Tyrosinase gene polymorphism in genetic susceptibility towards Vitiligo

Submitted in partial fulfillment of the requirement for the Degree of Bachelor of Technology

In

Biotechnology

Submitted by

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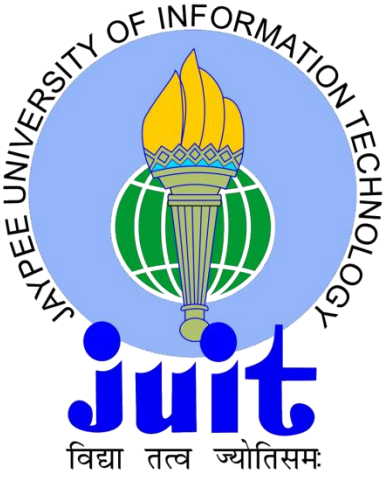

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CERTIFICATE

This is to state that the work titled "**Role of TYR gene variants (rs6336 and rs61753185) in genetic susceptibility to vitiligo**", submitted by "**Ankit Sharma and Sachin Sharma**" in partial indulgence for the award degree of **BachelorsofBiotechnology**of Jaypee University of Information Technology, Waknaghat has been conceded out under my direction. This work has not been submitted moderately or exclusively to any other Institute for the honor of this or any other degree of diploma.

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Date:

Place: Waknaghat

SUMMARY

Vitiligo, a common depigmentation disorder, caused by loss of melanocyte from the lesion site. Variousaspects are thought to play a role in inducing Vitiligo. Up regulated expression of tyrosinase in the melanocytes from perilisonal skin as compared to normal skin of vitiligo patient. Genetic variations in genes may contribute to disease pathogenesis. In this study, we investigated the association of tyrosinase gene polymorphism rs6336and rs61753185 with vitiligo susceptibility among north India population.

For the study we recruited 103 patients with vitiligo and 92 matched control samples without any history of vitiligo or any other autoimmune disorder .Informed consent from all the patients were collected. We showed that the two variants in TYR gene do not influence the susceptibility to vitiligo in an individual. We took help of many techniques which made our work convenient. Through the advantages of polymerase chain reaction- restriction fragment length technique, genotypes were effectivelyattained for the cases and controls.

Accordingly, appropriate statistical approaches were applied to explain the relation of variants with the susceptibility of the allelic frequencies of these SNPs between cases and control were, rs6336: p=0.0236; rs61753185: p=0.5787, which suggested no association of minor allele of both the SNP with the disease. The results showed that the SNP rs6336 may have role in protection against disease while, the SNP rs61753185 did not show any association with vitiligo susceptibility

Signature of StudentSignature of Supervisor

Name **Name**

Date **Date**

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ABBREVIATION

ABBREVIATION	
bp	Base pair
ºC	Degree Celsius
CI	Confidence interval
DNA	Deoxyribonucleic acid
dNTP	Deoxyribose nucleotide triphosphate
EDTA	Ethylene diamine tetra acetic acid
EtBr	Ethidium Bromide
DHI	di-hydroxy indol
MITF	microphthalmia associated transcription
µl	Microlitre
mins	Minutes
mM	Millimolar
M	Molar
OR	Odds ratio
PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism
ROS	Reactive oxygen species
secs	Seconds
SNP	Single nucleotide polymorphism
TE buffer	Tris – EDTA buffer
UVR	Ultraviolet rays

CHAPTER 1

INTRODUCTION

INTRODUCTION

Vitiligo is an idiopathic skin depigmentation disorder affecting only 1% of the total population. It is characterized by milky white patches on the skin. It is caused by destruction of pigment-forming cells known as melanocytes at the lesional site. Melanocytes are the cells which are responsible for the color of the skin and their destruction leads to depigmentation of the skin.The exact cause of loss of these cells is still not known. But various theories which are further covered in the thesis explain the pathogenesis of vitiligo. The consequences of this disease are not life threatening but can have profound psychological consequences which may range from mild embarrassment to severe loss ofself-esteem and self-confidence.Various research groups are finding the main reason for the occurrence and cause of mechanism of skin pigmentation with a goal of developing some treatment of the disease. Various biological and chemical agents have been developed, that target this disease. Moreover, similar type of treatment cannot be given to all the patients as the disease has various types. Therefore, there is need to develop new biomarkers and various types of methods to predict the outcome of the therapy.

The goal of our work was to identify potential vitiligo susceptibility genes in the hope that this knowledge may lead to better understanding of disease pathogenesis and more targeted disease treatment.This project is an approach, small yet significant towards the new discoveriesabout vitiligo pathology. Aimingpredominantly over the genetics of the disease, we are presently, looking into the existence of the non-synonymous SNP as well as their association with vitiligo susceptibility.

Significance of the study: In India, incidence rate of vitiligo has been reported more than any other country around 8.8% (Sehgal and Shrivastava 2007). And treatments which are given to the patients are not satisfactory and are not cost effective. Moreover, vitiligo affects the life of patient and presence of milky white patch is just not the diagnosis of disease. Modification in single nucleotide in the DNA sequence may disturb how human progresses any kind of disease. If there is some relationship of the SNP in a gene related with the disease it can act as a prognostic marker for the estimation of a treatment result in an individual undergoing a vitiligo therapy.

CHAPTER 2

REVIEW OF LITERATURE

REVIEW OF LITERATUTE

Vitiligo is an acquired achromia of the skin, the etiology of which is obscure (A. M. A. Arch. Derm 1958). It is mainly characterized by appearance of milky white patches on skin caused by destruction of melanocyte which result in lack of melanin at the lesion site.

The following section explains the structure and function of skin, giving a clear view about melanocyte and the process of formation of melanin, which is the center of attraction of the project.

2.1 Skin- Melanocyte& Melanogenesis

Human skin is the largest organ and it consists of two main layers:

- **Epidermis**
- **Dermis**

Epidermisis a stratified squamous epithelium layer consists of keratinocyte along with melanocyte, dendrocytes (Langerhans cells and Granstein cells) and the dermis, which consist a layer of vascular tissues. Skin act as a physical and chemical barrier and provide protection from harmful environmental stress such as DNA damaging harmful UV rays (Costin and hearing 2007). Melanocytes are one of the cells which help the skin to play its role and protect us. (Lin and Fisher 2007). Melanocytes, located at the basal layer of epidermis, functionally linked to corefibroblasts in the dermis and to keratinocyte in overlying epidermis, are largelyaccountable for the pigment of the skin and hair, and thus contribute to the skin presence and provide defense against ultraviolet radiation (Lin and Fisher 2007).

Melanocytes are obtained from the precursor cells called melanoblast which are present at the outer sheath of hair follicle. Melanoblasts basically originate from the neural crest of the embryonic ectoderm (Rawles 1947). Studies related to human embryogenesis demonstrates that undifferentiated melanoblast migrate towards developing epidermis around seventh week of gestation, and thenexist in in the basal epidermal layer (Holbrook, Underwood etal.1989; Suder and Bruzewic 2007).

2.1.1Melanogenisis:

Melanocyte cells are basically responsible for production of melanin by the process melanogeneis. Melanogenesis is a highly complex process which involves various enzymes and co-factors. Two different types of melanin are produced, Eumelanin and Pheomelanin. Eumelanin which is the major pigment,initiate in dark skin and black hair and Pheomelanin, which is related with the red hair and freckled skin phenotype.Theprocess of Melanogenisis occurs in the melanosomes. The first step which is rate limiting step in the process leads to conversion of L-tyrosine to DOPA (dihydroxyphenylalanine) catalyzed by tryosinase. Tyrosinase is regulated by the transcription factor named MITF. DOPA endures oxidation to form dopaquinone which further converted into dopachrome and then to DHI (5, 6 dihydroxy indole carboxylic acid). DHI and DHICA polymerizes to form eumelanin. Cysteine and glutathione reacts with dopaquinone to produce cysteinyldopas which undergo further cyclization to form benzothiazines and higher condensates giving rise to pheomelanins (Taieb 2000; Denat, Kadekaro et al. 2014).

Melanin production is triggered by ultraviolet rays present in sunlight. UV rays fall on the epidermal layer consisting of keratinocytes which leads to the activation of p53 tumor suppressor protein. Expression of POMC peptides is increased by activation of p53 in keratinocytes (Cui, Widlund et al. 2007).POMC is a precursor of melanotrophic peptides and adrenocorticotrophic hormone.α- MSH secreted by keratinocytes , competitively bind to melanocortin 1 receptor(MC1R) on melanocyte which activate cell signaling pathways, i.e. cAMP and MAP kinase pathway. cAMP fabrication leads to phosphorylation of cAMP reactive element binding protein (CREB) transcription factor family members. (Videira, Moura et al.2013).

"(CREB) transcriptionally activates various genes, encoding microphthalmia transcription factor (MITF), master regulator of melanogenesis. It acts as a transcription factor that is crucial to expression of numerous pigment enzymes i.e.tyrosinase. Melanocytes are highly in contact with approximately 36 keratinocytes. Figure 1 shows the melanocyte role and different signaling pathways that regulates melanogenesis.(Melanosomes containing the melanin migrate from the center of melanocyte cell body to the end of dendrites and deposit at keratinocytes"(Shajil, chatterjee et al .2006).

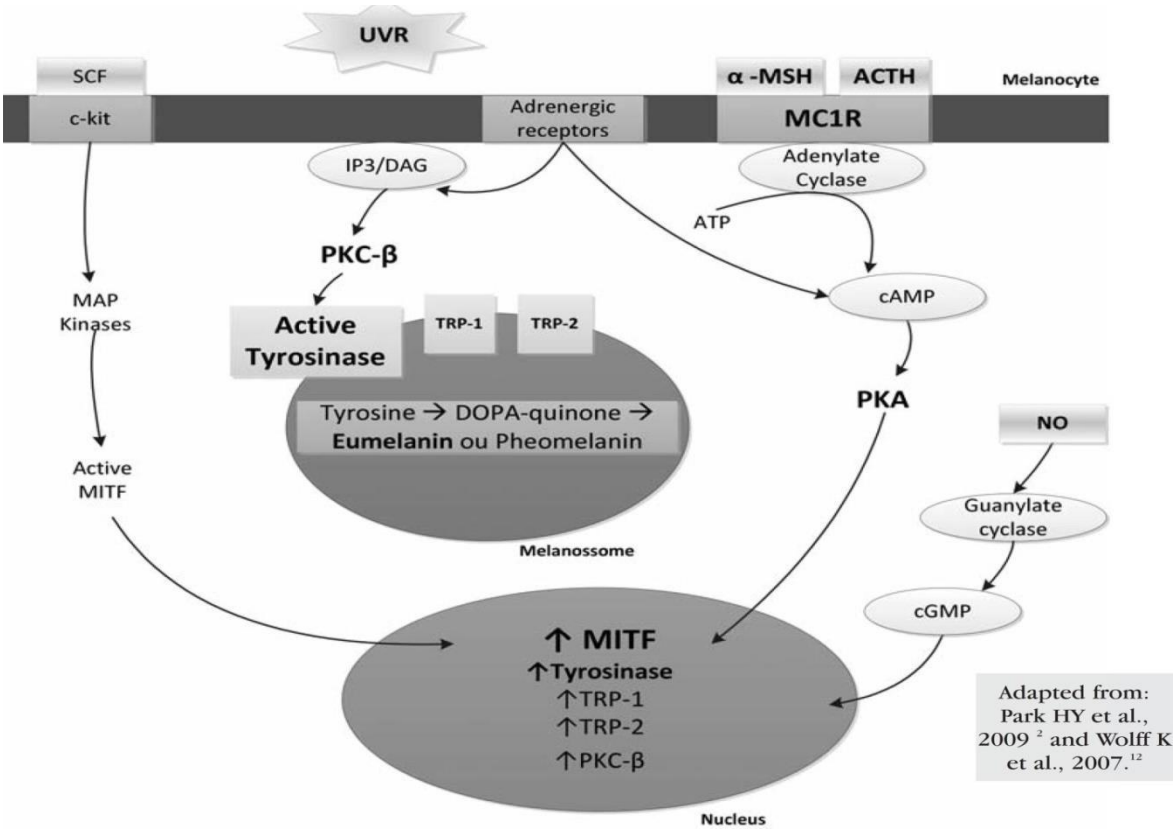

FIGURE 1: Melanocyte role and depiction of the different signaling pathways regulating melanogenesis: activation factors, receptors, second messengers and melanogenic enzymes. (Park HY et al., 2009 and Wolff K et al., 2007)

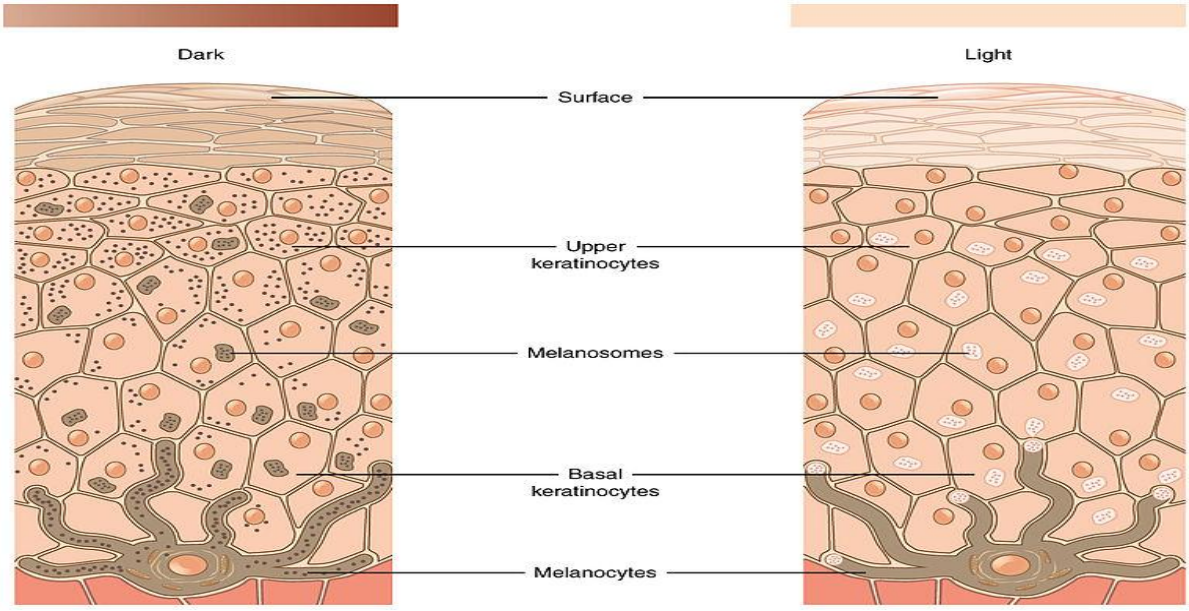

FIGURE 2: Melanocytes in dark & light skin.

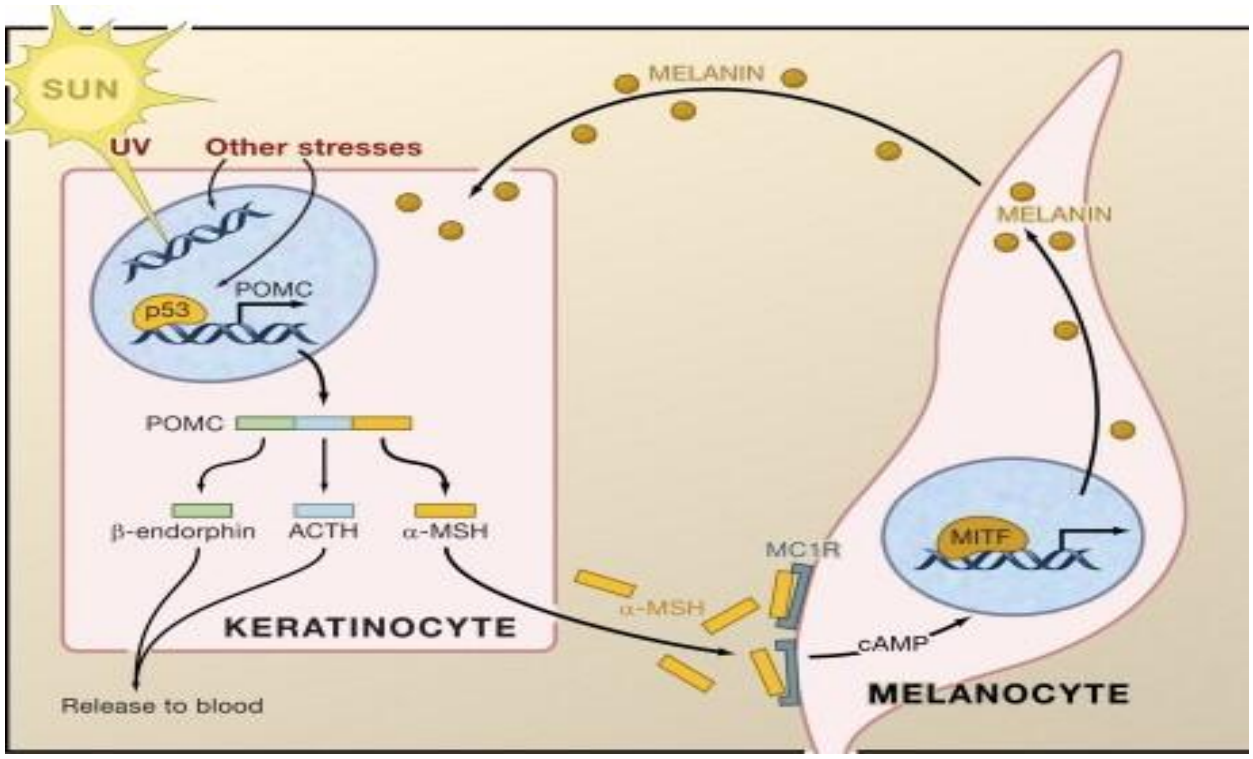

FIGURE 3: Overview of the melanogenesis process from the melanin production trigger to transfer of melanosome to keratinocyte.

2.1.2Melanocyte Distribution and Melanin content

Melanocyte density in the skin of different individuals 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 range of 12.2 to 12.8 melanocytes\mm. Though differences in melanocyte are described in dissimilar sites of the body in Asians. Melanin content responsible for skin color. Total of melanin existingin unirradiated skin from Asian and from White subjects are very alike, however the amount in Black skin it is found to be four folders higher. Difference in melanin production of various ethnic groups may be due to the difference in melanin synthesis, the type of pigment produced, and the mode, howmelanin is distributed within the keratinocytes. Iozumi et.al.reported higher melanin content in melanocytesin cultured cell from black skin compared to white skin and high melanin content and it is due to 10-fold higher catalytic efficiency of tyrosinase. Tyrosinase, the

rate limiting enzyme for synthesis of melanin is higher in black skin melanocytes than in Caucasian skin but there is no difference in amount of enzyme.

FIGURE 4: Human coloration (the main skin types): African, Asian, Caucasian, and American. (Left to right). (Costin and Hearing 2007)

A large number of genes (approximately 125) are involved in melanogenesis and mutations in any of them accounts to developmental pigmentary disorder (Bennett and Lamoreux 2003). In accumulation to the genes conveyed by melanocytes, signaling issues originating from adjacent tissues play important role in controlling melanogenesis (Tadokoro, Yamaguchi et al.2005). Change in any factor involved in the melanin production or in the regulation of melanocyte will lead to diseases such as albinism, vitiligo etc. (Videria, Mora et al.2013). Vitiligo is one of the example of such diseases where depigmentation of the skin occurs and both genetic and non-genetic factors play an important role.

2.2 VITILIGO: DEPIGMENT AND DISORDER

Vitiligo is an acquired, idiopathic and dermatological disorder which is characterized is seen by presence of white patches of different shape and seizes. It affects both genders male and female equally 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 20-30 years, approximately 65-75% earlier the age of 35 years. (Jaigirdar, Alam et al.2002)

2.2.1Prevalance and Incidence

The overall occurrence of this disease is around 1% however its incidence ranges from 0.1 to > 8.8% across the country and the world. Mainly thechiefpresence of thisdisorder has been recorded in Indians followed by Mexico and Japan. This may be because in India, there is a disgracelinked with vitiligo and affected person and their families particularly girls are easilydisliked for marital drive, so people out here go for early consultation(Cho,Kang et al.2000; Handa and dogra 2003; Shajil, Chatterjee et al. 2006). In India high rate of the disease was reported in Rajasthan and Gujarat that is around 8.8% (Shajil, Chatterjee et al. 2006)

2.2.2 Consequences

Vitiligo, considered as a depigmentation disease, is not life threatening disorder but is life challenging disorder which has many psychological consequences which may range from mild embarrassment to loss of self-confidence and self-esteem. (Ongenae, Beelaert et al. 2006) These symptoms are mostly are being observed in the racially darked skinned people where these white parches have become very prominent and can be seen clearly.

During social gatherings, vitiligo patients can experience feelings such as stress, anxiety and loss of self-consciousness and more embarrassment is faced by women as regarding their marriage. (Prasad and Dogra et al.2003; Dolatshi, Ghazi et al.2008).

2.2.3Clinical Classification of vitiligo

There is no uniform classification of this disease, appearance of white milky white patches on the skin of different shapes and sizes. Mostly, vitiligo is classified according to their distribution, pattern and classification. Majorly, the disease is best considered into segmental vitiligo and non-segmental vitiligo mentioned below in table1.

SEGMENTAL VITILIGO	NON SEGMENTAL VITILGO
Begins in childhood	Later onset
Autoimmunity rare	Autoimmunity associated
Frequently facial	Trauma prone site
No family history	Family history is present
Most affected area: Neck and trunk	Affected area neck elbow
Dermatomal, unilateral distribution	Bilateral distribution

Table1: Clinical subtypes of vitiligo. (Shajil, Chatterjee et al.2006)

Generally segmental vitiligo is considered as localized vitiligo and non segmental vitiligo is 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.

2.2.4 Vitiligo Pathogenesis -Theories of Vitiligo

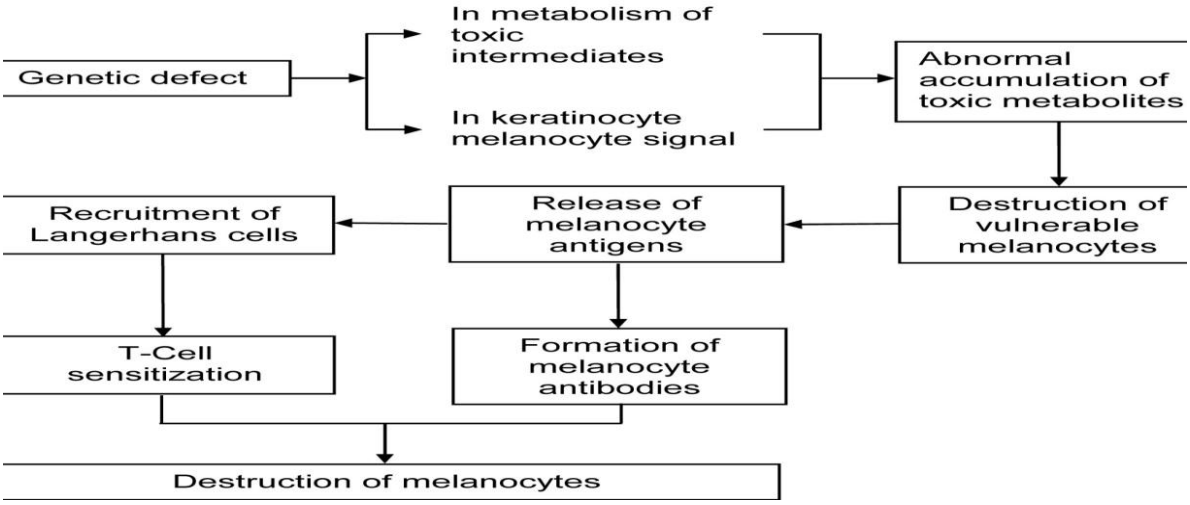

FIGURE 5: Pathogenesis of vitiligo (Mohammed GF et al (2015)

Neurochemical hypothesis:

Neurochemical hypothesis states that there is release of neurochemical factor such as norepinephrine and acetylcholine from the peripheral endings that stops the melanin production and gives rise to vitiligo. Both genetic and non-genetic factors contribute for high amount of neurochemical (Shajil, Chatterjee et al.2006; Panja, Bhattacharyaet al.2013). In vitiligo patients high concentration of norephrine is because of reduction of phenyl ethanolamine-N-methyl transferase activity and increase in amount of tyrosine hydroxylase activity.

Basically, this theory explains the segmental type of vitiligo which shows the unilateral distribution of vitiligo. Taieb suggested that segmental vitiligo is due to the cholinergic sympathetic nerves.

Oxidative stress theory:

This theory suggests that there is overproduction or inadequate removal of ROS. Oxidative stress theory is said to be the initial stage of vitiligo pathogenesis as the hydrogen peroxide is accumulated on epidermis of active vitiligo patients. Melanogenisis process in which large amounts of ROS are generated and removal of these species are controlled by antioxidant enzymes (Denat, Kadekaro et al.2014).

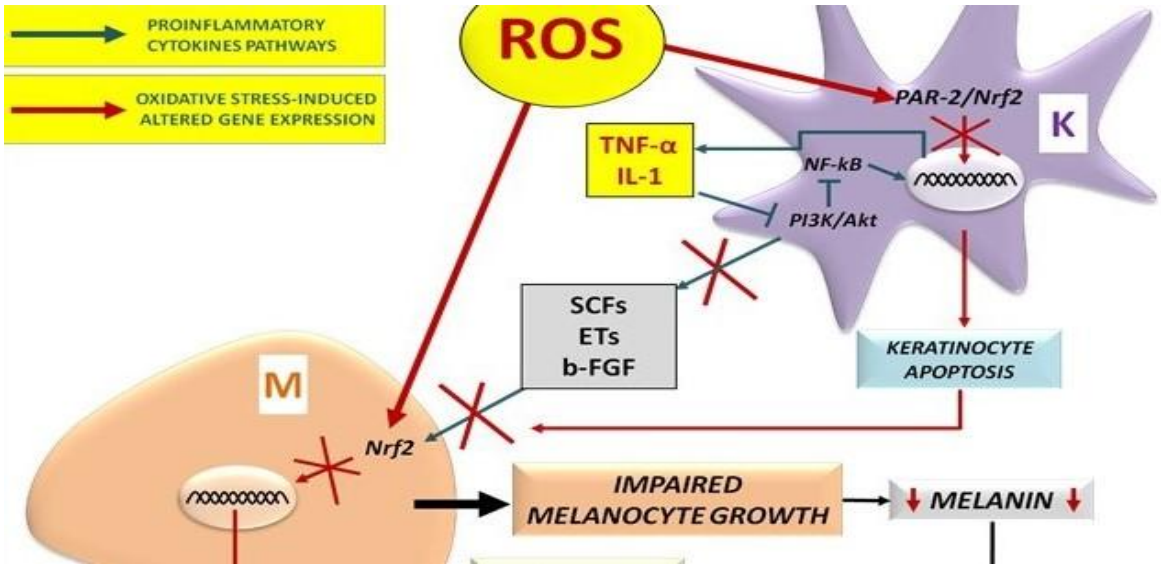

FIGURE 6: Induction of (ROS). (Denat, Kadekaro et al.2014)

If there is any defect in any of these anti-oxidant enzymes there will be accumulation of large radical species which will lead to production of vitiligo patients and also leads to occurrence of this hypothesis. It can be said that the biochemical defects in the melanin synthesis pathway, are cause of generation of reactive oxygen species (Passi, Grandinetti et al.1998).

Autoimmune hypothesis:

As, said earlier neural theory explains the segmental form of vitiligo, similarly, generalized vitiligo or non-segmental vitiligo occurrence are explained by this hypothesis. Epidemological studies reported that there is presence of autoantibodies against melanosomalproteins such as tyrosine the blood of vitiligo patients which supported this theory. This theory is also associated to other autoimmune diseases.

2.2.5 Genetics of Vitiligo:

Genetic factors show a great part in the etiology of the disease. Vitiligo is considered to be complex genetic disorder. Various studies revealed that a family history for vitiligo occurs in 38% of patients. (Njoo and Westerhof 2001). However, the strict mode of inheritance remains uncertain. Hereditary origin of the disease is regulated by a cluster of recessive alleles located at several unlinked autosomal loci which might betangled in the generation of oxidative stress, melanin biosynthesis, autoimmunity, that mightmutually confer the vitiligo phenotype. (Nath, Majumder et al.1994).

2.3 Tyrosinase gene:

The TYR gene deliversdirectionsfor making an enzyme called tyrosinase. This enzyme is existing in melanocytes, which are the cells that produce a pigment called melanin. Melanin is a substance that gives pigment to skin, hairs and eyes. Melanin is likewise originate in the light-sensitive tissue at the back of the eye (the retina), where it shows an important role in normal vision. The main step of tyrosinase is the production of melanin. It changes a protein (amino acid) called tyrosine to another compound called dopaquinone. Asequenceof reactions convert dopaquinone to melanin in the skin, hair follicles, the colored part of the eye (the iris), and the retina.

Oculocutaneous albinism

"More than 100 mutations in the TYR gene have been known in people with oculocutaneous albinism type 1 (King RA, Pietsch.2003). These mutations alter the normal production of melanin. Most TYR mutations reduce the activity of tyrosinase, preventing melanocytes from producing any melanin throughout life. These mutations cause a form of oculocutaneous albinism called type 1A (OCA1A). People with this form of albinism have white hair, light-colored eyes, and very pale skin that do not tan".

Chromosomal Location

"Cytogenetic Location: 11q14.3, which is the long (q) arm of chromosome 11 at position14.3. Molecular Location: base pairs 89,177,565 to 89,295,759 on chromosome 11" (Homosapiens Annotation Release 108, GRCh38.p7) (NCBI)

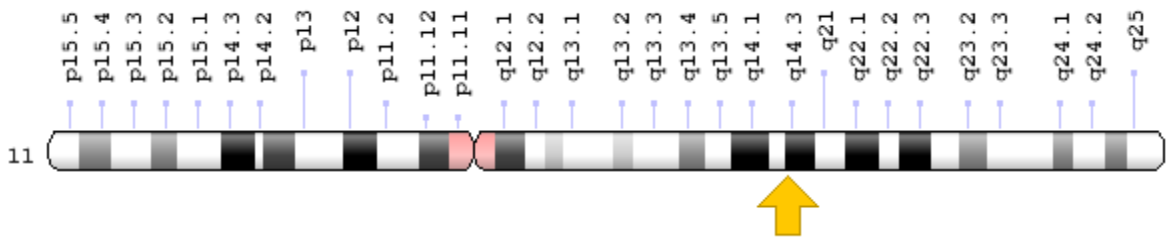

FIGURE 7:TYR gene in genomic location

(Source:http://atlasgeneticsoncology.org/Genes/TYRID42738ch11q14.html)

Other Terms for This Gene

- LB24-AB
- Monophenol monooxygenase
- OCA1A
- OCAIA
- SK29-AB
- Tumor Rejection Antigen AB
- TYRO_HUMAN

2.3.1 Single Nucleotide polymorphism:

Single Nucleotide polymorphism is a change in single nucleotide inDNA sequence that is present at a frequency of greater than 1% in a population. These are single base variation in coding regions that cause an amino acid substitution in the correspondent proteins. These missense variants constitute the most similar group of SNPs. By SNP genotyping information, researchers can investigate disease-associated genes and analyze the genetic structure of a particular population.

SNPs may be synonymous or can be silent or simply occur in the non-coding regions. Therefore, documentation of various variations in many genes and investigation of their effects may lead to better understanding of their impact on gene function. We have picked two SNPs (rs6336 and rs61753185) for genotyping by PCR-RFLP and to assess the association of these with vitiligo.

S.No	SNP ID	Transition	Ancestral	MAF
1	rs6336	C>T	C	T=0.0244/122
2	r61753185	G>A	G	A=0.0004/2

Table 2:SNPs (rs6336 &rs61753185) with their minor allelic frequency.

2.3.2 SNP Genotyping: PCR RFLP:

SNP genotyping is usually accomplished by many ways which include hybridization, allele-specific polymerase chain reaction, DNA sequencing, oligonucleotide ligation and endonuclease cleavage. Each of the above mentioned approaches has specific advantages and disadvantages. To perform genotyping of SNPs by applying PCR-RFLP, at SNP position there should be restriction site for any restriction enzyme that is easy and cost-effective method and can be used in small research laboratories. The PCR-RFLP methods allow very simple way of detecting point mutations.

PCR-RFLP involves steps:

- PCR: Biochemical reactions to form alleles-specific products
- RFLP: Detection procedures to identify the products

Polymerase chain reaction

The polymerase chain reaction is a technique in molecular biology that helps to amplify a part of a template DNA molecule. The technique depends upon thermal cycling which consists of cycles of repeated heating and cooling of reaction for DNA melting and enzymatic replication of the DNA. Basically there are three major steps in polymerase chain reaction, which are repeated for 35 cycles. This is done on an autosomal thermo cycler, which can heat and cool the tubes with the reaction mixture in a very short interval of time.

- **DENATURATION:**

  In this step, reaction is heated to 94-98^{0}C for 15-25 seconds. It results in melting of DNAby breaking the hydrogen bonds among the complementary bases, givingssDNA molecules.

- **ANNEALING**:

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

- **EXTENSION:**

  72^{0}C is the ideal 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 initation of DNA synthesis occurs at 3'-hydroxyl end of each primer. TAQ polymerase enzyme is used to extend the primers by joining the bases complementary to DNA strands.

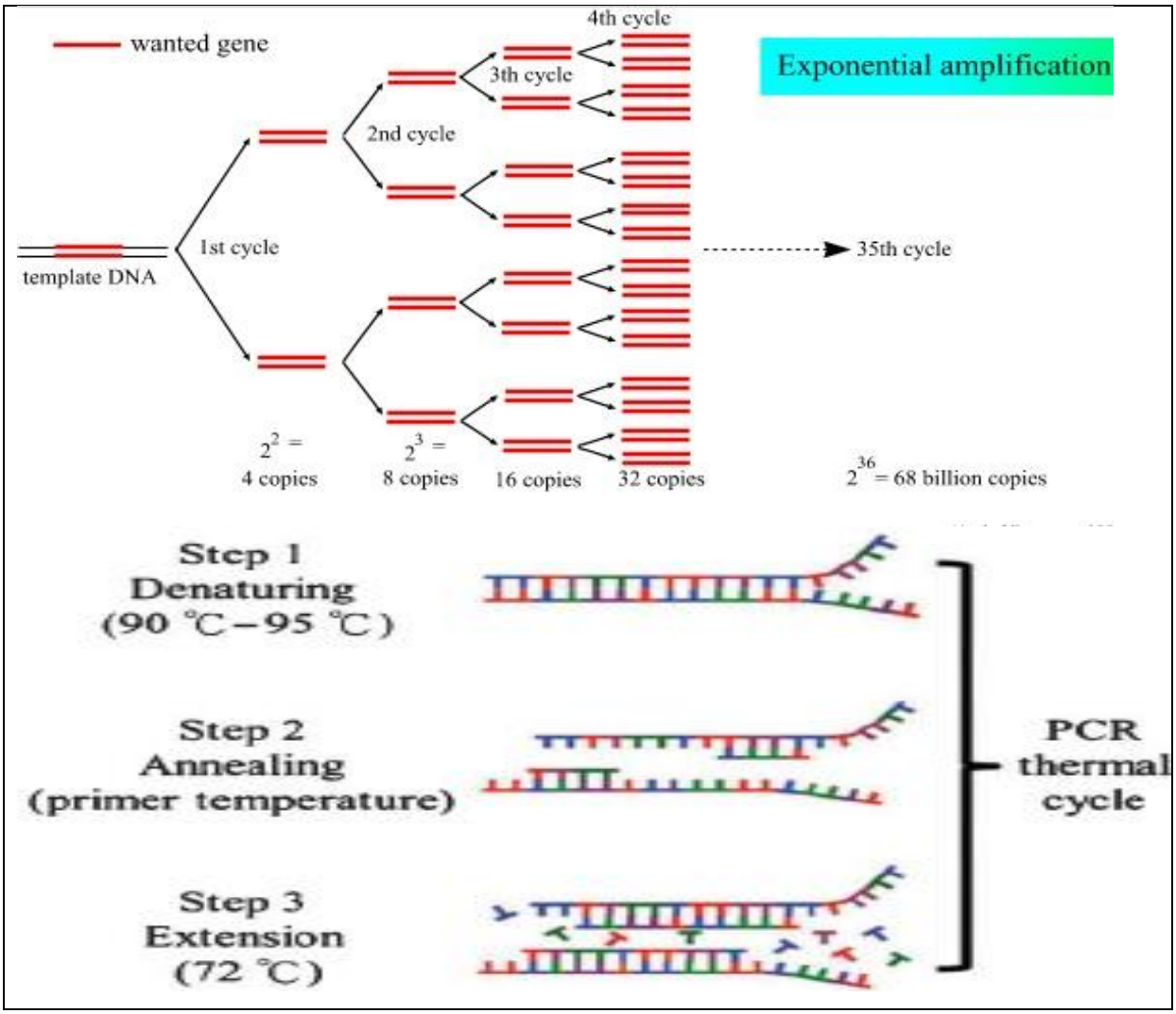

FIGURE 8: PCR Amplification cycle and major steps.

Restriction fragment length Polymorphism (RFLP):

RFLP arise because single base pair change can either generate or terminate the sites recognized by specific restriction enzymes, which leads to variation among individual in the length of the fragment formed from identical regions of genome. A single base pair change that is SNP is detectable genetic marker by RFLP because a mutated site is no longer cleaved by enzyme in question. Two chromosomes differ by such a mutation are then distinguishable o the basis of RFLP, which arises because a particular cleavage site is present in DNA molecule.

CHAPTER 3

OBJECTIVE

OBJECTIVE:

In our study we would discover the association of tyrosinase gene polymorphisms (rs6336 and rs61753185) with vitiligo susceptibility which might be used as prognostic markers for vitiligo susceptibility.

Objectives of study are:

- To forecast non-synonymous SNPs through in-silico exploration of the nsSNPs of *TYR* gene.
- Sample collection from vitiligo and healthy controls individuals and DNA isolation.
- Optimization and standardization of PCR cycling conditions for the SNPs rs6336 and rs61753185.
- To genotype SNPs rs6336 and rs61753185by PCR-RLFP in cases and control individual residing in North India
- To express the genetic relationship, if any, among polymorphism in tyrosine gene and exposureto vitiligo by statistical analysis.

CHAPTER 4

MATERIALS AND METHODS

OBJECTIVE 1:

PREDICTION OF nsSNPS BY SIFT

SIFT (Sorting Intolerant from Tolerant) acquired a query sequence and used multiple alignment evidence to forecast tolerant and deleterious substitutions for each position entered as the query sequence. SIFT server was accessible at (http://sift.jcvi.org/).

OBJECTIVE 2:

Study Population:

The study group comprised103 infected cases and 92healthy control samples. The diagnosis of vitiligo was clinically based on the presence of milky white patches on the skin. A overall of control persons with no history of vitiligo were included; they corresponds to patients with respect to sex, age and geographicdissemination. Conductance of study was approved by the ethics Institutional Review Board of the Dr. Rajendra Prasad Governmental College and Jaypee University of Information Technology. The significance of the study was described to all the fellows and written consent was achieved from all the cases and controls.

Sample Collection:

Venous blood was collected in EDTA coated vials from both infected and healthy subjects and carefully transported to laboratory under cold chain. Genomic DNA from blood samples was isolated using a standard inorganic method proposed by *Miller et al* and stored at -20^{0}C. Our study includes two SNPs (rs6336 and rs61753185).

Isolation of Genomic DNA from Whole Blood Sample

- 300µl blood sample was pippetted in 2ml eppendorf. Further Red blood cells lysis buffer was added and was kept for incubation on a rocker at a room temperature until RBCs completely lysed. (Appendix 2.6)
- Centrifugation was done at 13,000 rpm for 1 min to attain a creamish White blood cell pellet.

- Discard the supernatant and add 400µl of TE buffer and incubate for 56^0C for 30 min on water bath.(appendix 2.7).
- Subsequently 160 µl of 7.5 M ammonium acetate was added to solution and was mixed vigorously for about 1min on vortexer. Centrifuge the mixture at 13,000 rpm for 15 mins at RT, which results in separation of the precipitated protein as pellet.
- Supernatant then wastransferred into sterile micro-centrifuge. Absolute ethyl alcohol was added to this chilled native analysis.
- DNA obtained was further centrifuged at 13,000 rpm for 10 mins. The latter were subsequently washed into 150 µl of 70% ethanol.
- The dried pellet was dissolved IN 40µL TE Buffer by incubating at 65◦C for 10 mins. The dissolved DNA was stored at 20^0C till further use. (Appendix 2.8).
- The DNA quantification was done using the Nano Drop plus Spectrophotometer.
- The concentration of DNAwas checked by determining the absorbance of sample at A_{260}on a spectrophotometer.

OBJECTIVE 3:

Genotyping SNPs (rs6336 and rs61753185)

Genotyping of polymorphisms was carried out by PCR-RFLP method as both the SNPs consists naturalrestriction site.PRIMER3 software was used for the designing of primers (Forward and Reverse) mentioned in **Table 3.**

Primer Sequences		
	rs6336	**rs61753185**
Forward Primer	5’GAACCATGGGCTGTCTCTGGT3’	5’CCCTAGAGCCTGTGTCTCCT3’
Reverse Primer	5’TCCCGGTGCACAAAATGCAGA3’	5’TGGTCCCCAAAAGCCAAACT3’
Amplicon Size	364bp	271bp

TABLE 3: Nucleotide sequences of the Primers used

Polymerase chain reaction was performed in total volume of 12.5µl for both the SNPs. All the constituents used in the reaction for two SNPs (rs6336 and rs61753185) are mentioned in Table 4.

Reaction component	rs6336 per reaction volume (µl)	rs61753185 per volume reaction (µl)
Master mix	5	5
Primer (Forward)	0.4	0.4
Primer (Reverse)	0.4	0.4
MQ Water	5.7	5.7
DNA Template	1	1
Total	12.5	12.5

Table 4: Constituents used in PCR reaction for the amplification of DNA

PCR Cycling Conditions:

PCR cycling conditions for both the SNPs are mentioned in Table 5.

STEP	TEMPERATURE	TIME	CYCLES
Initial Denaturation	95^0C	2min	
Denaturation	95^0C	30sec	30cycles
Annealing	(rs61753185) 58^0C (rs6336) 58^0C	40sec 40sec	
Extension	72^0C	30sec	
Final Extension	72^0C	5min	
Final Hold	4^0C	Infinite	

Table 5: PCR cycling conditions for the amplification of genomic DNA.

Analysis of PCR products:

Agarose gel electrophoresis was conducted for analyzing of PCR products by using 1.5% gel containing ethidium bromide (0.5mg\ml). DNA marker of 100 bp was used. Electrophoresis was conducted for 30 to 45 min at 100 or 150 Volts in electrophoresis unit containing 1X TAE buffer. Bands were visualized under U.V. trans illuminator.

Restriction fragment length polymorphism (RFLP):

Amplified products were digested using restriction enzyme to detect which genotype is existing in specific individual. PCR products of rs6336 & rs61753185 are digested using NcoI & MspI respectively. Reaction conditions of RFLP for both the SNPs are given below in Table 6.

SNPs	Enzyme used	Restriction site	PCR-product	Cut smart buffer	Restriction enzyme	Nuclease free water
rs6336	NcoI	5′...C▼CATGG...3′ 3′...GGTAC▲C...5′	10	1.5	0.02	3.4
rs61753185	MspI	5′...C▼CGG...3′ 3′...GGC▲C...5′	10	1.5	0.05	3.4

Table 6: Components used for RFLP with respective restriction enzymes.

rs6336 (C>T)		rs61753185 (G>A)	
Genotype	**Band size (bp)**	**Genotype**	**Band size (bp)**
CC	224+140	**GG**	170+101
CT	224+140+364	**GA**	170+101+271
TT	364	**AA**	271

Table 7: Digested band pattern of SNP rs6336 and rs61753185.

OBJECTIVE 4: Statistical Analysis:

Statistical analysis was done using online tool (https://www.medcalc.org/calc/odds_ratio.php) to measure the relation of tyrosinase gene polymorphism towards vitiligo susceptibility. Fisher exact test was used to study the significant differences in genotype and allele frequencies between cases and controls (http://www.quantpsy.org/fisher/fisher.html). A chi-squared test was used to governobserved control genotype frequencies followed to Hardy-Weinberg equilibrium (HWE) expectation using online HWE calculator (http://www.had2know.com/academics/hardy-weinberg-equilibrium-calculator-2alleles.html). If $p<0.05$ observed frequency is considered to be in disequilibrium. Odds ratios and 95%CI wereconsideredtocalculate the risk associated with variant allele. Software Review Manager V5.3 was used to calculate odds ratio. The importance of odd ratio was determined by Z test and $p<0.05$was measured statistically significant.

CHAPTER 5

OBSERVATIONS & RESULTS

Observations & Results:

"103 diseased patients and 92 control subjects were genotyped for rs6336and rs61753185 in *tyrosinase* gene. The genotype distribution for both the SNP revealed no deviation from Hardy-Weinberg equilibrium in control population (p>0.05). Genotyping of the SNPs in the *TYR* gene revealed that the mutant allele T of SNPrs6336 was found in 6% of controls and 1% of cases. The mutant allele A of SNP rs61753185 was found in 7% of controls and 6% of cases. The allelic frequencies of these two SNPs between vitiligo and control were, rs6336: p= 0.0236; rs61753185: p= 0.5787, which suggested no association of minor allele of both the SNPs with vitiligo.**rs6336** and **rs61753185** variant alleles were found to have no relation to the risk of vitiligo".

Genotype /Allele	**Vitiligo (n/N[%])**	**Control (n/N[%])**	**Odds ratio (OR)**	**95% CI**	**p-value**
rs6336(C>T)					
CC	101/103[98]	83/92[90]	0.17	0.036 to 0.7891	0.0236
CT	2/103[2]	8/92[9]			
TT	0/103[0]	1/92[1]			
C	204/206[99]	174/184[94]			
T	2/206[1]	10/184[6]			
rs61753185(G>A)					
GG	92/103[89]	78/90[87]	0.79	0.3530 to 1.788	0.5787
GA	10/103[10]	11/90[12]			
AA	1/103[1]	1/90[1]			
G	194/206[94]	167/180[93]			
A	12/206[6]	13/180[7]			

Table 8: Allele and genotype distribution of the tyrosinase polymorphism in cases (N=103) and control (N=92)

PCR GRADIENT IMAGES:

rs6336

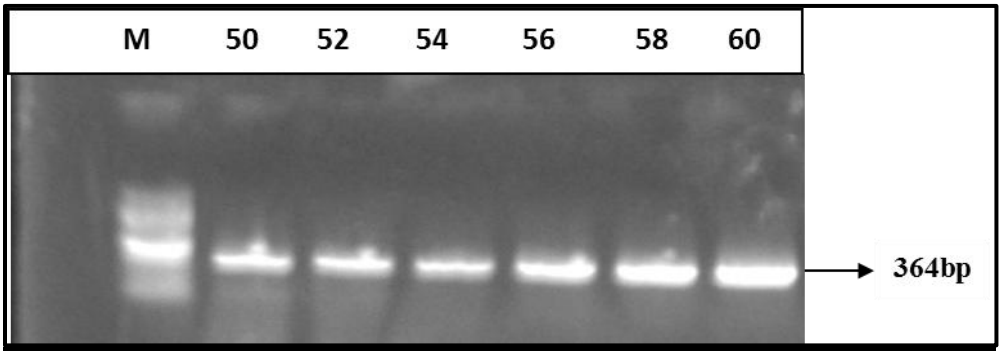

Figure 9(a): Amplification of DNA sample at different annealing temperatures 50ºC - 60ºC **Lane 2-Lane 7**; **Lane 1:** 100 bp ladder

rs61753185

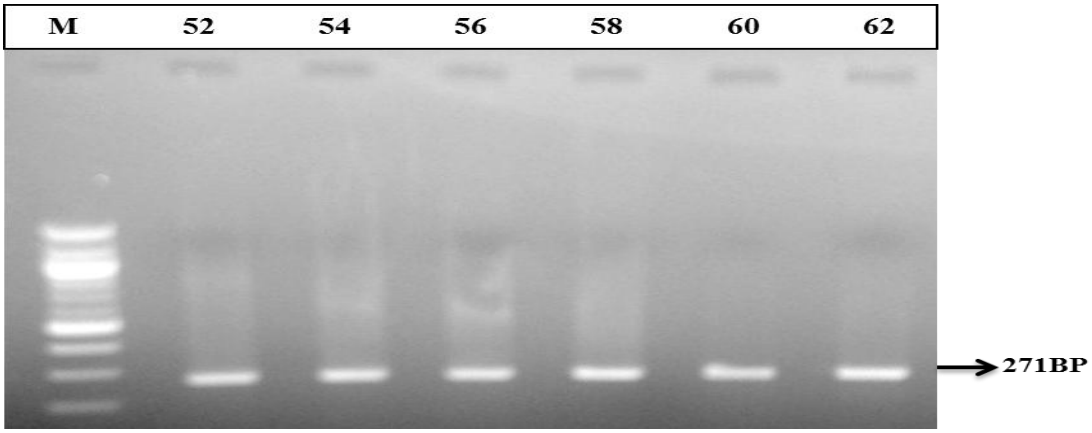

Figure 9(b): Amplification of DNA sample at different annealing temperatures 52ºC - 62ºC **Lane 2-Lane 7;Lane 1:** 100 bp ladder

AMPLIFIED PRODUCT

rs6336

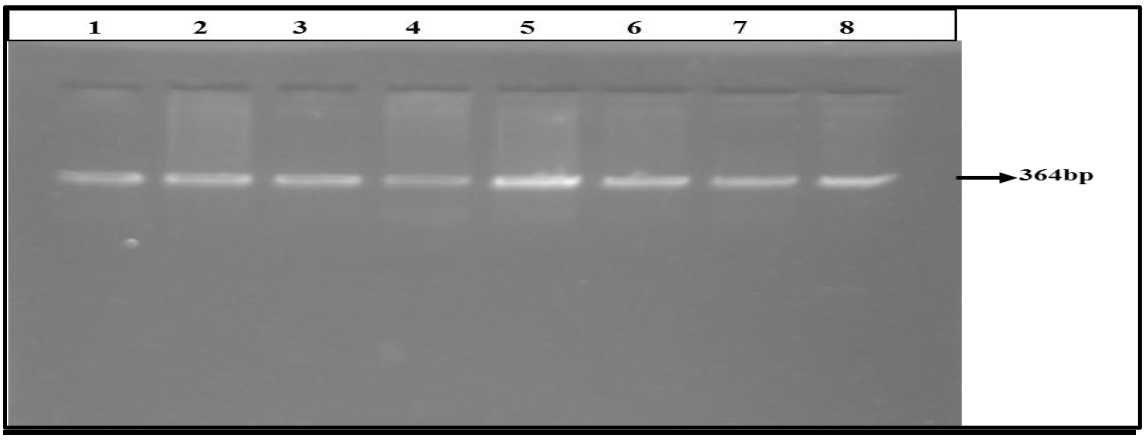

Figure 10(a): Amplification of 8 DNA samples at single annealing temperature 58ºC.**Lane 1-Lane 8**: DNA samples: 3-10.

rs61753185

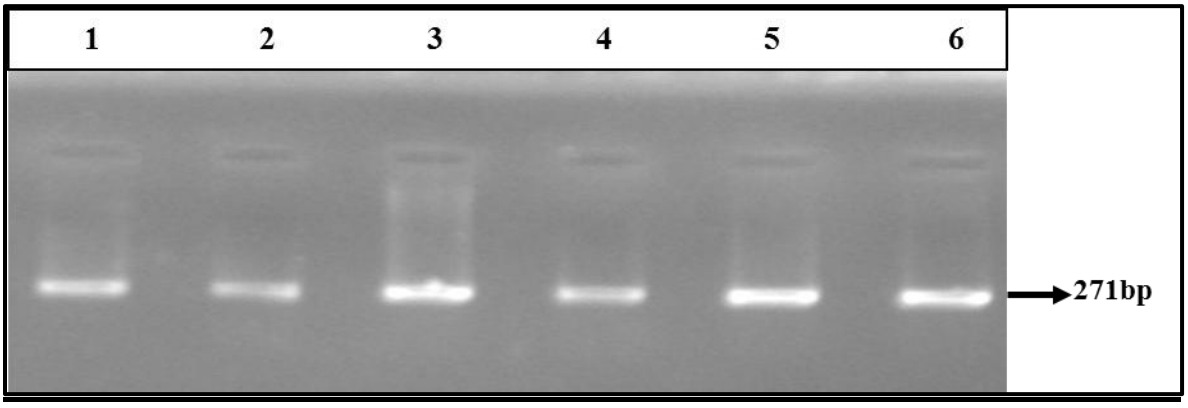

Figure 10(b): Amplification of 6 DNA samples at single annealing temperature 58ºC.**Lane 1-Lane 6**: DNA samples: 63-68.

RESTRICTION DIGESTION GEL IMAGES

rs6336

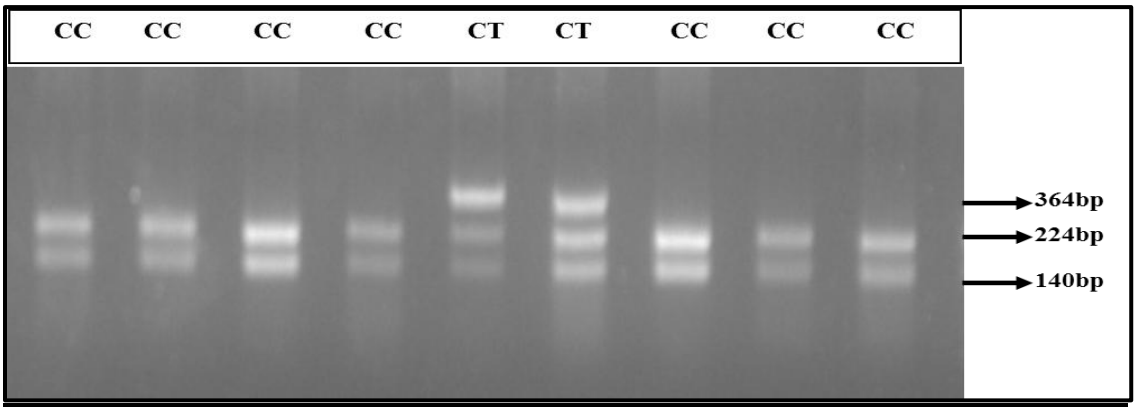

Figure 11(a): Digestion of 9 DNA samples at single annealing temperature 70ºC**.Lane 1-Lane 9:** DNA samples 268-276.

rs61753185

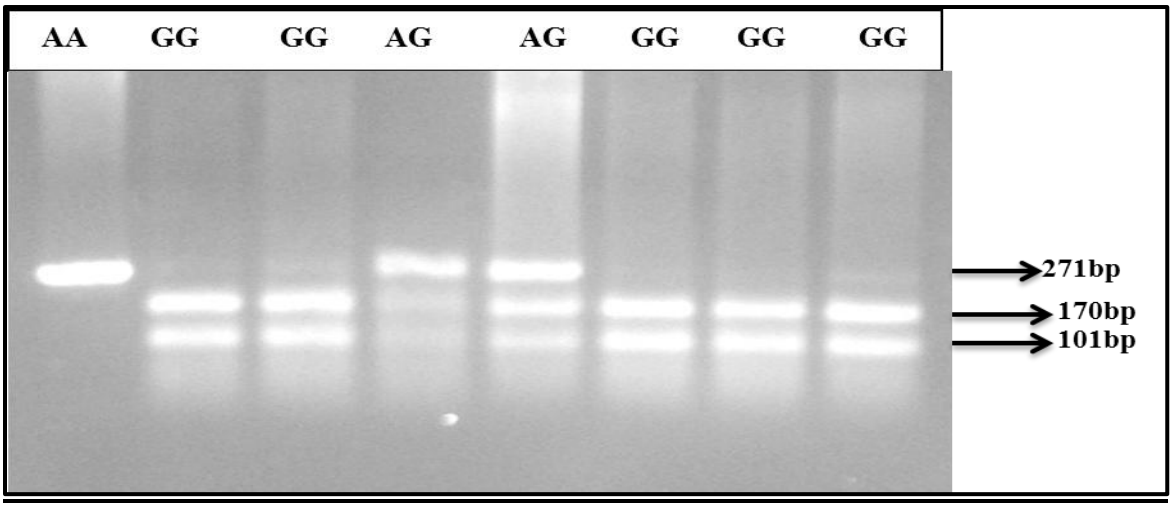

Figure 11(b): Digestion of 9 DNA samples at single annealing temperature 70ºC**. Lane 1-Lane 8:** DNA sample: 23-30.

CHAPTER 6

DISCUSSION

DISCUSSION:

Vitiligo is a prolongeddefaming disease which mainly affects melanocytes from epidermis layer, leading to the growth of white (de-pigmented) spots on the skin. It is a complex disorder with multiple genes and environmental triggers playing a role in inducing disease expression. A family based study with vitiligo pathogenesis tells that genetic factor is considered to be one of the determinants in vitiligo.

In the current study, we examined vitiligo patients for genetic variations in TYR gene. In our research, we focused on the association of SNP rs6336 and rs61753185 in TYR gene with the risk of vitiligo in north India population. The two SNPs considered here were chosen on the basis of earlier identified genetic relations with other physiopathological conditions. We showed that the two variants in TYR gene do not influence the susceptibility to vitiligo in an individual. However, the SNP rs6336 may have role in protection as seen from the observed odds ratio and p value. To the best of my knowledge, no previous report on association of TYR gene variant with vitiligo in any population is present. This is the first report linking TYR gene and it's SNP with vitiligo in north India population.

We took help of many techniques which made our work convenient. Through the advantages of polymerase chain reaction – Restriction fragment length polymorphism technique, genotypes were effectively obtained for the cases and controls. Subsequently, appropriate statistical methods were applied to explain the relation of variants with the susceptibility towards the disease.

The allelic frequencies of these SNPs between cases and control were, rs6336: p=0.0236; rs61753185: p=0.6226, which proposed no relation of minor allele of both the SNP with the vitiligo. The corresponding statistical parameters as given below;

rs6336: odds ratio (OR) =O.17; 95% confidence interval (CI) =0.0369-0.7891

rs61753185: odds ratio = 0.79and CI=0.3530 to 1.788. Therefore, the results showed that the SNP rs6336 may have role in protection against vitiligo while, the SNP rs61753185 did not show any association with vitiligo susceptibility

CHAPTER 7

CONCLUSION & FUTURE PROSPECTS

CONCLUSION & FUTURE ROSPECTS:

Computational study has now got very much importance to screen the disease specific SNP at molecular level. In this study *in silico* analysis has been done to predict deleterious nsSNP in the TYR gene. Also we genotyped the predicted nsSNPs in 103 vitiligo samples and 92 healthy samples to find out their role in vitiligo. The results from our study suggests that the nsSNP rs6336 may provide protection against vitiligo while, the other nsSNP rs61753185 has no role in vitiligo.

Documentation of novel genes that are related towards vitiligo susceptibility would be a great benefit and can act as one of the prognostic marker that will help identifying the individual at the risk of vitiligo at a former stage of disease. Besides, it will also assist to regulate the appropriate therapeutic and prophylactic approaches.

The work which we have done provides support for additional prospective studies to confirm the contribution of TYR gene variants in vitiligo. Therefore, it has been found that both the SNPs are not deleterious in their effects and do not have any potential to increase the risk of vitiligo. This approach paved the way of researchers in prioritizing SNPs, and also in identifying and short listing “candidate ns-SNPs" for further confirmatory analysis.

Moreover, as vitiligo is a multifarious disease, both gene-gene interactions and gene-environment relations may occur, and a single genetic variation is questionable to be sufficient to predict the overall risk. Consequently, additional research is desirable to explain the role of other functional SNPs of TYR gene and other genes tangled in similar biological pathways that may be involved in etiology of vitiligo.

APPENDIX

➢ GLASSWARES AND INSTRUMENTS

Glassware's

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

INSTRUMENTS

- PCR Tube stand
- Micro pipette
- Thermo-cycler
- Spinner
- Weighing balance
- Autoclave
- Incubator
- Laminar Air Flow
- Hot air oven
- pH meter
- Rocker
- Refrigerator (-80ºC,-20ºC,-4ºC)
- Vortex
- Centrifuge
- Water bath
- Agarose Gel Electrophoresis chamber
- UV trans illuminator

2. REAGENTS

- **Di-sodium ethylene diamine tetra acetate (0.5M,Ph 8.0)**
    - Take 186.1g of Na_2EDTA 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.

- **Tris (hydroxymethyl) aminomethane-chloride, Tris-Cl (1M,pH8.0)**
    - Take 121.2g Tris Base in 800ml of distilled water.
    - pH 8 was adjusted by adding 1N HCl.
    - Make up the volume to 1L by adding MQ water.

- **Tris-Cl (1M:pH 7.3)**
    - In 800ml of distilled water add 121.2g of Tris base.
    - ph 7.3 was adjusted by adding 1N HCl.
    - Make up the volume to 1L.
- **Ammonium Chloride, NH_4Cl (1M)**
    - In 800 MQ water dissolve 53.5g of ammonium chloride .
    - Make up the volume to 1L.

- **10% SDS**
    - In 70ml of distilled water dissolve 10g of SDS.
    - Heat to 68*C to mix the solution.
    - Make up the volume to 1L.
- **Red Blood Cell Lysis Buffer**
    - **COMPOSITION :**Tris 10mM , ph – 8.0;EDTA 1mM ; NH_4Cl 125mM, ph 8.0
        i. EDTA (0.5M) 2ml
        ii. Tris (1M,ph-8.0) 10ml
        iii. NH_4Cl(1M) 125ml

    Mix the above reagent in MQ water to obtain final volume of 1L.

- **Tris – EDTA(TE) buffer (pH 8.0)**
    - **COMPOSITION :** Tris 10Mm;EDTA 1mM , pH 8.0
        i. EDTA (0.5M) 2ml
        ii. Tris (1M , pH8.0) 10ML

Mix the above reagent in distilled water to obtain absolute volume of 1L

- **Tris – EDTA (TE) buffer (pH7.3)**
    - **Composition : Tris 10Mm;EDTA 1Mm,pH7.3**
        i. EDTA(0.5M) 2ml
        ii. Tris (1M, pH7.3) 10ml

Mix the above reagents in distilled water to obtain absolute volume 1L.

- **Ammonium Acetate (7.5M)**
    - In 20ml of MQ water dissolve 28.9g of ammonium acetate salt.
    - Final volume was adjusted to 50ml.

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