2.0%. Most of the studies conducted on vitiligo suggest slightly higher prevalence in females and that in 50% of the cases the onset of the disease occurs in childhood. However exceptions to these general observations exist. Childhood vitiligo has been correlated to halo nevi, atopic diathesis, and family history of vitiligo and autoimmunity whereas postpubescent vitiligo has been linked to acrofacial disease and thyroid disease.

It has also been observed that the disease reduces as we move to regions away from the equator[29].

2.4 ETIOLOGY OF VITILIGO

The exact causes behind the occurrence of vitiligo are still unclear, although it is suggested to be a complex idiopathic disease which involves several factors. Researchers have come up with a lot of theories which might explain the reason why vitiligo occurs, but have concluded that the pathogenesis of vitiligo is a result of all the possible factors, there is no one cause leading to this effect, hence it is called the 'Convergence Theory' of pathogenesis. The involved causative theories have been explained individually in this section [30].

1. Genetic Theory
2. Neural Theory
3. Autoimmune Theory
4. Reactive Oxygen Species Model
5. Melanocytorrhagy Hypothesis

GENETIC THEORY

Vitiligo has been attributed to multiple genes and might follow a multifactorial inheritance pattern[31]. In most cases, it shows 50% inheritance[32]. Genome-wide association studies, candidate gene studies, and linkage analysis have been used to identify key genes which might participate in contributing towards the occurrence of vitiligo. Genes such as HLA, PTPN22, NALP1, and XBP1 have shown a greater susceptibility towards the condition [33]. In recent studies, around 30 susceptibility loci have been identified with definitive relation to vitiligo and usually correspond to pathways involved in immune system or destruction of melanocytes.

Genome-wide linkage studies and candidate gene studies among different population groups have shown that different genes are responsible for depigmentation in various ethnic populations and also most of these genes participate in the pathogenesis of other autoimmune conditions.

Different patterns of vitiligo also consist of different participating genes. The major genes have been listed in Table 1[33-40].

Table 1: Major genes associated with vitiligo

Type	CHR	Gene	SNPs#	Context	*p*	OR (95% CI)	Population
HLA-related	6p21.32	*HLA*	rs7758128	UTR-5	8×10^{-11}	NR	European ancestry
	6p21.32	*HLA*	rs3806156	Intron	7×10^{-19}	1.42 (1.32–1.54)	European ancestry
	6p21.33	*HLA*	rs11966200	Intron	1×10^{-48}	1.9 (1.74–2.07)	Chinese
	6p21.33	*HLA*	rs9468925	UTR-5	2×10^{-33}	1.35 (1.28–1.41)	Chinese
	6p22.1	*HLA*	rs6904029	ncRNA	1×10^{-21}	1.49 (1.37–1.61)	European ancestry
Melanin-related	11q14.3	*TYR*	rs1393350	Intron	2×10^{-18}	1.53 (1.39–1.68)	European ancestry
	11q21	*TYR*	rs4409785	UTR-3	2×10^{-13}	1.34 (NR)	European ancestry
	12q13.2	*PMEL-IKZF4*	rs10876864	UTR-3	8×10^{-12}	1.18 (1.13–1.24)	Chinese
	12q13.2	*IKZF4*	rs2456973	Intron	3×10^{-14}	1.29 (NR)	European ancestry
	15q13.1	*OCA2*	rs1129038	UTR-3	4×10^{-8}	1.22 (NR)	European ancestry
Immune-related	2q24.2	*IFIH1*	rs2111485	UTR-5	5×10^{-15}	1.3 (NR)	European ancestry
	3p13	*FOXP1*	rs17008723		1×10^{-8}	1.33 (NR)	European ancestry
	3q13.33	*CD80*	rs59374417	UTR-5	4×10^{-10}	1.34 (NR)	European ancestry
	3q28	*LPP*	rs9851967	Intron	9×10^{-8}	1.14 (1.09–1.19)	Chinese
	3q28	*LPP*	rs1464510	Intron	1×10^{-11}	1.31 (1.21–1.41)	European ancestry
	6q15	*BACH2*	rs3757247	Intron	3×10^{-8}	1.2 (NR)	European ancestry
	6q27	*RNASET2-FGFR1OP*	rs2236313	Intron	1×10^{-16}	1.2 (1.15–1.25)	Chinese
	8q24.22	*TG-SLA*	rs853308	Intron	2×10^{-8}	1.2 (NR)	European ancestry
	10p15.1	*IL2RA-RBM17-PFKB3*	rs706779	Intron	3×10^{-9}	1.27 (1.17–1.37)	European ancestry
	10q25.3	*CASP7*	rs3814231	Intron	4×10^{-8}	1.23 (NR)	European ancestry
	11p13	*CD44*	rs10768122	UTR-3	2×10^{-9}	1.22 (NR)	European ancestry
	11q23.3	*CXCR5-DDX6*	rs638893	UTR-5	2×10^{-9}	1.22 (1.14–1.30)	Chinese
	12q24.12	*SH2B3*	rs4766578	Intron	4×10^{-18}	1.32 (NR)	European ancestry
	14q12	*GZMB*	rs8192917	Missense	3×10^{-8}	1.28 (1.17–1.39)	European ancestry
	21q22.3	*UBASH3A*	rs11203203	Intron	1×10^{-9}	1.27 (1.18–1.38)	European ancestry
	22q12.3	*C1QTNF6*	rs229527	Missense	2×10^{-16}	1.38 (1.28–1.50)	European ancestry
	22q13.2	*TOB2*	rs4822024		7×10^{-10}	1.28 (NR)	European ancestry
	1p13.2	*PTPN22*	rs2476601		1×10^{-7}	1.39 (1.23–1.57)	European ancestry
	6q27	*CCR6*	rs6902119	UTR-5	4×10^{-7}	1.23 (NR)	European ancestry
Others	1p36.23	*RERE*	rs4908760	Intron	7×10^{-15}	1.36 (1.26–1.48)	European ancestry
	2p16.3	*KCNK12*	rs6544997	UTR-5	7×10^{-6}	NR	Romanian
	4p16.1	*CLNK*	rs16872571	UTR-5	2×10^{-8}	1.21 (NR)	European ancestry
	6q27	*SMOC2*	rs13208776	Intron	9×10^{-8}	NR	Romanian
	10q22.1	*SLC29A3-CDH23*	rs1417210	UTR-3	2×10^{-8}	1.14 (1.09–1.19)	Chinese
	10q22.3	*ZMIZ1*	rs11593576	Intron	8×10^{-7}	1.14 (1.09–1.20)	Chinese
	16q12.2	*KIAA1005*	rs3213758	Missense	6×10^{-11}	2.77 (2.04–3.76)	Korean
	16q24.3	*MC1R*	rs9926296	Intron	2×10^{-13}	1.27 (NR)	European ancestry
	18q21.31	*ATP8B1*	rs10503019	Intron	3×10^{-6}	NR	Romanian
	19p13.3	*TICAM1*	rs6510827	Intron	9×10^{-8}	1.19 (NR)	European ancestry
	21q22.11	*HUNK*	rs2833607	UTR-3	2×10^{-6}	NR	Romanian

CI = confidence interval; CHR = chromosome regions; GV = generalized vitiligo; GWAS = genome-wide association study; NR = not reported; OR = odds ratio.

A study by Majumder et al (1988) demonstrated that recessive alleles at multiple unlinked loci could be involved in the genetic pathogenesis of vitiligo [41]. This hypothesis was further confirmed by Nath 1994, where it was found that a patient with vitiligo had at least one first-degree relative also with vitiligo. In many other studies, a family history of vitiligo was noticed.

Apart from the genes mentioned in the table, there have been pieces of evidence of the participation of other genes involved in other pathways which are highly expressed in vitiligo patients. Autoimmune diseases are often noticed along with vitiligo, the susceptibility of which can be determined by several gene loci associated with the autoimmune disease such as autoimmune susceptibility (AIS)-1, -2 (chromosome 7) and systemic lupus erythematosus vitiligo-related gene (SLEV1) (chromosome 17)[42].

FOXP3 gene was found be associated with X-linked recessive multiple autoimmune disease syndrome. Moreover, CTLA4 and AIS3 locus have also depicted slight association[43].

Transforming growth factor beta-receptor II (TGFBR2) inhibits the inflammatory pathways and activates lymphocytes targeted for melanocyte destruction [44].

Autophagy in melanocytes is carried out by the ultraviolet radiation resistance-associated gene (UVRAG) which resists photo-damage. SNPs present in this gene positively contribute to the occurrence of vitiligo[45].

NEURAL THEORY

Given by Lerner in 1959 [46] the neural theory explains that the occurrence of vitiligo can be contributed to emotional stress with the sympathetic nervous system playing a key role in pathogenesis. A dysfunction in sympathetic nervous system's activity results in changes in melanin synthesis which in turn leads to depigmentation. In addition to that, expression of specific neuropeptides and neuronal markers such as Neuropeptide Y (NPY), calcitonin gene-related peptide (CGRP), vasoactive intestinal polypeptide (VIP), and polyclonal general neuronal marker (PGP) were higher in patients with vitiligo [47]. Stress is known to induce high expression of these neuropeptides. A major population of vitiligo patients reported that their mental stress was primarily stemmed from personal and financial dissatisfaction, and hence it was stipulated that a stressful life can trigger the onset of vitiligo. Stressors lead to the discharge of catecholamines (such as melatonin) which show binding with receptors present in the skin arteriolar wall, ultimately resulting in vasoconstriction, hypoxia, and overproduction of oxygen radicals which target destruction of melanocytes. Melatonin also functions by lightening the pigment and inhibiting the formation of new melanin. The production of catecholamines by mental stress is triggered by stimulation of the hypothalamic-pituitary-adrenal axis[48]. Electron microscopic analysis of nerve fibers of vitiligo patients showed that the patients have thicker Schwann cell basement membranes surrounding the nerve fibers when compared to a control. Also, it was observed that the dermal nerves, nerve endings and sweat glands of approximately half of the patients under study were degenerated. Gokhale & Mehta (1983) who were conducting these experiments came to the conclusion that since melanocytes originate out of the neural crest, the degeneration of dermal nerves and nerve endings could play a role in the development of vitiligo[49].

AUTOIMMUNE HYPOTHESIS

Due to the simultaneous occurrence of vitiligo and autoimmune diseases, and the responsiveness of vitiligo patients to immunosuppressive treatments, scientists have framed an auto-immune hypothesis to explain the pathogenesis of vitiligo.

The neural hypothesis discussed above is often used to explain the pathogenesis of segmental-type vitiligo, while the autoimmune theory is more appropriate for non-segmental, or "generalized" vitiligo. The mechanisms of immunity are humoral (antibody-mediated), cell-mediated, or mediated by cytokines. Autoantibodies and their respective target cells are also relevant to the pathogenesis of vitiligo.

The role of humoral immunity: In some patients with segmental vitiligo, antibodies against MCHR1 (melanin-concentrating hormone receptor 1), tyrosinase and pigment cell-surface antigen were observed. Also, the presence of immunoglobulin G (IgG) and immunoglobulin M (IgM) in vitiligo patients against melanocytes was observed. In control groups, a lower concentration of IgA was observed. Besides, antibodies usually related to autoimmune conditions such as thyroid were present in most vitiligo cases which include anti-thyroglobulin antibodies, antithyroid antibodies, anti-thyroperoxidase, and anti-smooth muscle antibodies [50].

The role of cell-mediated immunity: The evidence of CMI was investigated by immunohistochemical analysis of the inflammatory perilesional vitiligo skin using single and double immunostaining for melanocytes, the results showed a higher concentration of melanocytes in normal skin contrary to vitiligo skin. The studied T cells were found to show increased production of interleukin IL-2R and an enhanced CD8: CD4 ratio. It was thus concluded that the destruction of melanocytes may be caused via the CD8 T-cell cytotoxicity pathway. The evidence of T-cell reactivity was the display of HLA-DR (MHC class II receptor) in all of the patients with vitiligo, especially along suprabasal and basal keratinocytes. Macrophages were in higher amount in vitiligo patients with an exception of the CD36 subset of macrophages which were higher in the control [51].

The role of cytokines in vitiligo: The immune system has a complex interplay of many cytokines and in vitiligo, there is a considerably higher expression of tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and IL-10. As IFN-γ and TNF-α are T helper cell-1 (Th1) cytokines, so vitiligo is mediated by the Th1 response.

BIOCHEMICAL THEORY

This theory suggests that vitiligo skin has an imbalanced redox (reduction-oxidation) state which results in the excessive synthesis of reactive oxygen species (ROS), as H_2O_2. These ROS oxidize components of the cell which leads to the destruction of melanocytes and creation of depigmented macules.

The redox status of vitiligo patients was examined by checking the levels of factors involved in redox status which include selenium, malondialdehyde (MDA), vitamins A and E, and glutathione peroxidase (GPx) in the erythrocyte activities, catalase (CAT) and superoxide dismutase (SOD). Superoxide dismutase scavenges free radicals and negates their toxicity by converting O_2- to H_2O_2 and O_2, and catalase transforms (H_2O_2) to (H_2O) and (O_2). Selenium is required for GPx activity and vitamins A and E are important in antioxidant activity.

Briefly, there are at least 5 important pathways enrolled in H_2O_2 overproduction in vitiligo[52]:

(1) Defective recycling of 6BH4

(2) Catecholamine formation increased as levels of monoamine oxidase A (MAO) increased

(3) Inhibition of thioredoxin/thioredoxin reductase by calcium

(4) NADPH oxidase activities increased by the cellular infiltrate

(5) Nitric oxide synthase (NOS) activities increased.

MELANOCYTORRHAGY

Electron microscopy, reverse transcription PCR (polymerase chain reaction) and southern blotting experiments were used to study melanocyte loss and two pathways have been supposed for melanocytes loss: highly programmed death by apoptosis and accelerated cell senescence.

Apoptosis and accelerated cell senescence: Apoptosis occurs at least in the traumatized vitiligo skin and hence they show degeneration because of swelling of the membrane-bound organelles, formation of vacuoles and cytoplasm condensation.

Koebner's phenomenon suggests that vitiligo can be induced by trauma. Lower expression levels of the antiapoptotic Bcl-2 and FLIP proteins are apparent in vitiliginous skin. On the other hand, there was an increased level of the proapoptotic Bax and p53 proteins and of the active forms of caspase-3, 8 and 9. *NALP1* gene, which plays role in cellular apoptosis, is associated with vitiligo susceptibility.

The triggers of apoptosis and senescence of epidermal keratinocytes are different but their effector pathways are similar and are regulated by similar molecular regulators. ROS accumulation and DNA damage are reasons behind triggering both apoptosis and aging.

Melanocytorrhagy theory: The Melanocytorrhagy Theory suggests that melanocyte loss occurs due to defective cell adhesion coupled with friction or other types of stress. Other stressors include catecholamines, ROS, or autoimmune elements. This theory combines all previously mentioned concepts. Defective cell adhesion has shown key role in pathogenesis of vitiligo, as the synthesis of extracellular matrix components by keratinocytes may be impaired, the presence of focal gaps in the basement membrane and affect formation of basement membrane. These abnormalities weaken the basal attachment of melanocytes. Trauma could further enhance this susceptibility with subsequent chronic melanocyte loss, known as melanocytorrhagy[53].

INTEGRATED THEORY (CONVERSION THEORY)

Hence it can be concluded that vitiligo is caused by a convergence of all these pathways and vitiligo may be a syndrome with a multi-factorial etiology rather than a single entity [54].

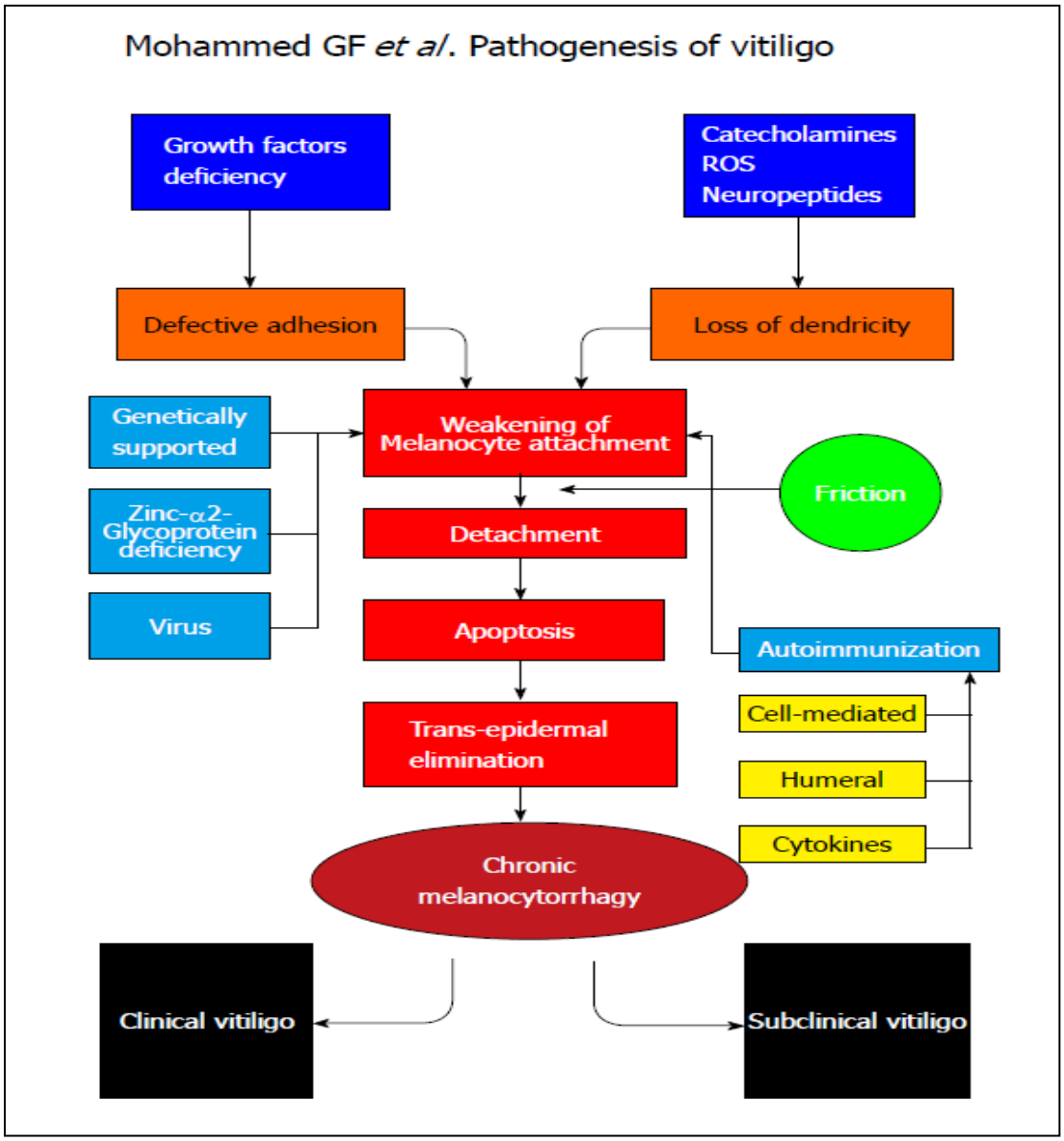

Fig 4: Illustrating the pathogenesis of vitiligo

2.5 *TYROSINASE* (*TYR*) GENE

The *TYR* gene encodes the enzyme tyrosinase, which is located in melanin producing melanocytes. A segment of the enzyme is hooked to the melanocyte membrane and the remainder of the enzyme is present within the melanocyte in a soluble form. Melanin is responsible for providing the skin, hair and eyes their colour and tyrosinase catalyzes the first step in melanin production. It converts tyrosine to dopaquinone and subsequent reactions convert dopaquinone to melanin in the skin, hair follicles, the colored part of the eye (the iris), and the retina. There is enough evidence to suggest that TYR gene is related to occurrence of oculocutaneous albinism and numerous mutations in the TYR gene have been recognized that are related to oculocutaneous albinism type 1. Disruption of the gene by these mutations, inactivation of tyrosinase and resulting disruption of melanogenesis forms the basis of the discoloration caused by this disease[55].

Tyrosinase

Tyrosinase can be generally categorized as a polyphenol oxidase enzyme and it plays important roles in catalyzing melanogenesis in humans. It is a 75kDa, copper-containing enzyme with activities involving o-hydroxylation of monophenols into their corresponding di-phenols and the oxidation of o-diphenols to o-quinones using molecular oxygen, which readily polymerizes to form melanin (brown/black pigmentation).

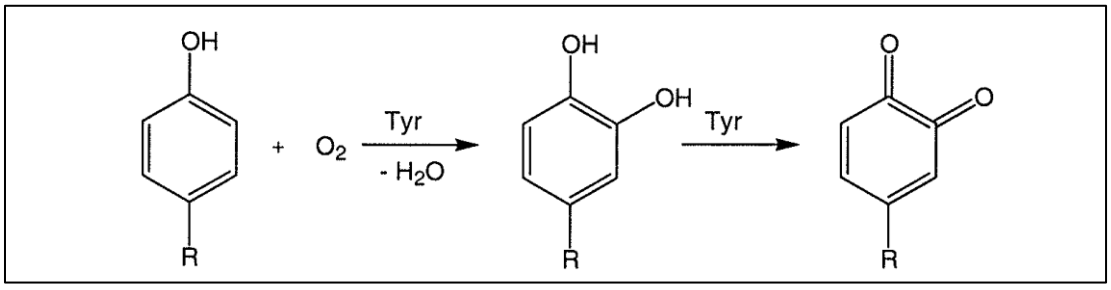

Fig 5: Reactions catalyzed by tyrosinase

Mechanism of Action:

Tyrosinases are copper containing metalloproteins and the primary function of copper ions is to facilitate electron transfer, which is the basis of its catalyzing action. Tyrosinase has been classified as a Type 3 copper protein with absorption maxima at about 600 and 330nm and it gives no EPR signal due to the presence of a pair of copper ions which are antiferromagnetically coupled.

The copper atoms (CuA and CuB) are located in a helix bundle, coordinated by three conserved histidine residues.

Tyrosinases are bifunctional enzymes and the two types of reactions they catalyze include the *ortho*-hydroxylation of monophenols to its corresponding o-diphenol (monophenolase, cresolase activity) and the oxidation of diphenols to its correspondening *ortho*-quinones (diphenolase, catecholase activity) both in the presence of molecular oxygen.

Gene Map

Location: 11q14.3, which is the long (q) arm of chromosome 11 at position 14.3

Molecular Location: base pairs 89,177,565 to 89,295,759 on chromosome 11 (Homo sapiens Annotation Release 108, GRCh38.p7) (NCBI)

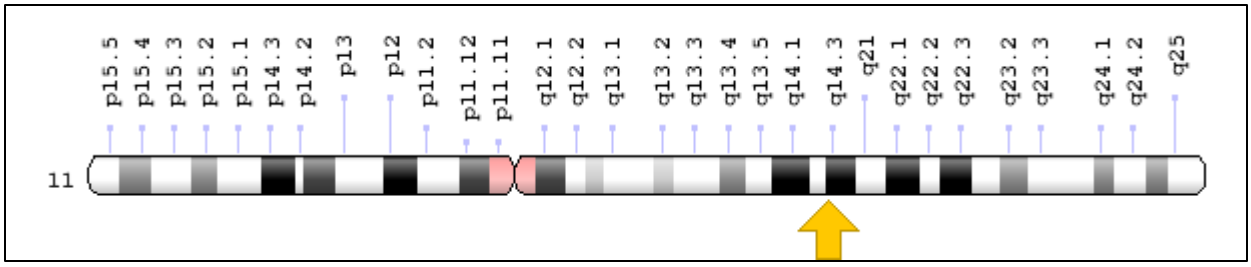

Fig 6: *TYR* Gene on the 11th chromosome of genome

ROLE OF *TYR* IN VITILIGO

The sequences spanning the TYR gene contain numerous non-synonymous SNPs which may result in formation of a dysfunctional enzyme disrupting melanogenesis and hence shows strong association with vitiligo. Tyrosinase is also a major autoantigen in GV which was first detected in an immunoblotting experiment and was present in 61% of the studied vitiligo patients. To support the experiment, ELISA was carried out with mushroom tyrosinase and 39% of vitiligo patients were found positive for antityrosinase antibodies[56].

2.6 SINGLE NUCLEOTIDE POLYMORPHISMS (SNPs)

Single nucleotide polymorphisms, or SNPs (pronounced "snips"), are the most frequently occurring kind of genetic variation among populations. Each SNP represents a change of a single nucleotide at a specific site in a DNA sequence. These variations occur naturally throughout a person's DNA, once in every 300 nucleotides on an average, which means there are roughly 10 million SNPs in the human genome. These are most prevalent in introns and can be called biological markers which can assist scientists in locating genes that are associated with disease. But when a variation occurs in the coding region of a genome, it is probable that it will affect the phenotype more seriously and may play a major role in causing or regulating a diseased condition. Usually SNPs don't have much effect on health or development but some of these have proven to be critical in studying human health and susceptibility towards diseases. An individual's SNP profile is now used to predict responses to specific drugs, susceptibility to environmental factors such as toxins, and risk of developing particular diseases. They have also found an application in tracking inheritance of diseased genes within affected families. Ongoing researches are attempting to identify critical SNPs associated with pathogenesis of complex diseases such as heart disease, diabetes, and cancer.

Analyzing genetic susceptibility to Vitiligo by studying SNPs present in TYR gene

A large proportion of genetic variation among populations is a result of a random mutation at specific nucleotide positions, called SNPs. The effects of these mutations vary, in some cases they can be synonymous while in some they might be deleterious and these genetic differences largely affect the individual phenotypically. Synonymous mutations don't affect much as the nucleotide change doesn't change the amino acid it codes for, on the contrary, a non synonymous SNP changes the amino acid being coded and hence the resultant protein.

For instance, presence of an SNP in the 3′ UTR region can affect mRNA stability by changing secondary structure or by changing binding sites thus making it more or less susceptible to degradation while an SNP in the 5′ region can affect promoter binding affinity to the site. The most severe and deleterious are the non-sense SNPs found in the coding region, which are the mutations that cause the formation of a premature stop codon which results in a truncated inactive polypeptide which is not functional. Another type of mutation includes missense mutations lead to change into an amino acid at that specific codon position. These SNPs can be

deleterious if the resultant amino acid is drastically different from the original one in terms of charge or polarity. As susceptibility to Vitiligo has a moderate genetic component, analysis of polymorphisms may contribute significantly to understanding the role of TYR gene in disease pathogenesis[57].

SNPs under study: rs28940876 and rs61754379

Tyrosinase: 529 amino acids

Location of gene: 11q14.3

Domains: Signal peptide, **Copper Binding**, Transmembrane

A bioinformatics tool called SIFT, ie Sorting Intolerant From Tolerant was used to identify the SNPs in the given gene TYR and a classification was made between the SNPs that would be tolerated by the protein and it will remain unaffected, and the SNPs that would cause inactivation of the protein and disturbance of its binding site and function.

The SIFT score of both the SNPs indicate that these are deleterious non synonymous SNPs.

1. **rs28940876**

Table 2: **rs28940876** summary

Position	**11:89178195**
Alleles	**C/T**
cDNA Position	**744 (out of 2485)**
Protein position	**81 (out of 529)**
Codons	**cCt/cTt**
Amino acid	**P/L**
Consequences	**Missense variant**
SIFT	**0**

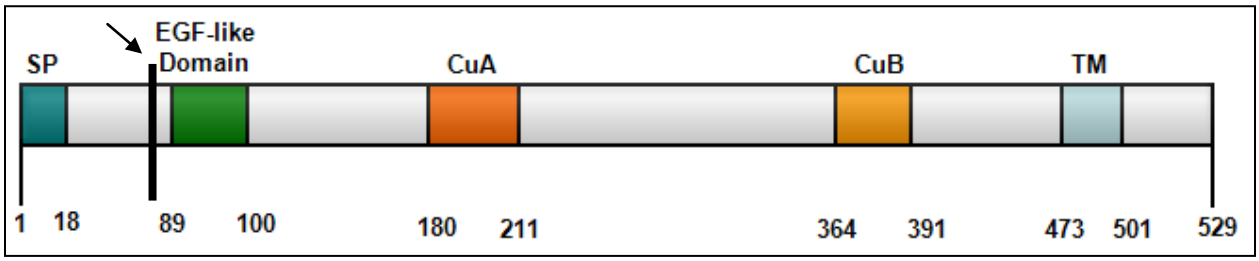

Fig 7: Position of change in amino acid (81) in the tyrosinase protein due to the presence of SNP- **rs28940876**

SNP present at position 81.

SNP falls in "Uncharacterized domain, di-copper centre"

SNP causes replacement of Proline by Leucine at the 81st position in the protein tyrosinase. There are major chemical differences between the two amino acids which might affect the structure of the resultant peptide negatively; hence this SNP is a potential candidate to study correlation with a dysfunctional TYR gene and susceptibility towards vitiligo.

2. **rs61754379**

Table 3: **rs61754379** summary

Position	**11:89191355**
Alleles	**A/G**
cDNA Position	**1475 (out of 2485)**
Protein position	**325 (out of 529)**
Codons	**Acc/Gcc**
Amino acid	**T/A**
Consequences	**Missense variant**
SIFT	**0.75**

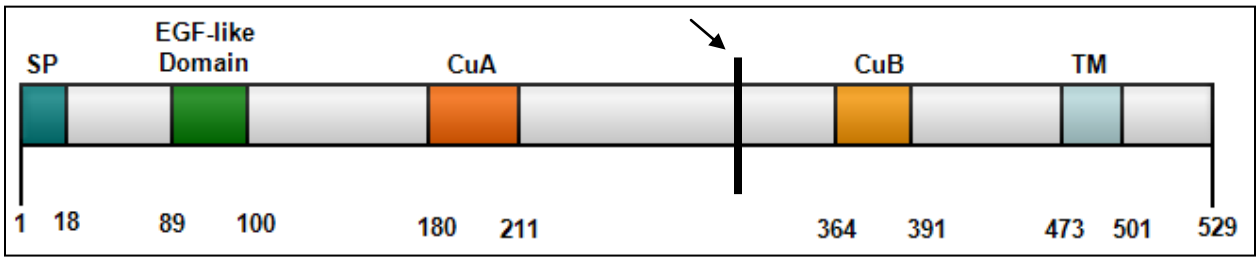

Fig 8: Position of change in amino acid (325) in the tyrosinase protein due to the presence of SNP- **rs61754379**

SNP present at position 325.

SNP falls in "Copper Binding domain"

The occurrence of this SNP causes Threonine to change into Alanine on the 325th position of the enzyme tyrosinase. These two amino acids differ chemically and since the SNP falls in the copper binding domain, its presence in the gene would definitely lead to an inactive enzyme resulting in a diseased condition.

2.7 SNP GENOTYPING: PCR-RFLP

PCR-RFLP is a relatively simple and cheap method for genotyping of SNPs. This method allows differentiation of homozygous and heterozygous samples at the target point mutation, along with the genotyping of multiple mutations such as HLA alleles. This method is especially convenient for simple experiments involving only qualitative confirmation of presence or absence of SNPs without additional experimental techniques.

It is a highly sensitive enzymatic technique, which first amplifies the DNA fragments which will be used for analysis by PCR. It is widely used for detection of genetic variants with biological significance, and in our study. Although there are many methods for genotyping, for example by PCR using hybridization, allele-specific PCR, primer extension, oligonucleotide ligation, direct DNA sequencing, MALDI-TOF etc, we have used PCR-RFLP method of genotyping to identify the presence of an SNP in our experiments.

PCR-RFLP is based on endonuclease digestion of PCR-amplified DNA. Cleavage at a specific restriction site is carried out by a specific restriction endonuclease and that restriction site must also contain the SNP of interest. So, in case variation occurs and the SNP changes the nucleotide at that specific position, the restriction site is no longer present and the endonuclease does not cleave at all. The SNP type is easily visualized by performing agarose gel electrophoresis and confirmation of genotypes is made by the number of bands visible after endonuclease digestion. This is a reliable method and requires the most basic instrumentation. This technique is limited by the restriction enzyme that is to be used which targets the SNP, and sometimes there are multiple sites for a particular restriction enzyme.

The first step is to design a primer pair that would amplify the product which contains the SNP under study and finding a specific restriction enzyme that will identify the SNPs in the PCR-amplified product. Both the primer and the enzyme must be optimized for good results. The template DNA could be from any starting material such as fresh or frozen tissue or blood etc. In our experiments we have used blood and isolated DNA using ethanol precipitation method. The purity of template DNA is evaluated by measuring its absorbance at 260 nm to absorbance at 280 nm. An A_{260}/A_{280}ratio of 1.7–1.9 means pure template DNA and is better suited for PCR procedure.[58]

CHAPTER 3: OBJECTIVES

The objectives of this study are:

1. To predict non-synonymous SNPs through in-silico analysis of the nsSNPs of *TYR* gene.

2. To optimize PCR for the predicted nsSNPs (rs28940876 and rs61754379).

3. To genotype predicted nsSNPs (rs28940876 and rs61754379) of TYR gene by PCR-RFLP in vitiligo patient and control individuals belonging to North India.

4. To look for genetic association, if any, between polymorphism in *TYR* and susceptibility to vitiligo by statistical analysis.

In our study, we would explore the association of *TYR* gene polymorphisms (rs28940876 and rs61754379) with vitiligo susceptibility which could be used as an informative marker for vitiligo susceptibility.

CHAPTER 4: MATERIASLS AND METHODS

4. A) PREDICTION OF DELETERIOUS nsSNPS BY USING SIFT

SIFT (Sorting Intolerant from Tolerant) took a query sequence and used multiple alignment information to predict tolerant and deleterious substitutions for every position entered as the query sequence. SIFT server was available at (http://sift.jcvi.org/).

4. B) EXPERIMENTAL PROCEDURES

Study population

The study group comprised of total 108 vitiligo patients. A total of 92 healthy individuals 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. Written consent was signed by all the individuals before drawing the blood sample, in addition to imparting knowledge about the project to all.

4. B.1) Sampling

2 ml venous blood was collected from the patients and healthy subjects in NA_2EDTA coated tubes. Blood samples were stored at $-20^{O}C$ till further use.

4. B.2) Isolation of Genomic DNA from Whole Blood Sample [31]

- 300 µl blood sample was mixed 1000 µl RBC lysis buffer in a 2ml autoclaved micro centrifuge tube. Kept for incubation on rocker at RT for 30-60 minutes until it turned shiny.
- The tube was centrifuged at 13,000 RPM for 1 min to obtain a white pellet.
- The supernatant was discarded and the pellet was thoroughly suspended in 300 µl of TE Buffer (pH 8.0) using a vortexing machine. 20 µl of 10% SDS solution was added, and the mixture was incubated at $56^{O}C$ for 30 mins in water bath.

- 160 µl of 7.5M ammonium acetate was added and mixed vigorously by vortexing for 1 min. Centrifuged at 13,000 RPM for 15 mins which resulted in separation of the proteins as a pellet.
- The supernatant was transferred to fresh sterile 1.5 ml micro centrifuge tube. Chilled ethyl alcohol twice the volume of the supernatant was added and the tube was rocked gently for 2-3 times to allow precipitation of gDNA.
- The tube was centrifuged at 13,000 RPM for 10 min to pellet down the gDNA. The pellet was washed in 150 µl of 70% ethyl alcohol and air dried at RT for 10-15 min.
- Dried DNA pellet was dissolved in 60 µl of TE Buffer (pH 7.3) by incubating at 65^{O}C for 10 mins. Dissolved DNA was stored at -20^{O}C for further use.
- The isolated genomic DNA was checked by Agarose gel (0.8%) and quantified using Nano Drop plus Spectrophotometer (GE Healthcare, US). The concentration of DNA was read by measuring the absorbance of a sample at 260 on a spectrophotometer.

4. B.3) Primer Designing

Designing of oligonucleotide primers is a crucial step for successful molecular biology experiments that require the PCR. The goals of primer design include good primer specificity, high annealing efficiency, appropriate melting temperature, proper GC content and the prevention of primer hairpins or primer dimers.

4. B.3.1) Protocol for designing primer

- NCBI (National Center of Biotechnology Information) webpage was opened.
- Primer BLAST option was selected and the nucleotide sequence of TYR neighboring the selected nsSNPs was entered into the query box.
- 'Get Primers' option was selected.
- The primer pair (forward and reverse) from the generated list of primers was selected.

Table 4: Primers for selected nsSNPs

NsSNPs	**rs28940876**	**rs61754379**
Forward Primer (5'- 3')	GCCTCAATTTCCCTTCACAG	TTATGCAATGGAACGCCCGA
Reverse Primer (5'-3')	TTCTCTGGGGCACTCAAATC	TGTCAGCTAGGGTCATTGTCG
Tm (^{O}C)	59.94	57.05
Amplicon size (bp)	194	330

4. B.3.2) Reconstitution of Primers (Lyophilized)

For 100µM, following amount of nuclease free water (NFW) was added.

Table 5: Amount of distilled water added to the primers for reconstitution

Primers	**NFW (µl)**
rs28940876F	201
rs28940876R	329
rs61754379F	242
rs61754379R	269

4. B.4) PCR Optimization

4. B.4.1) Gradient PCR

Gradient PCR was performed for both sets of primers to identify the best annealing temperature for the successive amplification of the specific product.

4. B.4.2) PCR: Biochemical reaction to form allele specific product

PCR technology was used for the amplification of the desired *TYR* gene segments. PCR amplification of the genomic DNA isolated from blood sample was carried out using primers

(Table 4) specific for the *TYR* gene sequence. Polymerase chain reaction for the *TYR* gene amplification was performed using the following protocol:

- 12.5 µl reaction mixture was prepared as per the composition given in the Table 6. Samples mixture was kept into the Thermocycler (Thermo scientific thermocycler) for amplification and parameters (as given in Table 7) were set.

Table 6: Reagent Mixture Composition used in the PCR for DNA Amplification

Reaction components	**Per reaction volume (µl)**	
	For rs28940876	**For rs61754379**
Master mix	6.25	6.25
DNA template (40ng/ µl)	1	1
Forward Primer (10µM)	0.25	0.25
Reverse Primer (10µM)	0.25	0.25
Nuclease free water	4.75	4.75
Total	12.5	12.5

Table 7: PCR Cycling conditions for the amplification of genomic DNA

- **For nsSNP: rs28940876**

Step	**Temperature**	**Time**	**Cycles**
Initial Denaturation	95^{O}C	2 min	1
Denaturation	95 ^{O}C	30 s	30
Annealing	54 ^{O}C	40 s	
Extension	72 ^{O}C	30 s	
Final extension	72 ^{O}C	5 min	1
Total Hold	4 ^{O}C	infinite	-

- **For nsSNP: rs61754379**

Step	**Temperature**	**Time**	**Cycles**
Initial Denaturation	$95^{O}C$	2 min	1
Denaturation	$95^{O}C$	45 s	35
Annealing	$61.5^{O}C$	1 min	
Extension	$72^{O}C$	45 s	
Final extension	$72^{O}C$	5 m	1
Total Hold	$4^{O}C$	infinite	-

4. B.5) <u>Genotyping PCR – RFLP</u>

Restriction Fragment Length Polymorphism (RFLP) was used to identify the allele specific product.

Amplified product was digested with the restriction enzyme to identify the genotype present in a particular individual. PCR products of rs28940876 and rs61754379 were digested with HaeIII. Reaction condition of RFLP for both the nsSNPs are mentioned in Table 8.

<u>Table 8: Reaction Conditions for RFLP Genotyping Method</u>

Reaction Components	**Reaction volume (µl)**	
	rs28940876	**rs61754379**
Restriction enzyme	HaeIII	HaeIII
Restriction site of the enzyme	5’ GG|CC 3’	5’ GG|CC 3’
	3’ CC|GG 5’	3’ CC|GG 5’
Enzyme (1 Unit)	0.1	0.1
Buffer	1.5	1.5
Water	3.4	3.4
DNA	10	10
Total	15	15

Analysis of Digested PCR Product:

Agarose gel electrophoresis was done to visualize the digested band pattern. Expected digested band pattern for different genotype is as shown in Table 9.

RFLP digested product was analyzed on 3% w/v (rs28940876) and 2% (rs61754379) agarose gel containing Eithidium bromide (0.5mg/ml). A NEB 100 bp marker was used as ladder. 15 µl of amplified and restriction enzyme treated product was loaded into the wells along with ladder into separate well. The gel was then run for 40-50 minutes at 100 Volts in 1X TAE buffer. Bands were visualized using U.V. transilluminator.

Table 9: Expected digested band pattern of SNP rs28940876 and rs61754379

rs28940876		**rs61754379**	
Genotype	**Band size (bp)**	**Genotype**	**Band size (bp)**
CC (WT)	43 + 151	AA (WT)	330
CT	43 + 151 + 194	GA	112 + 218 + 330
TT (MT)	194	GG (MT)	112 + 218

4. B.6) Statistical Analysis

Statistical tools were applied to assess the association of TYR gene polymorphism with vitiligo susceptibility.

A chi-squared test was used to determine whether observed control genotype frequency confirmed to Hardy-Weinberg equilibrium (HWE) expectation using HWE calculator (http://www.had2know.com/academics/hardy-weinberg-equillibrium-calculator-2alleles.html). Observed frequency considered to be in disequilibrium if p-value < 0.05. Odds ratios and 95% confidence intervals were calculated to assess the risk associated with the variant allele. Odd Ratio was calculated using MedCalc software (http://www.medcalc.org/calc/odds_ratio.php). The significance of the odd ratio was determined by Z-test if p-value was considered statistically significant.

CHAPTER 5: OBSERVATIONS AND RESULTS

➢ PCR GRADIENT GEL IMAGES

a) rs28940876

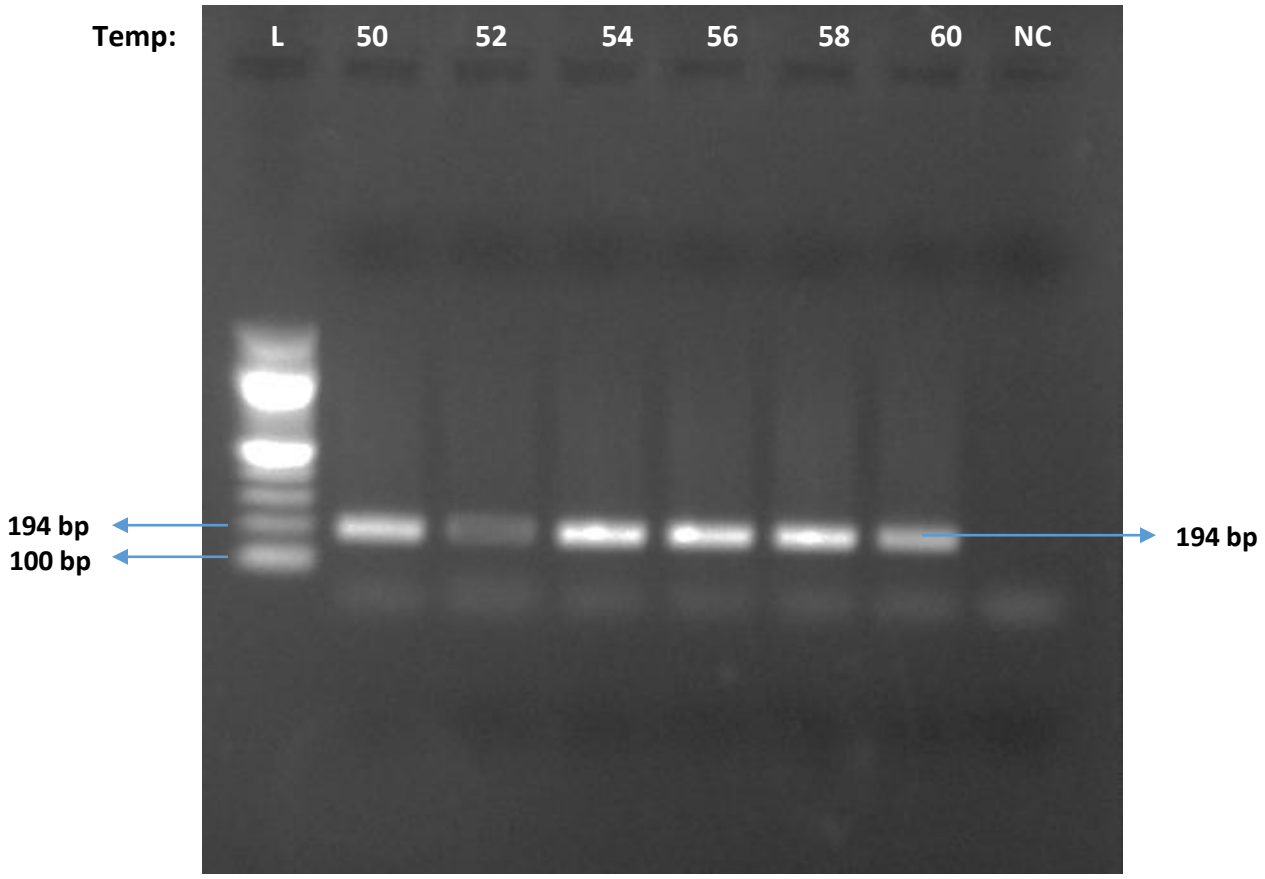

Fig 9.a) Agarose gel electrophoresis (2% agarose w/v) illustrating amplification of single DNA sample at different annealing temperatures $50^{O}C$ – $60^{O}C$. (Expected band size = 194 bp)

Lane 1: 100 bp Ladder; **Lane 2**: DNA sample at temperature $50^{O}C$; **Lane 3**: DNA sample at temperature $52^{O}C$; **Lane 4**: DNA sample at temperature $54^{O}C$; **Lane 5**: DNA sample at temperature $56^{O}C$; **Lane 6**: DNA sample at temperature $58^{O}C$; **Lane 7**: DNA sample at temperature $60^{O}C$; **Lane 8**: Negative control.

b) rs61754379

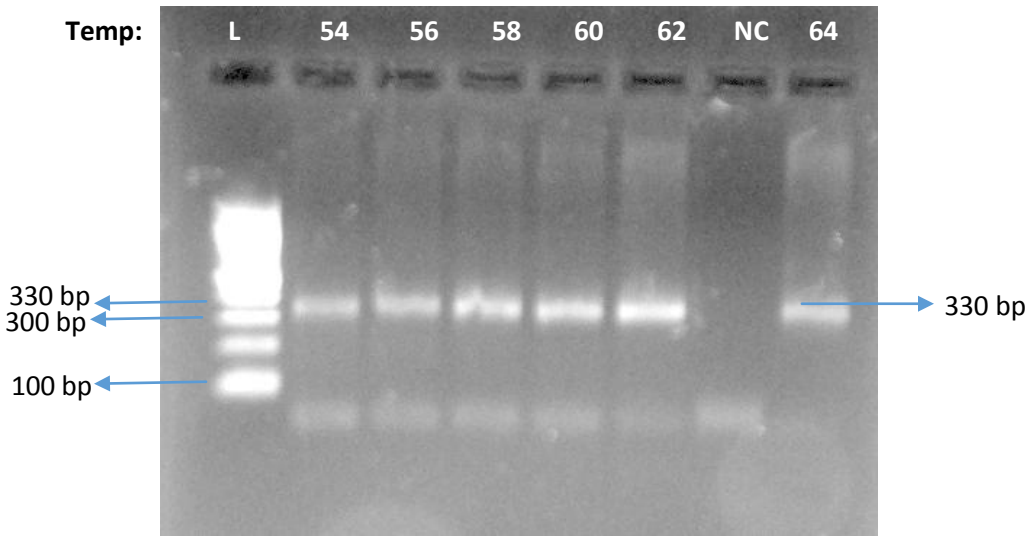

Fig 9.b) Agarose gel electrophoresis (2% agarose w/v) illustrating amplification of single DNA sample at different annealing temperatures 54^{O}C – 64^{O}C. (Expected band size = 330 bp)

Lane 1: 100 bp Ladder; **Lane 2**: DNA sample at temperature 54^{O}C; **Lane 3**: DNA sample at temperature 56^{O}C; **Lane 4**: DNA sample at temperature 58^{O}C; **Lane 5**: DNA sample at temperature 60^{O}C; **Lane 6**: DNA sample at temperature 62^{O}C; **Lane 7**: Negative control; **Lane 8**: DNA sample at temperature 64^{O}C.

➢ PCR AMPLIFIED PRODUCT

a) rs28940876

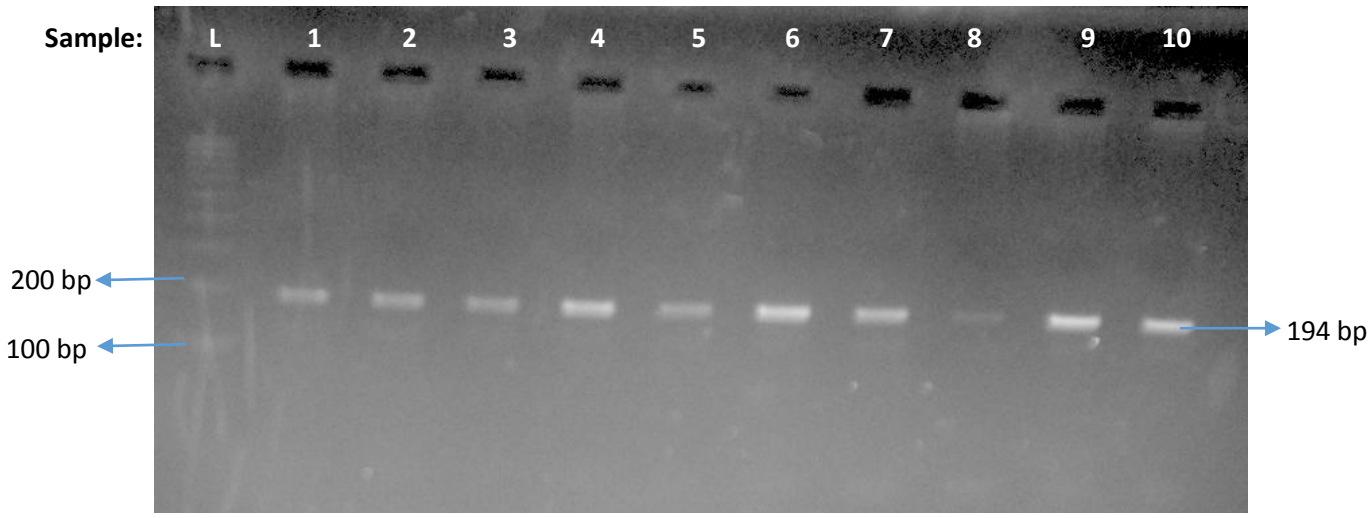

Fig 10.a) Agarose gel electrophoresis (2% agarose w/v) illustrating amplification of 10 DNA samples at a single annealing temperature 54^{O}C. (Expected band size = 330 bp)

Lane 1: 100 bp Ladder; **Lane 2**: DNA sample 1; **Lane 3**: DNA sample 2; **Lane 4**: DNA sample 3; **Lane 5**: DNA sample 4; **Lane 6**: DNA sample 5; **Lane 7**: DNA sample 6; **Lane 8**: DNA sample 7; **Lane 9**: DNA sample 8; **Lane 10**: DNA sample 9.

b) rs61754379

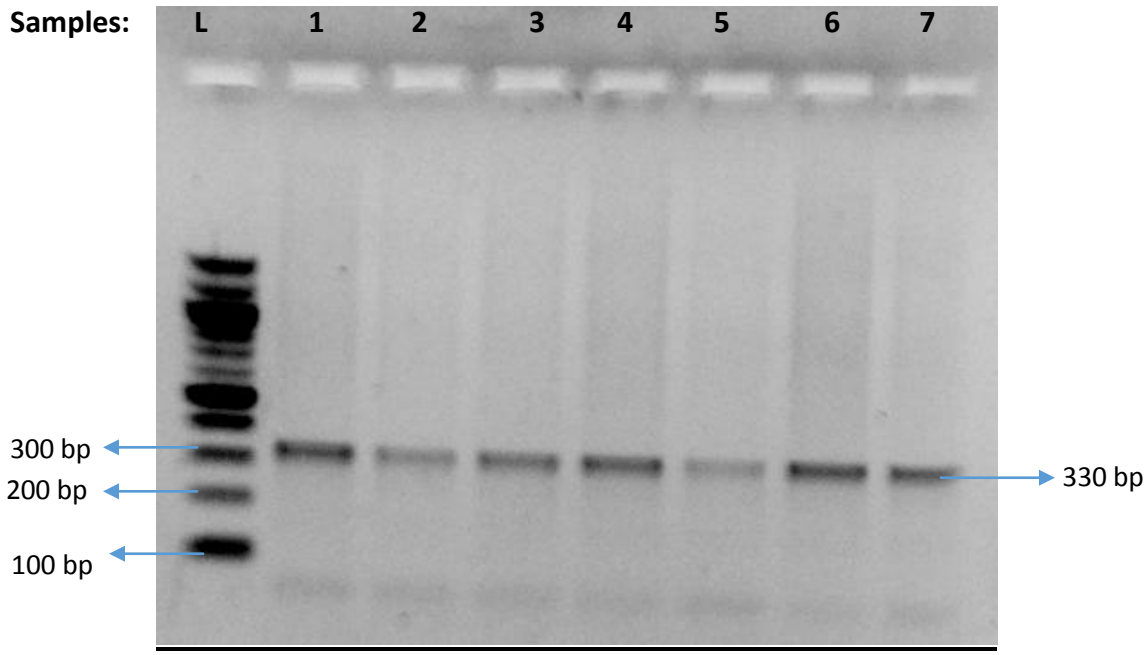

Fig 10.b) Agarose gel electrophoresis (2% agarose w/v) illustrating amplification of 7 DNA samples at a single annealing temperature 61^{O}C. (Expected band size = 330 bp)

Lane 1: 100 bp Ladder; **Lane 2**: DNA sample 1; **Lane 3**: DNA sample 2; **Lane 4**: DNA sample 3; **Lane 5**: DNA sample 4; **Lane 6**: DNA sample 5; **Lane 7**: DNA sample 6; **Lane 8**: DNA sample 7.

➢ RESTRICTION DIGESTION GEL IMAGE

a) rs28940876

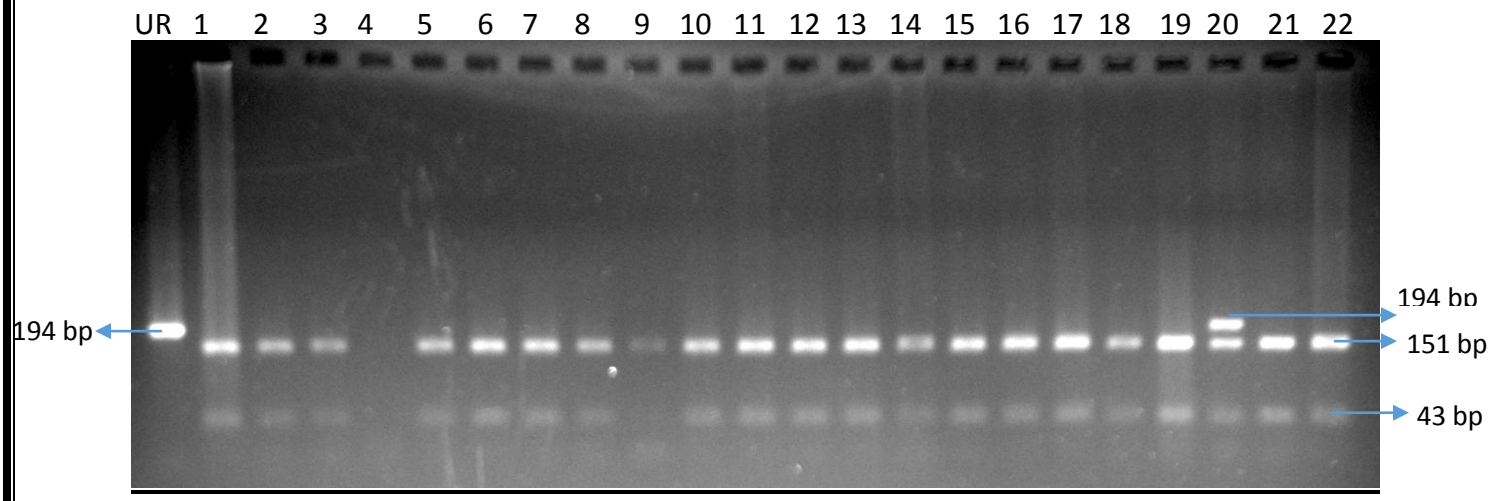

Fig 11.a) Agarose gel electrophoresis (3% agarose w/v) illustrating digestion of 22 DNA samples which were amplified at a single annealing temperature 54^{O}C. (Expected band sizes and genotype = CC: 151+ 43 bp; CT: 194+ 151+ 43 bp; TT: 194 bp)

Lane 1: Unrestricted amplified DNA sample; **Lane 2**: DNA sample 1; **Lane 3**: DNA sample 2; **Lane 4**: DNA sample 3; **Lane 5**: DNA sample 4; **Lane 6**: DNA sample 5; **Lane 7**: DNA sample 6; **Lane 8**: DNA sample 7; **Lane 9:** DNA sample 8; **Lane 10**: DNA sample 9; **Lane 11**: DNA sample 10; **Lane 12**: DNA sample 11; **Lane 13**: DNA sample 12; **Lane 14**: DNA sample 13; **Lane 15**: DNA sample 14; **Lane 16**: DNA sample 15; **Lane 17**: DNA sample 16; **Lane 18**: DNA sample 17; **Lane 19**: DNA sample 18; **Lane 20**: DNA sample 19; **Lane 21**: DNA sample 20; **Lane 22**: DNA sample 21; **Lane 23**: DNA sample 22.

b) rs61754379

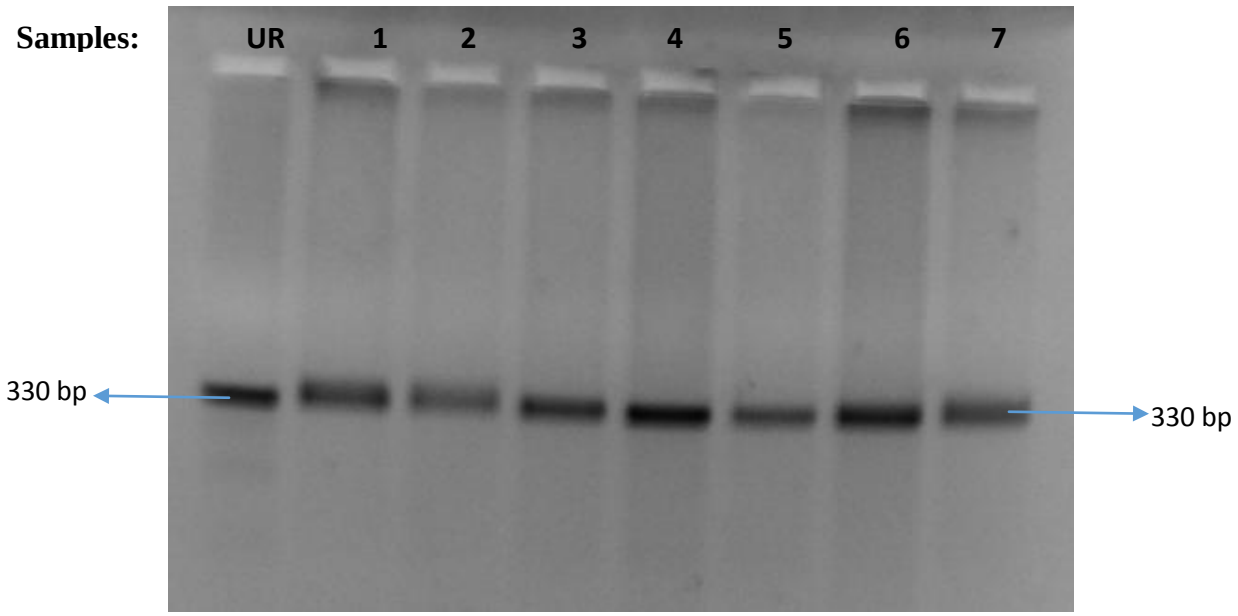

Fig 11.b) Agarose gel electrophoresis (2% agarose w/v) illustrating digestion of 7 DNA samples which were amplified at a single annealing temperature 61^{O}C. (Expected band sizes and genotype: AA – 330; AG – 330+ 218+ 112 bp; GG – 218+ 112 bp.)

Lane 1: Unrestricted amplified DNA sample; **Lane 2**: DNA sample 1; **Lane 3**: DNA sample 2; **Lane 4**: DNA sample 3; **Lane 5**: DNA sample 4; **Lane 6**: DNA sample 5; **Lane 7**: DNA sample 6; **Lane 8**: DNA sample 7.

EXPERIMENTAL RESULTS

108 vitiligo patients and 92 control subjects were genotyped for rs28940876 and rs61754379 in *TYR* 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 *TYR* gene revealed that the variant T allele of rs28940876 was found in 0.5% of controls and 0% of cases. The variant G allele on the other hand of rs61754379 showed no occurrence in the control as well as vitiligo cases taken under study. The allelic frequencies of these two SNPs between vitiligo and control were, rs28940876: $p= 0.4398$; rs61754379: $p= 0.9363$, which suggested no association of minor allele of both the SNPs with vitiligo. **rs28940876** and **rs61754379** variant alleles were found to have no relation to the risk of vitiligo.

(**rs28940876:** Odds Ratio (OR)= 0.2825; 95% confidence interval (CI)= 0.0232 - 59.4334; **rs61754379:** OR= 1.173495; 95% CI= 0.0114 to 6.9777)

Table 10: Allele and Genotype distribution of the *Tyrosinase* polymorphism in vitiligo cases (N=108) and control (N=92)

Genotype/Allele	Vitiligo (n/N[%])	Control (n/N[%])	Odds Ratio (OR)	95% CI	p-value
rs28940876					
CC	108/108[100]	91/92[98.91]	0.28	0.0232 to 59.4334	0.44
GT	0/108[0]	1/92[1.09]			
TT	0/108[0]	0/92[0]			
C	216/216[100]	183/184[99.5]			
T	0/216[0]	1/184[0.5]			
rs61754379					
AA	108/108[100]	92/92[100]	1.17	0.0114 to 6.9777	0.93
AG	0/108[0]	0/92[0]			
GG	0/108[0]	0/92[0]			
G	0/216[0]	0/92[0]			
A	216/216[100]	184/184[100]			

CHAPTER 6: DISCUSSION

Vitiligo is a chronic stigmatizing disease which mainly affects melanocytes from epidermis basal layer, leading to the development of white (depigmented) patches on the skin.

Autoimmune response against tyrosinase enzyme as well as mutations in *Tyrosinase (TYR)* gene have been speculated to have a role in vitiligo pathogenesis. In the present study, we investigated vitiligo patients for genetic variation in the *Tyrosinase* gene which may show association with vitiligo susceptibility.

We predicted nsSNPs using SIFT. The selected SNPs - rs28940876 and rs61754379 in *TYR* gene were investigated for showing any association with the risk of vitiligo in North Indian population. Using the technique of PCR-RFLP, genotypes were successfully obtained for the cases and control samples. Genotyping of the SNPs in the *TYR* gene revealed that the variant T allele of rs28940876 was found in 0.5% of the control population and was not found in the case population. The variant allele G of rs61754379 was not found in either of the two populations.

Consequently, appropriate statistical methods were applied to elucidate the association of variants with susceptibility of the disease.

The allelic frequencies of these two SNPs between vitiligo and control were rs28940876: p= 0.4398; rs61754379: p= 0.9363, which suggested no association of minor allele of both the SNPs with vitiligo. As a result, **rs28940876** and **rs61754379** variant alleles were found to have no relation to the risk of vitiligo with respective statistical parameters as given below:

rs28940876: Odds Ratio (OR)= 0.2825; 95% confidence interval (CI)= 0.0232 - 59.4334, p-value= 0.44 ; **rs61754379:** OR= 1.173495; 95% CI= 0.0114 to 6.9777, p-value= 0.93 .

Therefore, the results showed that the selective variants were not significantly associated with the risk of vitiligo.

CHAPTER 7: CONCLUSION AND FUTURE PROSPECTS

The SIFT predicted SNPSs rs28940876 and rs61754379 in *TYR* gene when investigated for showing any association with the risk of vitiligo were not found to be associated with the same. The genotyping allelic frequencies of cases and control were not statistically significant, which results in concluding that there is no association of the selected variants with vitiligo susceptibility. Although insignificant, this data could further be utilized in studying the etiological factors of this disease by genotyping in more number of Vitiligo samples with different population sets.

APPENDIX

1) GLASSWARE AND INSTRUMENTS

1.1) Glassware

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

1.2) Instruments

- Micro pipette
- Thermo-Cycler
- Laminar Air Flow Hood
- 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 Spectrophtometer
- Vortexing machine
- Agarose Gel Electrophoresis Unit
- UV transilluminator

- Centrifuge
- Water Bath

2) REAGENTS

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

Dissolved 18.61g of Na_2EDTA in 50ml MQ water by magnetic stirring. Simultaneously dropwise added 10M NaOH till pH 8.0 was reached. Volume raised to 100ml when salt was completely dissolved in the solution.

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

Dissolved 12.11g Tris Base in 75ml of sterile MQ water. pH was set at 8.0 using 1N HCl. Volume raised to 100 ml with MQ water. Filtered using Whattmann filter paper and stored in a sterile tight screw capped reagent bottle.

2.3) Tris (hydroxylmethyl) aminomethane-chloride, Tris-Cl (1 M, pH 7.3)

Dissolved 12.11g Tris Base in 75ml of sterile MQ water. pH was set at 7.3 using 1N HCl. Volume raised to 100 ml with MQ water. Filtered using Whattmann filter paper and stored in a sterile tight screw capped reagent bottle.

2.4) Ammonium Chloride, NH_4Cl (1M)

Dissolved 5.35g of Ammonium Chloride in 80ml MQ water. Volume raised to 100 ml.

2.5) Sodium dodecyl sulphate, SDS (10%)

Mixed 10g in 70ml of MQ water. Volume raised 100ml.

2.6) RBC lysis Buffer

10mM Tris-Cl(1 M, pH 8.0) + 1mM EDTA + 125mM NH_4Cl (pH 8.0)

Mixed:

1M Tris-Cl, pH 8.0	**10 ml**
0.5M EDTA	**2 ml**
1M NH_4Cl	**125 ml**

Volume raised to 1000ml by MQ water.

2.7) Tris EDTA (TE) Buffer; pH 8.0

Mixed:

1M Tris Cl; pH 8.0	**10 ml**
0.5M EDTA	**2 ml**

Volume raised to 1000 ml by MQ water.

2.8) Tris EDTA (TE) Buffer; pH 7.3

Mixed:

1M Tris Cl; pH 7.3	**10 ml**
0.5M EDTA	**2 ml**

Volume raised to 1000 Buffer ml by MQ water.

2.9 Ammonium acetate (7.5M)

Dissolved 28.9g Ammonium acetate in 20 ml MQ water. Final volume raised to 50ml.

2.10) 70% Ethanol

70ml dehydrated ethyl alcohol mixed with 30ml sterile MQ water.

REFERENCES

1. Al-Mutairi, N. and K.H. Al-Sebeih, *Late onset vitiligo and audiological abnormalities: is there any association?* Indian J Dermatol Venereol Leprol, 2011. **77**(5): p. 571-6.
2. Akay, B.N., et al., *Epidemiology of vitiligo, associated autoimmune diseases and audiological abnormalities: Ankara study of 80 patients in Turkey.* J Eur Acad Dermatol Venereol, 2010. **24**(10): p. 1144-50.
3. Nair, B.K., *Vitiligo--a retrospect.* Int J Dermatol, 1978. **17**(9): p. 755-7.
4. Kopera, D., *Historical aspects and definition of vitiligo.* Clin Dermatol, 1997. **15**(6): p. 841-3.
5. Behl, P.N. and R.K. Bhatia, *400 cases of vitiligo. A clinico-therapeutic analysis.* Indian J Dermatol, 1972. **17**(2): p. 51-6.
6. Ezzedine, K., et al., *Revised classification/nomenclature of vitiligo and related issues: the Vitiligo Global Issues Consensus Conference.* Pigment Cell Melanoma Res, 2012. **25**(3): p. E1-13.
7. Alikhan, A., et al., *Vitiligo: a comprehensive overview Part I. Introduction, epidemiology, quality of life, diagnosis, differential diagnosis, associations, histopathology, etiology, and work-up.* J Am Acad Dermatol, 2011. **65**(3): p. 473-91.
8. Iannella, G., et al., *Vitiligo: Pathogenesis, clinical variants and treatment approaches.* Autoimmun Rev, 2016. **15**(4): p. 335-43.
9. Videira, I.F., D.F. Moura, and S. Magina, *Mechanisms regulating melanogenesis.* An Bras Dermatol, 2013. **88**(1): p. 76-83.
10. Brenner, M. and V.J. Hearing, *The protective role of melanin against UV damage in human skin.* Photochem Photobiol, 2008. **84**(3): p. 539-49.
11. Costin, G.E. and V.J. Hearing, *Human skin pigmentation: melanocytes modulate skin color in response to stress.* FASEB J, 2007. **21**(4): p. 976-94.
12. Tsatmali, M., J. Ancans, and A.J. Thody, *Melanocyte function and its control by melanocortin peptides.* J Histochem Cytochem, 2002. **50**(2): p. 125-33.
13. Wasmeier, C., et al., *Melanosomes at a glance.* J Cell Sci, 2008. **121**(Pt 24): p. 3995-9.
14. Raposo, G. and M.S. Marks, *Melanosomes--dark organelles enlighten endosomal membrane transport.* Nat Rev Mol Cell Biol, 2007. **8**(10): p. 786-97.

15. Park, H.Y., et al., *Cellular mechanisms regulating human melanogenesis.* Cell Mol Life Sci, 2009. **66**(9): p. 1493-506.
16. Ando, H., et al., *Melanosomes are transferred from melanocytes to keratinocytes through the processes of packaging, release, uptake, and dispersion.* J Invest Dermatol, 2012. **132**(4): p. 1222-9.
17. Slominski, A., et al., *Melanin pigmentation in mammalian skin and its hormonal regulation.* Physiol Rev, 2004. **84**(4): p. 1155-228.
18. Prota, G., *Melanins, melanogenesis and melanocytes: looking at their functional significance from the chemist's viewpoint.* Pigment Cell Res, 2000. **13**(4): p. 283-93.
19. Halaban, R., et al., *Abnormal acidification of melanoma cells induces tyrosinase retention in the early secretory pathway.* J Biol Chem, 2002. **277**(17): p. 14821-8.
20. Hearing, V.J. and M. Jimenez, *Mammalian tyrosinase--the critical regulatory control point in melanocyte pigmentation.* Int J Biochem, 1987. **19**(12): p. 1141-7.
21. Pillaiyar, T., M. Manickam, and V. Namasivayam, *Skin whitening agents: medicinal chemistry perspective of tyrosinase inhibitors.* J Enzyme Inhib Med Chem, 2017. **32**(1): p. 403-425.
22. Howitz, J., et al., *Prevalence of vitiligo. Epidemiological survey on the Isle of Bornholm, Denmark.* Arch Dermatol, 1977. **113**(1): p. 47-52.
23. Mehta, N.R., et al., *Epidemiological study of vitiligo in Surat area, South Gujarat.* Indian J Med Res, 1973. **61**(1): p. 145-54.
24. Majumder, P.P., J.J. Nordlund, and S.K. Nath, *Pattern of familial aggregation of vitiligo.* Arch Dermatol, 1993. **129**(8): p. 994-8.
25. Porter, J.R., et al., *Psychosocial effect of vitiligo: a comparison of vitiligo patients with "normal" control subjects, with psoriasis patients, and with patients with other pigmentary disorders.* J Am Acad Dermatol, 1986. **15**(2 Pt 1): p. 220-4.
26. Kent, G. and M. Al'Abadie, *Psychologic effects of vitiligo: a critical incident analysis.* J Am Acad Dermatol, 1996. **35**(6): p. 895-8.
27. Le Poole, I.C., et al., *Review of the etiopathomechanism of vitiligo: a convergence theory.* Exp Dermatol, 1993. **2**(4): p. 145-53.
28. Hercogova, J., R.A. Schwartz, and T.M. Lotti, *Classification of vitiligo: a challenging endeavor.* Dermatol Ther, 2012. **25 Suppl 1**: p. S10-6.

29. Van Driessche, F. and N. Silverberg, *Current Management of Pediatric Vitiligo.* Paediatr Drugs, 2015. **17**(4): p. 303-13.
30. Malhotra, N. and M. Dytoc, *The pathogenesis of vitiligo.* J Cutan Med Surg, 2013. **17**(3): p. 153-72.
31. Njoo, M.D. and W. Westerhof, *Vitiligo. Pathogenesis and treatment.* Am J Clin Dermatol, 2001. **2**(3): p. 167-81.
32. Alkhateeb, A., et al., *Epidemiology of vitiligo and associated autoimmune diseases in Caucasian probands and their families.* Pigment Cell Res, 2003. **16**(3): p. 208-14.
33. Jin, Y., et al., *Variant of TYR and autoimmunity susceptibility loci in generalized vitiligo.* N Engl J Med, 2010. **362**(18): p. 1686-97.
34. Birlea, S.A., et al., *Genome-wide association study of generalized vitiligo in an isolated European founder population identifies SMOC2, in close proximity to IDDM8.* J Invest Dermatol, 2010. **130**(3): p. 798-803.
35. Jin, Y., et al., *Common variants in FOXP1 are associated with generalized vitiligo.* Nat Genet, 2010. **42**(7): p. 576-8.
36. Quan, C., et al., *Genome-wide association study for vitiligo identifies susceptibility loci at 6q27 and the MHC.* Nat Genet, 2010. **42**(7): p. 614-8.
37. Jin, Y., et al., *Genome-wide analysis identifies a quantitative trait locus in the MHC class II region associated with generalized vitiligo age of onset.* J Invest Dermatol, 2011. **131**(6): p. 1308-12.
38. Jin, Y., et al., *Genome-wide association analyses identify 13 new susceptibility loci for generalized vitiligo.* Nat Genet, 2012. **44**(6): p. 676-80.
39. Tang, X.F., et al., *Association analyses identify three susceptibility Loci for vitiligo in the Chinese Han population.* J Invest Dermatol, 2013. **133**(2): p. 403-10.
40. Cheong, K.A., et al., *Three new single nucleotide polymorphisms identified by a genome-wide association study in Korean patients with vitiligo.* J Korean Med Sci, 2013. **28**(5): p. 775-9.
41. Majumder, P.P., S.K. Das, and C.C. Li, *A genetical model for vitiligo.* Am J Hum Genet, 1988. **43**(2): p. 119-25.

42. Spritz, R.A., et al., *Novel vitiligo susceptibility loci on chromosomes 7 (AIS2) and 8 (AIS3), confirmation of SLEV1 on chromosome 17, and their roles in an autoimmune diathesis.* Am J Hum Genet, 2004. **74**(1): p. 188-91.
43. Birlea, S.A., et al., *Comprehensive association analysis of candidate genes for generalized vitiligo supports XBP1, FOXP3, and TSLP.* J Invest Dermatol, 2011. **131**(2): p. 371-81.
44. Yun, J.Y., et al., *Transforming growth factor beta receptor II (TGFBR2) polymorphisms and the association with nonsegmental vitiligo in the Korean population.* Int J Immunogenet, 2010. **37**(4): p. 289-91.
45. Liang, C., et al., *Autophagic and tumour suppressor activity of a novel Beclin1-binding protein UVRAG.* Nat Cell Biol, 2006. **8**(7): p. 688-99.
46. Lerner, A.B., *Vitiligo.* J Invest Dermatol, 1959. **32**(2, Part 2): p. 285-310.
47. Al'Abadie, M.S., et al., *Neuropeptide and neuronal marker studies in vitiligo.* Br J Dermatol, 1994. **131**(2): p. 160-5.
48. Morrone, A., et al., *Catecholamines and vitiligo.* Pigment Cell Res, 1992. **5**(2): p. 65-9.
49. Gokhale, B.B. and L.N. Mehta, *Histopathology of vitiliginous skin.* Int J Dermatol, 1983. **22**(8): p. 477-80.
50. Ingordo, V., et al., *Vitiligo and autoimmunity: an epidemiological study in a representative sample of young Italian males.* J Eur Acad Dermatol Venereol, 2011. **25**(1): p. 105-9.
51. Le Poole, I.C., et al., *Presence of T cells and macrophages in inflammatory vitiligo skin parallels melanocyte disappearance.* Am J Pathol, 1996. **148**(4): p. 1219-28.
52. Mohammed, G.F., A.H. Gomaa, and M.S. Al-Dhubaibi, *Highlights in pathogenesis of vitiligo.* World J Clin Cases, 2015. **3**(3): p. 221-30.
53. Gauthier, Y., et al., *Melanocyte detachment after skin friction in non lesional skin of patients with generalized vitiligo.* Br J Dermatol, 2003. **148**(1): p. 95-101.
54. Halder, R.M. and J.L. Chappell, *Vitiligo update.* Semin Cutan Med Surg, 2009. **28**(2): p. 86-92.
55. Oetting, W.S., *The tyrosinase gene and oculocutaneous albinism type 1 (OCA1): A model for understanding the molecular biology of melanin formation.* Pigment Cell Res, 2000. **13**(5): p. 320-5.

56. Cui, J., Y. Arita, and J.C. Bystryn, *Characterization of vitiligo antigens.* Pigment Cell Res, 1995. **8**(1): p. 53-9.
57. Lemon, W.J., et al., *Single nucleotide polymorphism (SNP) analysis of mouse pulmonary adenoma susceptibility loci 1-4 for identification of candidate genes.* J Med Genet, 2003. **40**(4): p. e36.
58. Duan, X., et al., *An improved polymerase chain reaction-restriction fragment length polymorphism assay for the detection of a PON2 gene polymorphism.* Biomed Rep, 2016. **5**(1): p. 133-135.