Genetic Analysis, In silico Gene Identification and Drug Designing of Inherited Vitiligo in Pakistan

Researcher

Shagufta Kanwal.

Reg #: 07/FBAS/MSBI/F07

Supervisors

Dr. Naveeda Riaz.

Mr. Muhammad Ismail.

Department of Bioinformatics

Faculty of Basic & Applied Sciences

International Islamic University Islamabad

2009

RNA - Research
Genetic regulation - Research
Molecular dynamics
Genetic Technic ques - Trends
RNA - Genetics

A.E
8.4.11

Genetic Analysis, In silico Gene Identification and Drug Designing of Inherited Vitiligo in Pakistan

Researcher

Shagufta Kanwal.

Reg #: 07/FBAS/MSBI/F07

Supervisors

Dr. Naveeda Riaz.

Mr. Muhammad Ismail.

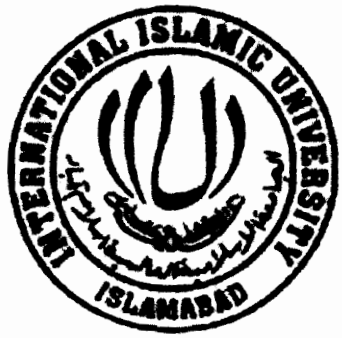
Department of Bioinformatics

Faculty of Basic & Applied Sciences

International Islamic University Islamabad

2009

Genetic Analysis and insilico Gene Identification and Drug Designing of Inherited Vitiligo in Pakistan



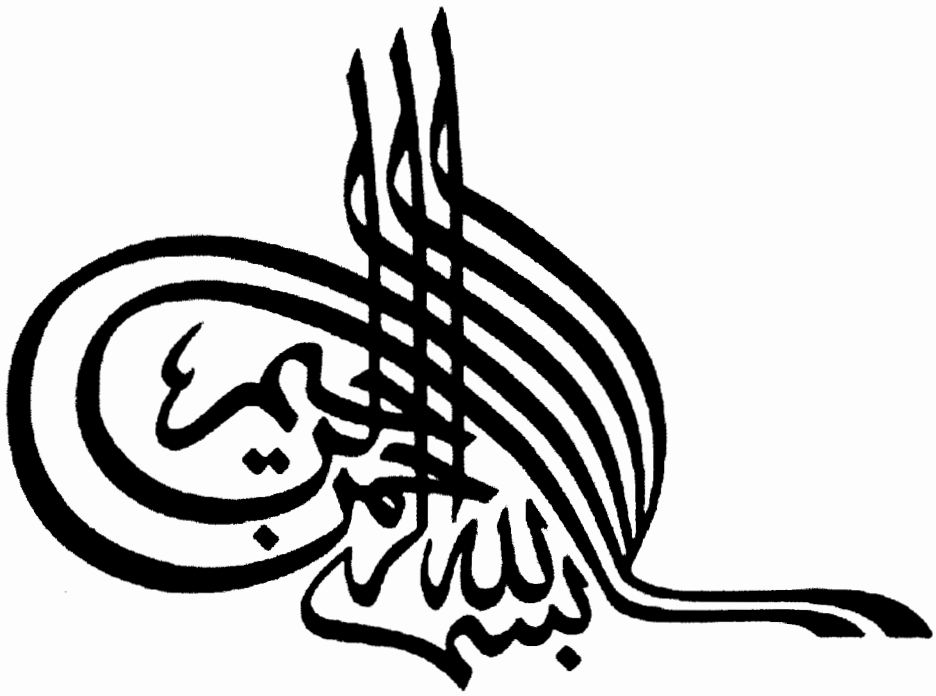
Shagufta Kanwal
Reg #: 07/FBAS/MSBI/F07

Submitted in partial fulfillment of the requirements for the Masters of Sciences in Bioinformatics at the Department of Bioinformatics, Faculty of Basic and Applied Sciences, International Islamic University Islamabad.

Supervisors: Dr. Naveeda Riaz.

Mr. Muhammad Ismail.

27th August, 2009



Verily, when He intends a thing, His Command is, "be", and it is!

(Ya-Sin: 82)

DECLARATION

I hereby declare that the work presented in the following thesis is my own effort, except where otherwise acknowledged, and that the thesis is my own composition. No part of the thesis has been previously presented for any other degree.

Shagufta Kanwal

Dedicated to my lovable and encouraging parents

Mr. Muhammad Rafique Mirza &

Mrs. Attiya Rafique

CERTIFICATE

Title of Thesis: Genetic Analysis and Insilico Gene Identification and Drug Desiging of Inherited Vitiligo in Pakistan

Name of Student: Shagufta Kanwal

Registration No: 07/FBAS/MSBI/F07

Accepted by the Department of Bioinformatics, Faculty of Basic and Applied Sciences, International Islamic University Islamabad, in partial fulfillment of the requirements for the Master of Sciences in Bioinformatics.

Viva Voce Committee

Chairman _____

(Dr. Irfan Khan)

Head of Department/ Supervisor _____

(Dr. Naveeda Riaz)

External Supervisor _____

(Mr. Muhammad Ismail)

Internal Examiner _____

(Dr. Asma Gul)

External Examiner _____

(Dr. Muhammad Ashraf)

(27th August, 2009)

ABSTRACT

Linkage or association studies of genetic disorders help us to find out candidate gene loci responsible for its inheritance throughtout a family. These genetic studies can be further analyzed by bioinformatics tools to broaden the vision of research. The splendours of bioinformatics have helped scientist to work out with a research of centuries in just few days in less time and cost. This research work reflects the genetic studies conducted on an inherited and progressive skin disorder called "Vitiligo" which is characterized by the destruction or inactivation of melanocytes, resulting in patches of depigmentation on skin. It is found in one study that vitiligo runs in families and mostly affected patients are 20% of the relatives especially their children and siblings are on highest risk. Linkage studies are conducted on three Pakistani families affected with vitiligo. These families were found to be affected with generalized vitiligo having white patches spread throughout the whole body. This disorder is found in these families in autosomal recessive fashion.

The linkage studies helped in the identification of a disease locus which carries a genetic factor responsible for this disorder. This disease linked locus 4q13-q21 is found, after scanning nine different chromosomal loci, which is further analyzed by the help of bioinformatics tools of gene finding to hit upon disease gene candidates depending upon the similarity with the known gene CATALASE. Six gene finding tools *i.e.* G2D, SUSPECT, PROSPECTR, POSMED, GENEDISTILLER and GENEWANDERER have been used to gather 250 genes that are linked to the disease locus on specific positions. Then these genes are prioritized on the basis of average weights or scores. Six genes are predicted to be responsible for causing vitiligo on this linked locus *i.e* nine members of Chemokine CXCL genes, cytokines IL8, IGJ and growth factors *i.e* FGF5, EREG and FRAS1.

Computational drug designing techniques have been applied to find any possible cure for vitiligo. Ligand screening has been done by searching in databases like drug bank and then docked with a mutated receptor protein to predict best possible ligand binding affinities on the basis of lowest energy values along with best confirmation in receptor binding pocket. In vitiligo, a defective antioxidant defence mechanism is suggested, according to which certain reactive oxygen species such as hydroxyl ions, super oxide ion radicals etc are produced in the body which show cytotoxic action on

melanocytes (skin cells) present in epidermis and also inhibit tyrosinase enzyme. In order to prevent free radical damage, the body must have antioxidant defence system. Catalase is an enzyme that effectively scavenges the toxic reactive oxygen species but its reduced production, in the skin cells of epidermis, has been found in patients affected with vitiligo. Antioxidant supplementation is required to boost the antioxidant body defence mechanism to prevent melanocyte damage by reactive oxygen species and this can cure vitiligo to provide hope to affectees. For this purpose psoralea coryfolia, a Chinese and an Indian herb, has been found which is being used as vitiligo cure for centuries. Its chemical constituents like psoralen, methoxalen and some other drug supplements like Tacrolimus (antioxidant), monobenzene and L-phenylalanine have been docked with mutated model of catalase to predict best binding affinities. I believe that the proposed drug targets will prove to be an effective cure for patients affected with vitiligo in future.

AKNOWLEDGMENTS

All praises are due to Almighty ALLAH , the Most Merciful and compassionate and His Holy Prophet Muhammad (Peace be upon him) , the most perfect and exalted among and of ever born on the surface of earth, who is for ever a torch of guidance and knowledge for humanity as a whole.

I am feeling pride to articulate some obsession about my honourable teacher and research supervisor Mr. Muhammad Ismail, Deputy Director, Institute of Biomedical and Genetic Engineering, Islamabad. He drove the work, asking for as much as could be delivered. His energy, optimism, intelligence and continuous encouragement at every step during the course of this whole project enabled me to achieve my goals. I must say that without his support and kind effort this task would have been impossible.

I am extremely obliged to record my feelings of thankfulness for my respected teacher and research supervisor Dr. Naveeda Riaz, Associate Professor, Department of Bioinformatics, Faculty of Applied Sciences, International Islamic University Islamabad, for her valuable contributions, critical insight, inspiring guidance, consistent encouragement and constructive suggestions throughout the course of this study.

Countless thanks to Ms. Amara Javed, Scientific Officer, Mr. Qaisar Mansoor, Scientific Officer and Mr. Syed Hasnain Haider, Principle Tech. II, Institute of Biomedical and Genetic Engineering, Islamabad, for their indispensable moral support and scholastic enthusiasm during my whole study.

I also wish thanks to Dr. Irfan Khan, Chairman, Department of Bioinformatics, International Islamic University Islamabad and Dr. Asma Gul, Associate Professor, International Islamic University Islamabad for their valuable suggestions.

I am thankful to my closest friends specially Ms. Sumaira Nishat because without her encouragement and assistence, this task was impossible for me, Ammara Jabeen, Maimoona Mushtaq, Tahira Noor, Saima Zubair, Adeela Safdar, Iffat Farzana Anjum and Awaisa Ghazal. The good time spent with them can never be erased from my memories.

No words can express my deepest gratitude to my dearest parents who have done and still doing their best to adorn me with the jewels of education, and for all they did for me; indeed everything. May they be included among whom, ALLAH loves (Ameen). Words become meaningless when I see them as icons of strength for being what I am today. I owe deep gratitude to my brothers, Mr. Shehzad Mirza, Mr. Kamran Mirza, Mr. Ali Raza and sisters in Law, Mrs. Sania Kamran for their unmatched support, love and prayers.

I also venerate the affected families, without their corporation and support the present research work could not be accomplished.

Road is long and life is short, may ALLAH give me the valor and opportunity to discover and face the truth (Ameen).

Shagufta Kanwal

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ACRONYMS

ACD	Acid Citrate Dextrose
ACE	Angiotensin Converting Enzyme
AIRE	Autoimmune Regulator
AIS1	Autoimmune Disease, Susceptibility to, 1
APS	Ammonium per Sulphate
ARG	Arginine
ASP	Aspartic Acid
CAT	Catalase
CHLC	Cooperative Human Linkage Center
COMT	Catechol-O-methyltransferase
CTLA-4	Cytotoxic T-Lymphocyte Antigen *4*
CXCL13	chemokine C-X-C motif ligand 13
DAG	Alpha-Dystroglycan
DigiCGA	Digital Candidate Gene Approach
EDTA	Ethylene Diamine Tetra Acetic Acid
EREG	Epiregulin
FBXO11	F-box protein 11
FGF5	Fibroblast Growth Factor 5
FOXD3	Forkhead box D3
FRAS1	Fraser syndrome 1
GLUE	Genetic Linkage User Environment
G2D	Genes2Diseases
GO	Gene Ontology
GTP-CH I	GTP-cyclohydrolase I
HIS	Histidine
HGMD	Human Genome Mutation Database
IGJ	Immunoglobulin J
IL8	Interleukin-8
OMIM	Online Mendelian Inheritance in Man
PCR	Polymerase Chain Reaction
R-Score	Relative Score

MHC	Major Histocompatibility complex
MDA	Malondialdehyde
OD	Optical Density
PosMed	Positional Medline
PROSPECTR	**PRiO**rization by **S**equence & **P**hylogenetic **E**xtent of Candida**Te** **R**egions
SLE	Systemic Lupus Erythematosus
T2D	Type 2 Diabetes
TAE	Tris Accetate EDTA
TAP1	Transporter Associated with antigen-Processing
TBE	Tris Boric EDTA
TEMED	Tetramethylehylenediamine
VAMAS1	Vitiligo-Associated Multiple Autoimmune Disease Susceptibility 1

INTRODUCTION

A disease is a condition of an organism that affects body functions, associated with specific symptoms and signs. It may be caused by either some external factor, such as invading organisms, or by internal malfunctions, such as autoimmune diseases. But the diseases which are caused by the mutations at gene level are normally heritable and may become lethal. Genetic disorders arise when one or both copies of a specific gene undergo an alteration known as mutation. Mutations in genes can be inherited directly from the parents. It happens by chance that two healthy copies of a gene are produced recessively or only one gene is defective showing dominant effect.

With the advent of Bioinformatics, the genetic studies got a big boost towards gene identification, structure prediction and drug designing. After the discovery of a disease locus, genes can be narrow down to find their association with the disease and then to find the possible cure for it. Such hypothetical predictions prove to be of great importance to conduct further verification and analysis in laboratories in very less time and cost. This signifies that bionformatics is the ardent need of modern era.

Vitiligo (MIM: 193200)

Vitiligo is an inherited and progressive skin disorder (commonly known as leukoderma), characterized by the destruction or inactivation of melanocytes, resulting in patches of depigmentation. In this disease condition, the immune cells attack melanocytes (skin cells) present on epidermis and destroy them which turns that area white in color. The mechanism of action of immune system is still unknown that how it attacks melanocytes in people affected with vitiligo. Small patches appear on the skin which with the passage of time, enlarge into larger patches. These patches are more prominent near the body parts like eyes, mouth, nose, arms, legs and genital organs. These patches are very much visible especially on the skin of the patient who has high contrast skin (Huggins *et al.,* 2005).

It is found in one study that vitiligo runs in families and mostly affected patients are 20% of the relatives especially their children and siblings are on highest risk. Like other hereditary diseases which run in families, it shows that some people

have got naturally affected genes which are susceptible to cause vitiligo. Sometimes some environmental factors like skin injury, sun burn or stress can also stimulate vitiligo causing genes in patients to develop vitiligo at any stage of life (Margaret and Wayne, 2007).

Vitiligo affects individuals of all ethnic groups with an estimated occurrence of 0.5% of the world population (Garsaud et al., 2001). It is believed that genetic factors are also responsible for the transmission of vitiligo (Majumder *et al.,* 1993; Kim *et al.,* 1996; Garsaud et al., 2001; Sun *et al.,* 2006). However, the genetic transmission of vitiligo follows a multifactorial pattern, involving genes that predispose to the expression of the disease after interaction with environmental factors (Garsaud *et al.,* 2001). Alkhateeb *et al.,* (2002) studied certain families affected with vitiligo in autosomal dominant with incomplete penetrance. However, a non-Mendelian pattern followed in familial aggregation of vitiligo shows its polygenic and mutifactorial inheritance (Spritz, 2007).

Onset of Vitiligo

According to Herane (2003), some people, approximately 30%, acquire this disease at the age of 20 years and some people about 14 % acquire it till the age of 10 years. The incidence decreases as the age grows, and some people approximately 10% acquire this disease at a later age *i.e.* 42 years. Many reports have showed that the mean age of onset is younger in females than in males. In certain communities, this disease frequency can run up to more than 20%. For most of the patients, it is found that vitiligo runs in the family, with more than 25% of patient's hereditary factors involved in it (Schallreuter *et al.,* 2008).

Associated Autoimmune Disorders

An immune response is thought to be involved in vitiligo progression in which melanocytes are attacked by the patient's own immune system, therefore, known as autoimmune disease (Margaret and Wayne, 2007). Le Poole *et al.* (2006) found a strong association of this disease in consanguinial marriages occurred in certain communities which can be the cause of vitiligo's transmission. Importance of genetic factors in vitiligo pathogenesis can be assessed by fact that the frequency of vitiligo found in fisrt degree relatives is approximately 7%. As vitiligo is a

polygenic inherited disorder, that's why it shows association with many other autoimmune diseases like alopecia, hashimoto's thyroiditis, pernicious anemia, adrenocortical insufficiency, diabetes mellitus and Addison's disease (Fain *et al.*, 2005). Hristakieva (2003) also suggested that vitiligo may be associated with leucotrichia, prematurely gray hair, halo nevi and psoriasis as well.

A syndrome, described by Rozycki *et al.* (1971), is of deafness which is associated with vitiligo. Witkop (1979) also reported a disorder named as black lock-albinism-deafness syndrome (BADS) which also confirms vitiligo association with deafness. Vitiligo is one of many other disorders associated with kabuki syndrome, a congenital mental retardation syndrome as described in family studies done by Ewart-Toland *et al.*, (1998), McGaughran *et al.*, (2001) and Genevieve *et al.*, (2004). Another syndrome associated with vitiligo is Vogt-Koyanagi-Harada with ocular and neurological abnormalities (Huggins *et al.*, 2005).

Studies of Abdallat *et al.*, (1980) and Lison *et al.*, (1981) showed evidences that spasticity syndrome is also associated with skin disorder like vitiligo, hair pigmentation, hyperpigmentation and premature graying of body hair. Labrune *et al.* (1992) reported a case of progressive vitiligo at the age of 12 years and retarded psychomotor development with urethral duplication.

Types of Vitiligo:

Huggins *et al.* (2005) separated vitiligo into two main categories; segmental and non-segmental types as shown in Table 1.1.

Non-segmental Vitiligo:

In this type of vitiligo, patient posses lesions and patches which are distributed symmetrically over whole body and remain getting enlarge throughtout life. Patches can either appear generalized or localized. Depigmented patches are usually widespread over the body in generalized vitiligo.

Generalized vitiligo is subdivided into:

i) Vitiligo Universalis:

A widespread type of vitiligo in which few pigmented patches stay over the body.

ii) Vitiligo Vulgaris: Vitiligo with scattered macules on the whole body.

iii) Acrofacial Vitiligo:

In acrofacial vitiligo, skin patches are found on the legs and arms and around mouth parts.

iv) Focal Vitiligo:

In this type of disorder, there is specific distribution of lesions or patches which are limited in quantity and location. At later stages, this type of vitiligo can be transformed into generalized vitiligo or can follow initial phases of clinical course.

Segmental Vitiligo:

Segmental Vitiligo shows has unilateral appearance and distributed over whole skin. Segmental vitiligo has early onset and spreads rapidly. According to Anna and Picardo (2006), Segmental vitiligo may be caused genetically due to either loss of production of melanocytes due to two reasons *i.e.* either anti-apoptotic proteins are lost or toxic neuromediators are released in higher concentration. Anbar *et al.* (2006) found that vitiligo affects both sexes almost equally in the non- segmental vitiligo group but females are found predominant in the segmental vitiligo group. Following are the major characteristics of vitiligo types as shown by Huggins *et al.* (2005).

	Segmental	**Nonsegmental**
Lesion or patch distribution	Unilateral,dermatomal lesions	Symmetrical,non-dermatomal lesions
Onset of Disease	Early	Any age
Course of disease	Rapid initial growth within 2 years with non-progression	Inconsistent rate of growth with new lesions throughout life
Autoimmune association	Rare	Strong

Table 1.1: Segmental versus non-segmental vitiligo (Huggins *et al.*, 2005)

Known Chromosomal Loci

Majumder *et al.* (1988) suggested a multiple recessive homozygous model, *i.e.* 4 diallelic unlinked loci which are responsible for the causation of vitiligo. Nath *et al.*, (1994) cross-validated the genetic model proposed by Majumder *et al.*, (1988)

to explain the nonmendelian inheritance of vitiligo. They postulated that 3 interacting autosomal diallelic loci are involved in the pathogenesis of vitiligo and that affected individuals were found to be recessive homozygotes at 3 loci. Arcos-Burgos *et al.* (2002) did linkage disequilibrium analyses and found 6p21.3–21.4 loci to be the major genetic factor of genes susceptible to vitiligo between markers D6S276 and D6S273.

Following are the vitiligo associated loci which have been discovered so far:

Autoimmune Disease, Susceptibility to, 1; AIS1 (MIM: 607836)

Alkhateeb *et al.,* (2002) and Fain *et al.,* (2003) performed genome-wide genetic linkage analysis and confirmed about AIS1 located at 14.4-cM interval on chromosome 1p32.2-p31.3 and found lod score of 2.90 located on chromosome 1p31as a major locus for vitiligo. Alkhateeb *et al.* (2005) found a mutation present in the promoter sequence of the FOXD3 gene, responsible for regulating melanoblast differentiation and suggested that increased transcriptional activity by the promoter gene variants can either suppress or alter the melanoblasts differentiaion in vitiligo patients.

Autoimmune Disease, Susceptibility to, 2; AIS2 (MIM: 608391)

Spritz *et al.* (2004) again confirmed the localization of AIS1 at 73.7 cM on chromosome 1p and two other loci were also found, out of which one is designated as AIS2 at 89.4 cM on chromosome 7 (lod = 3.73).

Autoimmune Disease, Susceptibility to, 3; AIS3 (MIM: 608392)

In the same study by Spritz *et al.* (2004), he identified another locus of susceptibility to autoimmune disease including vitiligo on chromosome 8 and designated it as AIS3 located at 54.2 cM on chromosome 8.

Autoimmune Disease, Susceptibility to, 4; AIS4 (MIM: 608392)

In a genome-wide linkage analysis, Chen *et al.* (2005) obtained a strong evidence for linkage of vitiligo to chromosome 4q13-q21. The multipoint linkage analysis yielded a maximum nonparametric lod score of 4.62 for the interval between markers D4S392 and D4S3042 and a maximum heterogeneity lod (hlod) score of

4.01 at the adjacent interval between D4S3042 and D4S2947 under a recessive model of inheritance.

Vitiligo-associated Multiple Autoimmune Disease Susceptiblity1; VAMAS1 (MIM: 606579)

Nath *et al.* (2001) hypothesized that Vitiligo and Systemic Lupus Erythematosus have some genetic effects and found linkage evidences at 17p13, where the parametric lod score was 3.64 and nonparametric linkage score was 4.02. Johansson *et al.* (2004) also performed a genomewide scan and found significant linkage to chromosome 17p12-q11, with lod score of 3.88 for marker D17S1294 and D17S1293 under a dominant inheritance model.

Jin *et al.* (2007) reported several mutations in the NALP1 gene located at 17p13. This gene regulates inflammation, cell death and its expression is very high in T cells and langerhan cells which play an important role in skin autoimmunity. Association analyses with vitiligo resulted in identification of NALP1 as a candidate gene. He identified association of NALP1 gene to vitiligo *i.e.* a nonsynonymous change in its coding region, leucine 155 to histidine, which is susceptible to both vitiligo alone and to autoimmune diseases.

Specific alleles of one or more genes of Major Histocompatibility Complex (MHC) at chromosome 6p21 are found associated with different autoimmune diseases. It is found that MHC is probably not a major locus susceptible to vitiligo in Caucasians, but there is also a weak evidence for linkage in Han Chinese families as well (Fain et al., 2005).

In addition to the genomewide linkage analyses, population based association analyses were used to investigate several candidate genes for vitiligo for example, CTLA-4 (Kemp *et al.,* 1999; Blomhoff *et al.,* 2005), CAT and TAP1 (Casp *et al.,* 2002, 2003), MC1R and ASIP (Na *et al.,* 2003), ACE (Jin *et al.,* 2004), and HLA (Zamani *et al.,* 2001; Tastan *et al.,* 2004; Zhang *et al.,* 2004). Linkage analysis evidences have been reported on 1p36, 4q13-q21, 6p21-p22, 6q24-25, 14q12-q13 and 22q12 (Chen *et al.,* 2005). Some other candidate genes associated with vitiligo are AIRE (Nagamine *et al.* 1997), GCH1 (Fernandez, 1997; Bandyopadhyay *et al.,*

2000), VIT1 (Le Poole *et al.,* 2001), COMT (Tursen *et al.,* 2002) and SLEV1 (Nath *et al.,* 2001).

Some of the HLA genes i.e genes of Transporter Associated with Antigen-Processing (TAP1) and immunoproteasome (LMP2/LMP7) are also found associated with vitiligo in early onset in Caucasian patients (Prahalad *et al.,* 2001). Blomhoff *et al.,* (2005) found that the cytotoxic lymphocyte antigen 4 (CTLA-4) genes, which regulates activation of T-cell, are also associated with vitiligo alongwith other autoimmune diseases.

Le Poole *et al.* (2001) found that reduced activity of the VIT1 gene (a fragment of FBXO11 gene containing a zinc finger motif) has been associated with increased susceptibility to vitiligo and is found on chromosome 2p16. As a result of nucleotide mismatch repair in melanocytes, he concluded that the decreased levels of FBXO11 in vitiligo cells were associated with increased levels of MSH6. The angiotensin converting enzyme (ACE) also shows association with vitiligo. The study done by Jin *et al.* (2004) suggested its strong association in Korean population.

One important candidate gene of vitiligo is catalase (CAT) whose decreased enzyme activity and increased production of hydrogen peroxide have been observed in the epidermis of vitiligo patients. The genetic association studies done by Casp *et al.* (2002, 2003) found that T/C single nucleotide polymorphism (SNP) present in exon 9 of the CAT gene is association with vitiligo. Then it was reconfirmed by Nikos *et al.* (2006) that a silent substitution at the third nucleotide in codon 389 in exon 9 of catalase gene *i.e.* GAC (Asp) → GAT (Asp) is responsible for causing vitiligo. The enzyme which is responsible for the synthesis of tetrahydrobiopterin is GTP-cyclohydrolase I, which regulates melanin biosynthesis and is also a candidate gene for vitiligo (Fernandez, 1997; Bandyopadhyay *et al.,* 2000).

BIOINFORMATICS ANALYSIS

Candidate Disease Gene Identification

Linkage analysis and association studies help scientists to generate increasingly large data sets suggesting all candidate genes responsible for causing a particular

disorder. Bioinformatics can be applied to analyse the data sets efficiently from the larger data sets to identify the best candidate disease genes for validation. Bioinformatics tools have been developed in this regard. They mine different data sources like sequence, biological, functional and expression data which help a researcher to narrow down search for best disease gene candidates (Kanehisa and Bork, 2003; Stein, 2003).

There are generally two categories of existing methods for candidate gene identification as follows:

i. Methods based on sequence functional annotation.
ii. Methods based on sequence similarity.

Different computational disease gene prioritization tools have been used by Tiffin *et al.* (2006) for the analysis of Type 2 diabetes (T2D) and obesity and generated a list of nine most appropriate candidate genes for T2D and five for obesity. The tools used were GeneSeeker, a web-based tool that filters positional candidate disease genes based on expression and phenotypic data from both human and mouse (van Driel *et al.*, 2005); G2D (Genes2Diseases; http://www.ogic.ca/projects/g2d_2/), a tool used to find candidate disease genes in any region of human genome (Perez-Iratxeta *et al.*, 2005); PROSPECTR and SUSPECTS, web-based servers used to prioritize candidate disease genes in large areas of interest (Adie *et al.*, 2006). Zhu and Zhao (2007) stepped towards advance research in the area of candidate gene finding approach, the strategies for breaking the information holdup by traditional candidate gene approach and also discussed a new candidate gene finding approach i.e. digital candidate gene approach (DigiCGA).

Docking Analysis of Different Compounds for Treating Vitiligo

Structure based or rational drug discovery has opened new ways of devising medications and drug targets in the market and are still progressing day by day with the growing speed of research. Structure based methods are used in drug development. The complete analysis of three-dimensional (3-D) structure of the target protein or complex is key point to focus upon because the structure and conformation of the target determines the binding pocket where the ligand binds. Computational methods have been developed to determine protein structures which

have enhanced the applicability, accuracy and speed of structural studies (Eswar N and Sali A, 2007).

The actual cause of vitiligo is still not known. The hypothesis of autoimmunity involved in vitiligo is most accepted one because autoantibodies have been discovered which affect melanocytes and tyrosinase (Fitzpatrick, 1997). In vitiligo, a defective antioxidant defence mechanism is suggested, according to which certain reactive oxygen species such as hydroxyl ions, super oxide ion radicals etc are produced in the body which show cytotoxic action on melanocytes (skin cells). They start a chain reaction to activate lipid peroxidation processes including malondialdehyde (MDA) in the body which can damage DNA and cell membrane finally leading to cytotoxicity, mutagenicity and cell death (Schallreuter *et al.*, 1994). The ultraviolet rays generate these reactive oxygen species which inhibit tyrosinase and induce damage to melanocytes (Schallreuter *et al.*, 1989; 1991).

Oxidative stress is built up due to increased production or decreased destruction of toxic reactive species. It is found that the destruction of melanocytes induce increased oxidative stress in body to activate an autoimmune. In order to prevent free radical damage, the body must have antioxidant defence system. Catalase is an enzyme that effectively scavenges the toxic reactive oxygen species but its reduced production has been found in cells of epidermis of patients affected with vitiligo. Hence, the antioxidant plays an important role in the damage caused by free radicals.

Antioxidant supplementation in vitiligo is postulated to boost up the working of antioxidant defence mechanism and prevent melanocyte damage by reactive oxygen species. Catalase is an enzyme that effectively scavenges the toxic reactive oxygen species. Low levels of catalase have been found in epidermis of patients affected with vitiligo. UV irradiation of the skin is known to trigger free radical generation and resultant melanocyte damage. Human melanocytes in cell culture are especially sensitive to oxygen radicals and melanocytes from vitiliginous skin require the addition of catalase to the culture medium to grow. The melanocytes from healthy skin proliferate without addition of catalase enzyme. Histological examination of normal and affected epidermis with vitiligo revealed the evidence for peroxidative damage to both keratinocytes and melanocytes.

In another study, it was found that antioxidants *e.g.,* alpha tocopherol, butylated hydroxytoluene etc, selectively inhibit the photochemical stage of erythema and hyperpigmentation (Potapenko *et al.,* 1989). It is very clear that the basis of these processes is the reaction of the psoralen-photosensitized oxidation of unsaturated lipids and the destruction of barrier functions of biomembranes, since the photochemical stage of these reactions is inhibited by the antioxidant. Thus, antioxidants can be used as a tool for improvement of psoralen photochemotherapy and to increase chances of vitiligo treatment (Yildirim *et al.,* 2003).

Psoralea corylifolia

Psoralea corylifolia, a Chinese and an Indian medicinal herb, is commonly used to improve general vitality. The seed and fruit contain psoralen. It is commonly known as "Babchi" in Pakistan. It has antibacterial and antifungal properties and therefore, used as laxative and stimulant. Modern research has shown that it is used in the treatment of skin disorders like vitiligo, leprosy, leucoderma and hair loss (Yeung, 1985; Duke and Ayensu, 1985; Chopra *et al.,* 1986).

Following compounds have been selected for computational analysis to find any possible cure for vitiligo.

i. Monobenzone

Monobenzone is the monobenzyl ether of hydroquinone which is medically used for skin depigmentation. Monobenzone is a white, tasteless crystalline powder which is soluble in alcohol but insoluble in water. It is found that the topical application of monobenzone in animals increases the excretion of melanin from melanocytes. The same depigmenting effect of the monobenzone is found in humans. Monobenzone is thought to cause destruction of melanocytes and permanent skin depigmentation; therefore, used to treat the loss of skin color in vitiligo. Monobenzone is used as a depigmenting agent when applied topically to the skin because it inhibits melanin produced by polymerization of oxidation products of tyrosine and dihydroxyphenyl compounds. Monobenzone permanently remove color from the normal skin located around skin affected with vitiligo (http://www.drugbank.ca/drugs/DB00600).

ii. Tacrolimus

Tacrolimus is known as an immunosuppressive drug, mainly used after organ transplant to combat against patient's immune system. It is used in the treatment of refractory uveitis after bone marrow transplants, atopic dermatitis and the skin condition like vitiligo (http://www.drugbank.ca/drugs/DB00864).

Taher *et al.* (2009) recently confirmed that tacrolimus is very effective in the curing vitiligo. It increases IL-10 expression in the lesion and patches of vitiligo and destroys melanocytes due to unchecked Th1 pathways. Radakovic *et al.* (2009) also showed that tacrolimus ointment appears to be more efficacious for facial vitiligo. A significant reduction of oxidative stress and an increase in antioxidant capacity in serum of patients treated with topical tacrolimus was observed by Lubaki et *al.* (2009) and found that tacrolimus is able to reduce the systemic oxidative stress independently from its repigmenting capacity.

iii. Methoxsalen

It is a natural compound of the type called furocoumarin which is found in Psoralea corylifolia. It is a photoactive compound so therefore generates small DNA segments or chunks in the presence of ultraviolet A. It is also known to treat psoriasis and vitiligo. Methoxsalen selectively inhibits the synthesis of DNA (http://www.drugbank.ca/drugs/DB00553).

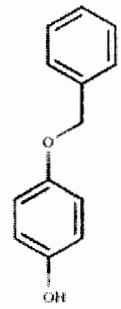
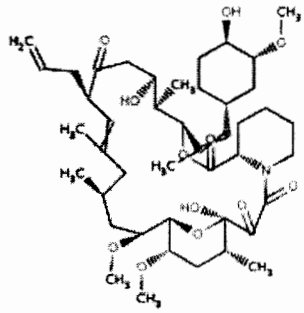
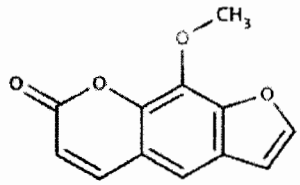
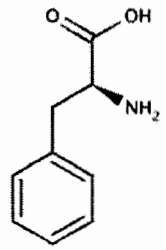
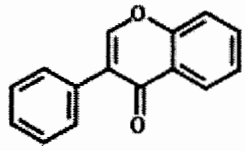
iv. L-Phenylalanine

L-Phenylalanine is a vital aromatic amino acid which is a precursor of melanin, dopamine, nor-adrenalin (also called nor-epinephrine) and thyroxine. The mechanism of antivitiligo activity of L-phenylalanine is not well understood. It is believed that L-phenylalanine stimulate the production of melanin in the vitiligo affected skin (http://www.drugbank.ca/drugs/DB00120).

v. Psoralen

It is also called psoralene. Psoralen is found naturally in the seeds of *Psoralea corylifolia.* It is mostly used in treatment for psoriasis, eczema and vitiligo as PUVA (Psoralen +UVA) therapy, due to high UV absorbance of psoralen. The psoralen is used to sensitise the skin first and then skin problem is cleaned up by applying UVA light.

Table 1.2: Potential drug targets for docking analysis

Drug Card/ Chem Spider	Name	Formula	Weight	Structure
DB00600	**Monobenzone**	$C_{13}H_{12}O_2$	200.2332	
DB00864	**Tacrolimus**	$C_{44}H_{69}NO_{12}$	804.0182	
DB00553	**Methoxsalen**	$C_{12}H_8O_4$	216.1895	
DB00120	**L-Phenylalanine**	$C_9H_{11}NO_2$	165.1891	
CS65255	**Psolaren**	$C_{15}H_{10}O_2$	222.2387	

Photochemotherapy with psoralens is an established first-line vitiligo treatment (http://en.wikipedia.org/wiki/Psoralen). Fitzpatrick (1997) also found that psoralens cause photosensitivity and on subsequent UV exposure of the affected skin, repigmentation results after many treatments.

In present study, Pakistani families with vitiligo have been ascertained. The data presented here includes linkage analysis carried out for all ascertained families, candidate disease gene identification for AIS4 by web based tools and computational analysis of psoralea corylifolia and other drug antioxidant and supplements used as possible treatment for vitiligo by the help of docking tools.

MATERIALS AND METHODS

The study was conducted in a very organized way. Informed consent was obtained from the parents of the affected children and all other family members who participated in the study. Written consent was also taken from the patients to publish their photographs showing their disease affected areas.

Families with Inherited Vitiligo

Three Pakistani families, referred hereafter as A, B and C affected with vitiligo, were studied from Punjab province of Pakistan. Information regarding family history of each family member was collected.

Pedigree Analysis

For genetic implication an extensive pedigree was constructed for each family by the standard methods described by Bennett *et al.,* (1995). The pattern of inheritance of Inherited Vitiligo was worked out by observing the segregation of these disorders within family. The exact genealogic relationships for all the affected individuals were obtained through personal interviews of elders of the families. Males were symbolized by squares and females by circles. The normal individuals were designated with unfilled symbols while the affected individuals by filled symbols. Each generation was indicated by Roman numeral. The individual within a generation were designated by Arabic numerals.

Blood sampling

Blood samples were drawn by 5 c.c. syringes and collected in vacutainers containing Acid Citrate Dextrose (ACD). The blood was stored at 4°C until DNA extraction.

Genomic DNA extraction

Genomic DNA was purified from peripheral blood lymphocytes according to phenol chloroform extraction method.

1. Five to 8 ml blood was collected in 50 ml falcon tube.
2. To the blood sample, three times of Cell lysis Buffer was added and was stored on ice for 30 minutes.

3. After chilling, centrifugation was carried out at 1200 rpm for 10 minutes at 4°C to separate white blood cells.
4. The supernatant was discarded and pellet was resuspended.
5. Firstly, 4.75 ml of STE was added and then 250 µl of 10% SDS was added drop-wise while vortex.
6. 10 µl of protinase-K (20 mg/ml) was added and was incubated overnight at 55°C.
7. On the following day, 5ml of mixture of equilibrated phenol and chilled chloroform-Isoamylalcohol (1:1) was added and tubse were shaken for 10 minutes and then were kept on ice for 10 minutes.
8. After chilling, centrifugation was carried out at 3200 rpm for 30 minutes at 4°C and aqueous layer was removed with cut tip and was transferred into separate labeled 15ml falcon tube.
9. 10 µl of RNase (10 mg/ml) was added to this aqueous layer and was incubated in water bath shaker at 37°C for 2 hours.
10. 250 µl of 10% SDS was added drop wise without vortex, then 10 µl of protinase-K (20 mg/ml) was added and incubated at 55°C for 1 hour.
11. Step 7 and 8 was repeated to obtain a clean supernatant containing genomic DNA.
12. The clear supernatant was transferred to a new 15ml falcon tube and DNA was precipitated by the addition of 500 µl of 10M Ammonium Acetate and 5 ml of Isopropanol.
13. The tube was placed at -20°C overnight.
14. On the following day, centrifugation was carried out at 3200 rpm for 60 minutes, supernatant was discarded and pellet was resuspended.
15. The pellet was washed with 5 ml of chilled 70% ethanol at 3200 rpm for 40 minutes at 4°C. Supernatant was discarded and pellet was dried.
16. The pellet was re-suspended in 10 mM Tris-HCl (PH 8.0).
17. Genomic DNA was quantified by taking Optical Density (OD) of sample at 260 nm and 280 nm by Spectrophotometer (Bio-Rad Laboratories, USA).
18. The dilution of 100 ng/µl of each stock sample was prepared for PCR amplification.

Genotyping

To determine the linkage or exclusion of the families for all known loci for vitiligo, a minimum of two microsatellite markers, each of the candidate regions of these loci were genotyped in all the available individuals of families. Microsatellite markers used for genotyping these families are shown in Table 2.1.

Microsatellite markers mapped by Cooperative Human Linkage Center (CHLC) were obtained from Research Genetics, Inc. (USA). The cytogenetic locations of these markers, their heterozygosity as well as the sizes of the amplified products were obtained from LDB Genetic Map (H.H. Stassen, Psychiatric University Hospital Zurich, Switzerland).

Polymerase chain reaction (PCR)

Polymerase chain reactions were performed in a total volume of 10µl, containing 40 ng/µl of genomic DNA from DNA dilutions, 0.25 µl of each primer (20 µM stock), 1 µl 10X PCR buffer with $MgCl_2$, (500 mM KCl, 100 mM of Tris-HCl pH 8.3, 15 mM $MgCl_2$, 0.01% (w/v) gelatin), 1 µl of dNTPs (2.5mM stock), 5.6 µl of dH_2O and 0.4 µl of Taq DNA polymerase (3U/µl) (BIOMATIK). The master mix was centrifuged for 10 seconds for thorough mixing. PCR was carried out in Gene Amp PCR system 9700 (Applied Biosystems, USA).

Horizontal gel electrophoresis

The amplification of the genomic region was checked on 2% agarose gel, which was prepared by melting 6 gms of agarose in 350 ml 0.5X TAE buffer (0.89 M Tris, 0.025 M Borate, EDTA PH 8.3) in a microwave oven for few minutes. Ethidium bromide (final concentration 0.5 µg/ml) was added to the gel to facilitate visualization of DNA after electrophoresis. PCR reaction products were mixed with Bromophenol blue dye (0.25% Bromophenol Blue in 40% sucrose solution) and loaded into the wells. Electrophoresis was performed at 100 Volts for half an hour in 1X TBE buffer. Amplified products were detected by placing the gel on UV transilluminator (Biometria, Germany).

Polyacrylamide Gel Electrophoresis

1. Master Mix was prepared as given below by adding reagents in sequential order.

Master Mix	Final Conc.	Required Volume per reaction µl
Deionizad H_2O		5.6
10X PCR Buffer	1 X	1
2.5 Mm dNTPs	100 µM	1
Taq Polymerase(2.5 U/µL)	2.5 U	0.4
20 µM Forward Primer	600 nm	0.25
20 µM Reverse Primer	600 nm	0.25
Total		8.5

2. 40ng/µl DNA and 8 µl master mix was added to each tube.
3. DNA was amplified under the above mentioned PCR thermal cycler conditions:

Step	Temperature	Duration	Cycles
Denaturation	95°C	5 min	1
Denaturation	95°C	45 sec	35
Annealing	55°C	1 min	
Extension	72°C	1 min	
Final Extension	72°C	10 min	1

4. 10% Polyacrylamide gel solution was prepared by adding 50 ml of 40% Acrylamide solution and 25 ml of 10X TBE q.s. to 250 ml with deionized water.
5. 300 µl of 25 % APS (Ammonium per Sulphate) and 300 µl of TEMED were added in 50 ml from the 250 ml of 10 % Acrylamide solution and was poured into the base of Biorad SeqiGen System and allowed to polymerize for 2-3 minutes.
6. To the remaining 200 ml of 10% Acrylamide solution, 850 µl of 25% APS solution and 150 µl of TEMED were added, then poured and allowed to polymerize for at least 2 hours.

7. 1X TBE Buffer was added in the upper and lower buffer chambers of the gel unit. After setting up the sequencing system, it was pre-run for 10-15 minutes at 100 watts constant power.
8. After taking out the comb, wells were washed with 1X TBE Buffer.
9. 5 µl gel loading dye was mixed with amplified product. DNA molecular weight marker was loaded in the first lane of the gel and 8 µl of the amplified products were loaded in rest of the appropriate wells. 1.4 µl of PCR products was mixed with 5µl gel loading dye containing Bromophenol Blue and Xylene Cyanol and analysed on 10% denaturing polyacrylamide gel.
10. Electrophoresis was performed at 100 watts for 4-5 hours. After cutting the gel according to expected band sizes, it was stained with Ethidium Bromide for few minutes.
11. Amplified products were detected by placing the gel on UV transilluminator and gel photographs were analyzed to determine alleles.
12. Fragment analysis was performed on SynGen using GeneScan (ver 3.1.2) software.

Reagents used in gel solution

1. **10X TBE** (Tris Boric Acid EDTA buffer; pH 8.2)
    1. 108 gm Tris, 55 gm Boric Acid and 7.44 gm EDTA were dissolved up to 0.8 L of deionized water on hot stirrer.
    2. Volume of solution was raised up to 1 L in a cylinder.
    3. pH of solution was adjusted at 8.03.
    4. It was filtered, transferred to labeled bottles and stored at room temperature.

2. APS 25% (w/v)

25 grams of Ammonium per Sulphate was dissolved in 100 ml of distilled water, labeled and stored in the refrigerator.

Linkage analysis

Pedigree and genotype data were managed and recorded for linkage analysis using Cyrillic 2.1.3. Two-point LOD scores were calculated using the MLINK program

of LINKAGE software package version 5.1 (Lathrop *et al.,* 1984). Analyses were automated by using linkage support programs (LSP, LCP, MAKEPED, PREPLINK). For Family A, B and C, an autosomal recessive model with a penetrance of 0.999 (phenocopy rate of 0.001 for homozygous normal individuals) and a disease allele frequency of 0.001 was assumed. The mutation rate was set to zero and equal recombination rates between males and females were assumed. Marker allele frequencies were taken from Marshfield human diversity panel (Asia-Pakistan population, based on approximately 190 individuals) or from CEPH database (http://www.cephb.fr/). For fine mapping, the marker allele frequencies were calculated from the family founders or assumed to be equal. Analyses were also conducted by using the online facility of GLUE (Genetic Linkage User Environment, UK HGMP Resource Centre; http://www.rfcgr.mrc.ac.uk/).

Candidate Disease Gene Identification

To select the disease causing gene, results of association studies in vitiligo affected family was used as a primary filter. Genomic locations and known genes in mapped loci were assembled into our starting set of candidate genes as defined by ENSEMBL (www.ensembl.org). Following six tools i.e. SUSPECT, PROSPECTR, G2D, GeneWanderer, GeneDistiller and PosMed were used to generate a list of genes on the basis of their similarity with known genes associated with vitiligo phenotype, their chromosomal position, P-Value and weighted Score etc.

G2D

G2D (Perez-Iratxeta *et al.,* 2005) make predictions of candidates on chromosomal regions by defining and scoring a number of BLASTX matches of that region against a scored database of genes. Depending upon GO Score and R-Score, a list of hundred Candidate disease genes, responsible for causing vitiligo, was generated by G2D. List was further refined and five best candidates were selected by the functional analysis of genes and their involvement in genetic disorders as mentioned in the disease database such as OMIM (Online Mendelian Inheritance in Man; http://www.ncbi.nlm.nih.gov/OMIM).

PROSPECTR and SUSPECT

The genes in mapped locus were scored by SUSPECT. The genomic area to be searched for candidate gene was limited by marker position. Nearby genes to the marker were selected and then scored by PROSPECTR (Adie *et al.,* 2006).

GeneWanderer

GeneWanderer is a web-based computational program to prioritise a set of candidate genes according to their probability of being involved in particular disease phenotype (Kohler *et al.,* 2008).

GeneDistiller

GeneDistiller is a web-based application which integrates information from various data sources such as gene-phenotype associations, gene expression patterns and protein-protein interactions into a central database (Seelow *et al.,* 2008).

PosMed (Positional Medline)

It prioritizes candidate genes by connecting phenotypic keywords to the genes through connections representing not only gene-gene interactions but also other biological interactions (e.g. metabolite-gene, mutant mouse-gene, drug-gene, disease-gene and protein-protein interactions) and ortholog data (Yoshida *et al.,* 2009).

Docking Studies

A detailed literature survey was carried out for finding ligands which can be used to treat vitiligo. Some potential drug targets were screened and retrieved from DrugBank (Wishart *et al.,* 2006). The structure of Monobenzone, Tacrolimus, Methoxsalen and L-Phenylalanine were obtained in pdb format alongwith its description, mechanism of action, toxicity and usefulness in order to treat vitiligo effectively. One of the structures of drug targets *i.e.* psoralen was retrieved from chemspider (http://www.chemspider.com) in mol file format which was converted in pdb file format by using Marvinbeans-5.2.2 (http://www.chemaxon.com). The target protein structure of human catalase (1QQW.pdb) was retrieved from Protein Data Bank (Bernstein *et al.*, 1977). One of mutations of catalase, as reported by Nikos *et al.* (2006), was assumed to be induced in protein model in order to dock a

mutated receptor protein with the potential drug ligands to cure it. This mutation has been found in Human gene mutation database (http://www.hgmd.cf.ac.uk/ac) with accession number (CM025323). It is a silent substitution *i.e.* GAC (Asp) → GAT (Asp) at the third nucleotide in codon 389 in exon 9 of catalase gene responsible for vitiligo susceptibility.

The mutated catalase receptor protein was docked with selected ligands by using Autodock tools version 3.0.5 (Morris *et al.,* 1998) which makes computational predictions of molecular interactions and calculate the docking energies of ligand-receptor complexes while Hex 5.0, developed by Ritchie and Graham (2000), is used to carry out Macromolecular Docking by using Spherical Polar Fourier correlations.

Table 2.1: Microsatellite markers used to test linkage to known Vitiligo Loci

Locus	Cytogenetic Location	Markers	Distance(cM) Rutgers Map Build 36	Amplified Length (bp)	Variation Type
AIS1 (FOXD3)	1p31	D1S1622	32.5	251	TNR
		D1S552	22.6	240	TTR
VIT1 (FBXO11)	2p16 2q13	D2S410	125.3	164	TNR
AIS4	4q13-q21	D4S2367	63.9	-	TTR
		D4S3243	67.5	-	TTR
AIS2	7q	D7S2212	79.0	193	TTR
		D7S3046	67.1	-	TTR
AIS3	8p	D8S1110	54.2	260	TTR
		D8S1477	34.9	145	TTR
GCH1	14q22.1-q22.2	D14S599	41.0	247	TNR
		D14S306	41.9	200	TTR
NALP1 (SLEV1)	17p13	D17S1294	28.3	260	TTR
	19	D19S586	37.8	238	TNR
		D19S433	-	210	TTR
COMT	22q12	D22S420	25.5	156	DNR

RESULTS

The different shades and colors of the human are due to the production of a brown pigment called melanin. A wide variety of skin pigment disorders are due the excessive production or absence of this pigment. Identification and characterization of the underlying gene(s) can increase our understanding of mechanism of autoimmune disorders. I got a chance to study three Pakistani families affected with Vitiligo.

VITILIGO

Vitiligo is an inherited and progressive skin disorder (commonly known as leukoderma), characterized by the destruction or inactivation of melanocytes, resulting in patches of depigmentation on skin. In this disease condition, the immune cells attack melanocytes (skin cells) present on epidermis and destroy them which turns that area white in color. The mechanism of action of immune system is still unknown that how it attacks melanocytes in people affected with vitiligo. Small patches appear on the skin which with the passage of time, enlarge into larger patches. These patches are more prominent near the body parts like eyes, mouth, nose, arms, legs and genital organs. These patches are very much visible especially on the skin of the patient who has dark colored skin (Huggins *et al.,* 2005).

It is found in one study that vitiligo runs in families and mostly affected patients are 20% of the relatives especially their children and siblings are on highest risk. Like other hereditary diseases which run in families, it shows that some people have got natural affected genes which are susceptible to developing genes. Sometimes some environmental factors like skin injury, sun burn or stress can also stimulate vitiligo affected genes in patients to develop vitiligo at any stage of life (Margaret and Wayne, 2007).

Vitiligo affects individuals of all ethnic groups with an estimated occurrence of 0.5% of the world population (Garsaud et al., 2001). It is believed that genetic factors are also responsible for the transmission of vitiligo (Majumder *et al.,* 1993; Kim *et al.,* 1996; Garsaud et al., 2001; Sun *et al.,* 2006). However, the genetic

transmission of vitiligo follows a multifactorial pattern, involving genes that predispose to the expression of the disease after interaction with environmental factors (Garsaud *et al.,* 2001). Alkhateeb *et al.,* (2002) studied certain families affected with vitiligo in autosomal dominant with incomplete penetrance. However, a non-Mendelian pattern followed in familial aggregation of vitiligo show its polygenic and mutifactorial inheritance (Spritz, 2007). Arcos-Burgos *et al.* (2002) studied the risk and the penetrance of the vitiligo population and seprated out two different categories i.e. one with the early onset, dominant mode of inheritance and without environmental effects while others with the late onset, recessive mode of inheritance and also influenced by environmental effects.

Alkahateeb *et al.,* (2002) and Fain *et al.,* (2003) performed a genome-wide genetic linkage analysis and confirmed that AIS1 is located at 14.4-cM interval on chromosome 1 locus (1p32.2-p31.3) and found lod score of 2.90 on chromosome 1p31 as a major locus for vitiligo. Spritz *et al.* (2004) again confirmed the localization of AIS1 at 73.7 cM on chromosome 1p and two other loci were also found, designated as AIS2 located at 89.4 cM on chromosome 7 (lod = 3.73) and AIS3 located at 54.2 cM on chromosome 8. In a genome-wide linkage analysis, Chen *et al.* (2005) obtained linkage evidence of vitiligo on chromosome 4q13-q21. The linkage analysis results yielded a lod score of 4.62. Nath *et al.* (2001) hypothesized that Vitiligo and Systemic Lupus Erythematosus have some genetic effects and found linkage evidences at 17p13, where the parametric lod score was 3.64 and nonparametric linkage score was 4.02.

Description of the familial studies

Family A

The family resides in Punjab, Pakistan. By proper interviewing the elders of the family, a five-generation pedigree of the family was constructed as shown in Figure 3.1. Three patients (IV-2, IV-2 and V-2) and five normal (IV-1, IV-5, IV-8, V-3 and V-4) of the family were physically examined. All affected subjects have generalized vitiligo i.e., irregular patches spread over whole body especially face, arms, legs etc. Pedigree represents an autosomal recessive mode of inheritance (Figure 3.1). The blood samples were taken from patients and normal subjects and processed for genomic DNA extraction.

Family B

The Family B also resides in Punjab, Pakistan. By proper interviewing the elders of the family, a five-generation pedigree of the family was constructed as shown in Figure 3.2. Vitiligo patients which are seven in number are mentioned as III-12, IV-2, IV-3, IV-10, IV-13, IV-15 and IV-18 and five normal labeled as III-4, III-5, III-9, IV-9 and IV-11. Pedigree represents an autosomal recessive mode of inheritance (Figure 3.2). The blood samples were taken from patients and normal subjects and processed for genomic DNA extraction.

Family C

The Family C also resides in Punjab, Pakistan. By proper interviewing the elders of the family, a four-generation pedigree of the family was constructed as shown in Figure 3.3. Three patients (IV-3, IV-5 and IV-7) and three normal subjects (III-7, III-8 and IV-6) of the family were physically examined. Pedigree represents an autosomal recessive mode of inheritance (Figure 3.3). The blood samples were taken from patients and normal subjects, then processed for genomic DNA extraction.

Linkage Studies

Exclusion for all known Vitiligo Loci

Family A, B and C were evaluated for the possibility that the phenotype is linked to the known loci for Vitiligo, named AIS1 (1p31), VIT1 (2p21), AIS4 (4q13-q21), AIS2 7q, AIS3 8p, GCH1 (14q22.1-q22.2), NALP1 (SLEV1) 17p13, 19, COMT (22q12), (Alkhateeb *et al.,* 2002, 2005; Fain *et al.,* 2003; Jin *et al.,* 2004; Le Poole *et al.,* 2001; Chen *et al.,* 2005; Spritz *et al.,* 2004; Spritz *et al.,* 2004; Fernandez, 1997; Bandyopadhyay *et al.,* 2000; Jin *et al.,* 2007; Spritz *et al.,* 2004; Y *et al.,* 2007).

No evidence of linkage was witnessed in Family A and C for all loci. Two point linkage analysis yielded significant negative LOD scores at $\theta = 0.0$ for all loci. However in Family B, results of two point linkage analysis provided strong evidence that a vitiligo locus is linked to marker on chromosome 4q13-q21. A maximum LOD Score calculated was 1.89 at recombination fraction of 0.0 and penetrance of 1.00 at D4S3243 locus. The results were excluded for all critical

regions of vitiligo susceptible loci flanked by markers D1S1622, D1S552, D2S410, D4S2367, D4S3243, D7S2212, D7S3046, D8S1110, D8S1477, D14S599, D14S306, D17S1294, D19S586, D19S433 and D22S420.

Candidate Disease Gene Identification

Lists of Candidate Disease genes were generated in mapped loci of Family B by using web based tools of G2D (Perez-Iratxeta *et al.,* 2005), PROSPECT and SUSPECT (Adie *et al.,* 2006), GeneDistiller (Seelow *et al.,* 2008), GeneWanderer (Kohler *et al.,* 2008) and PosMed (Yoshida *et al.,* 2009). The list was refined to best candidates depending upon their similarity with vitiligo associated CAT gene. The candidates were ranked by using weighted score of SUSPECT. The list of selected AIS4 associated candidate genes are presented in Table 3.2.

Docking Analysis

In the present study, certain antioxidants and supplements *i.e.* Psoralen, methoxalen (found in Psoralea corylifolia), Tacrolimus, Monobenzene and L-phenylalanine have been analyzed computationally and docked with mutated catalase protein model. The docking energies, Number of hydrogen bonds formed, number of clusters, Intermolecular energies, Internal energies of ligand and Torsional free energies are presented in Table 3.2.

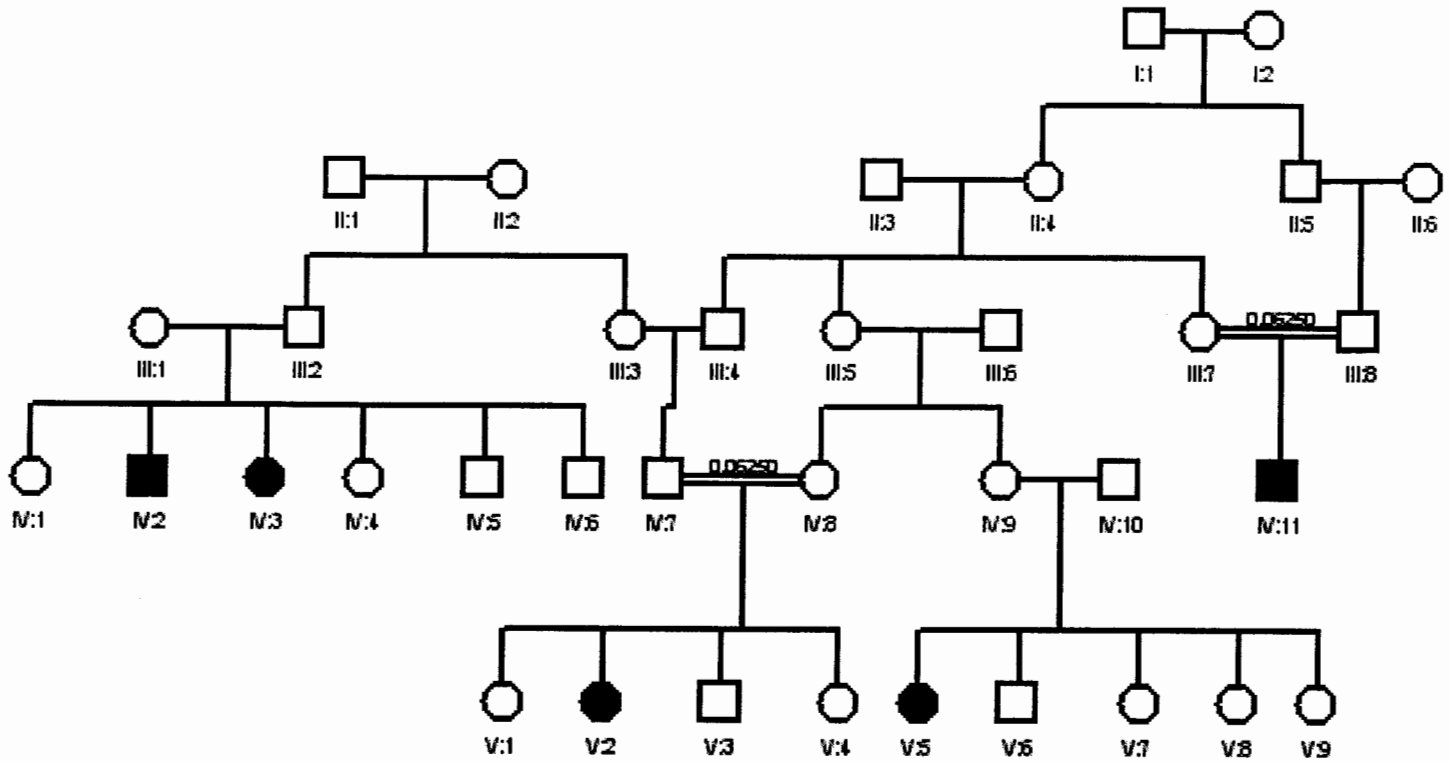


Figure 3.1: Pedigree of family A affected with Vitiligo.

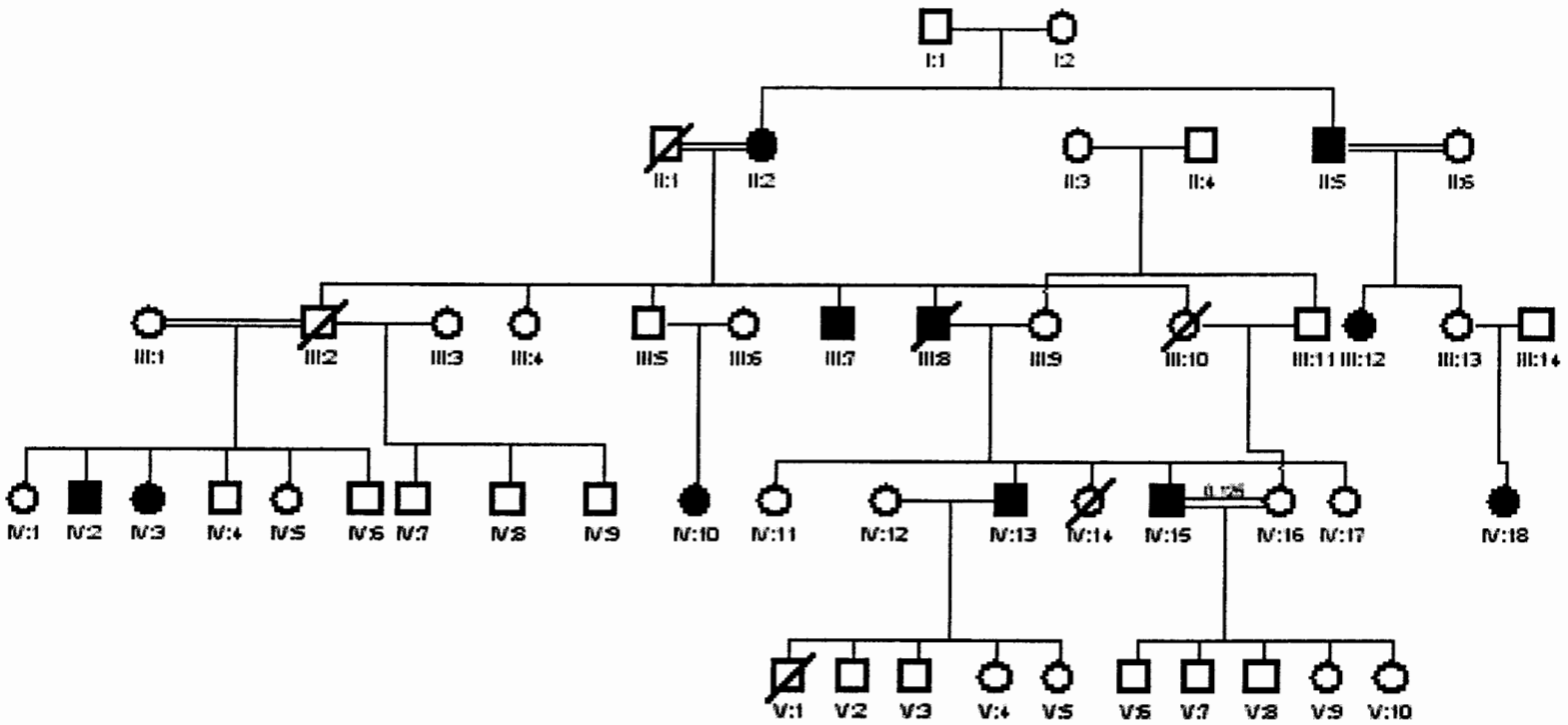


Figure 3.2: Pedigree of family B affected with Vitiligo.

Figure 3.3: An affected Individual (IV-13) from family B with Vitiligo.

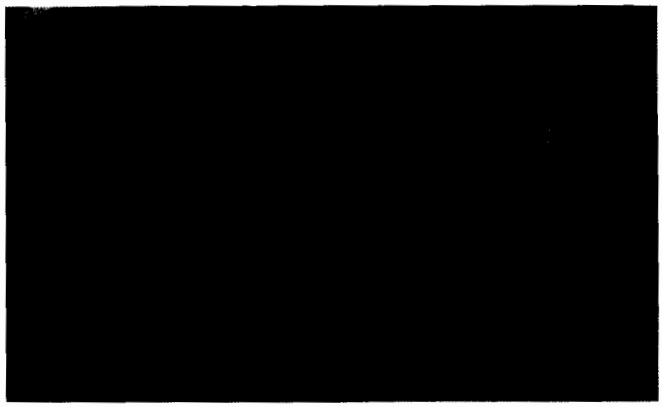


Figure 3.4: An affected Individual (IV-15) from family B with Vitiligo.

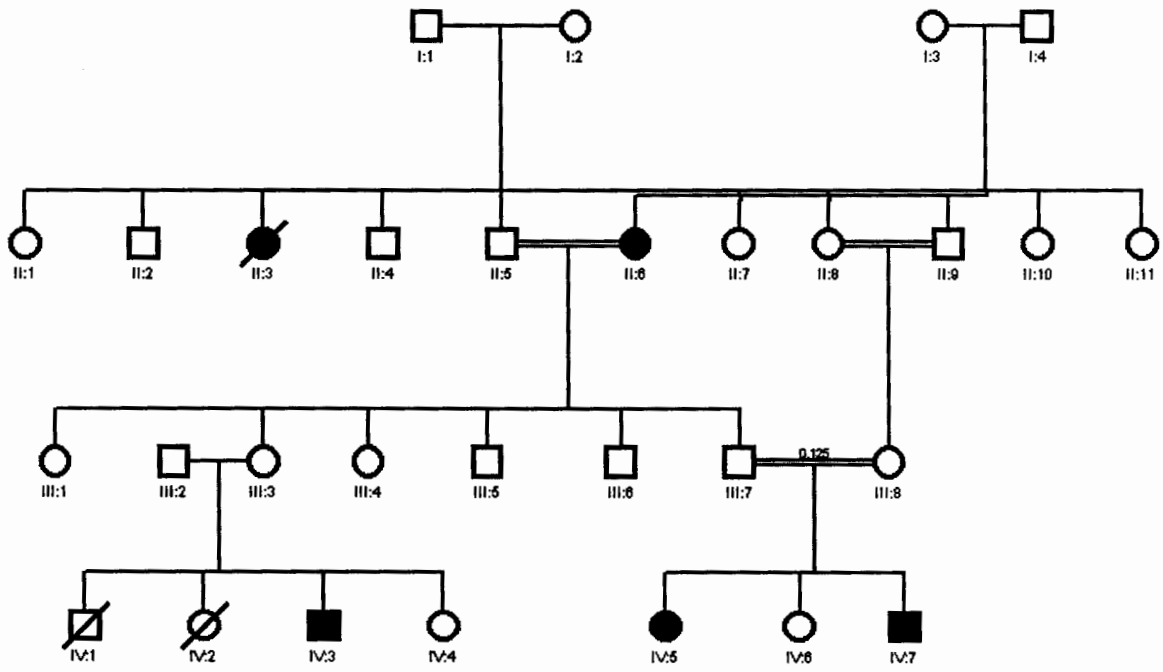
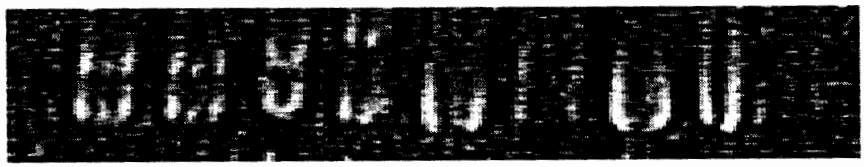


Figure 3.5: Pedigree of family C affected with Vitiligo.

1 2 3 4 5 6 7 8



Family A

Lane 1. IV-1 Lane 4. IV-5 Lane 7. V-3
Lane 2. IV-2 Lane 5. IV-8 Lane 8. V- 4
Lane 3. IV-3 Lane 6. V-2

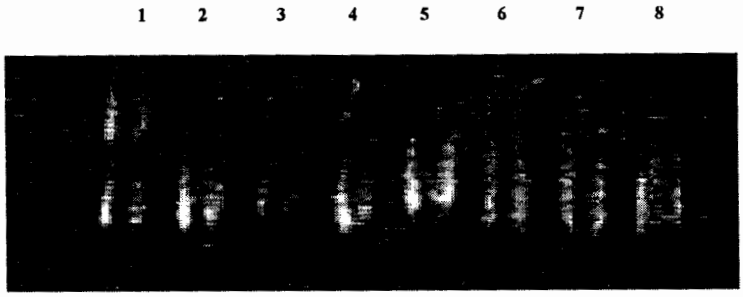
Figure 3.6: Gel Electropherogram of ethidium bromide stained 10% non-denaturing polyacrylamide gel for marker D4S2367 at 63.9cM chromosome 4q13-q21, of family A. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numbers indicate their positions within generation.

1 2 3 4 5 6 7 8

Family A

Lane 1. IV-1 Lane 4.IV-5 Lane 7. V-3
Lane 2. IV-2 Lane 5.IV-8 Lane 8.V- 4
Lane 3.IV-3 Lane 6. V-2

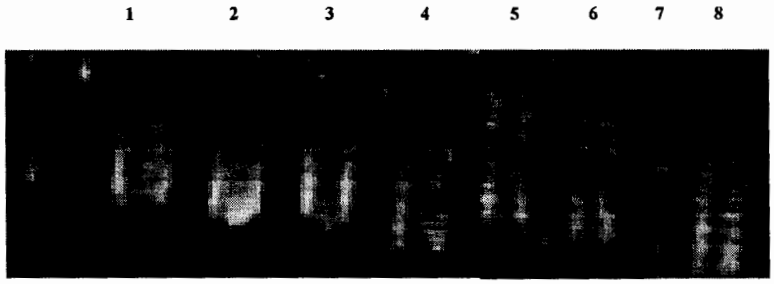
Figure 3.7: Gel Electropherogram of ethidium bromide stained 10% non-denaturing polyacrylamide gel for marker D7S3046, at 67.1 cM on chromosome 7q of family A. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numbers indicate their positions within generation.



Family A

Lane 1. IV-1 Lane 4.IV-5 Lane 7. V-3
Lane 2. IV-2 Lane 5.IV-8 Lane 8.V- 4
Lane 3.IV-3 Lane 6. V-2

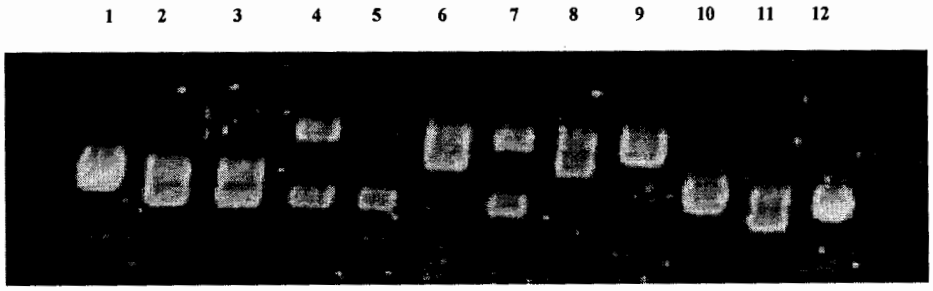
Figure 3.8: Gel Electropherogram of ethidium bromide stained 10% non-denaturing polyacrylamide gel for marker D7S2212 at 79.0 cM on chromosome 7q of family A. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numbers indicate their positions within generation.



Family A

Lane 1. IV-1 Lane 4.IV-5 Lane 7. V-3
Lane 2. IV-2 Lane 5.IV-8 Lane 8.V- 4
Lane 3.IV-3 Lane 6. V-2

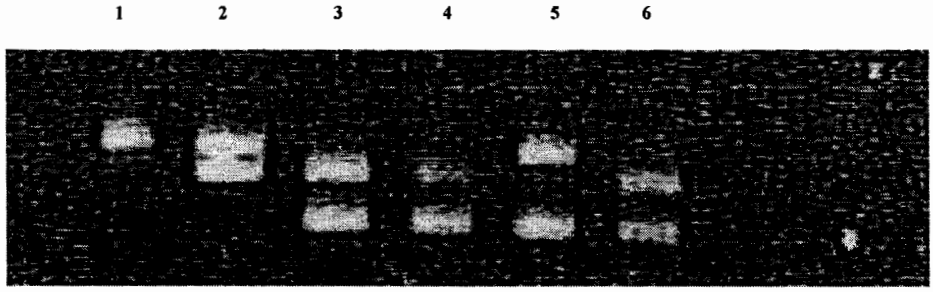
Figure 3.9: Gel Electropherogram of ethidium bromide stained 10% non-denaturing polyacrylamide gel for marker D17S1294, at 28.3 cM on chromosome 17p13 of family A. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numbers indicate their positions within generation.



Family B

Lane 1. III-4 Lane 5.IV-11 Lane 9. IV-18
Lane 2.III-5 Lane 6.IV-13 Lane 10.IV-2
Lane 3.IV-10 Lane 7.IV-15 Lane 11.IV-3
Lane 4.III-9 Lane 8.III-12 Lane 12.IV-9

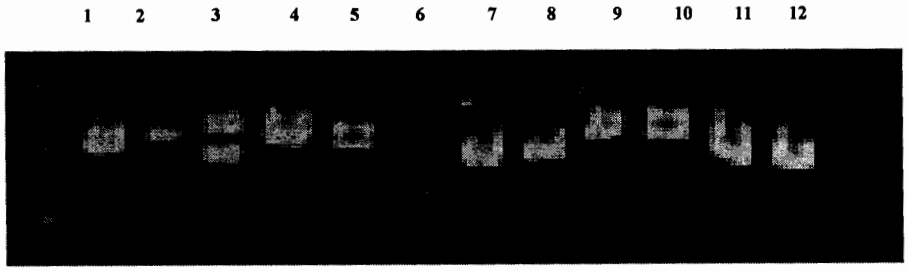
Figure 3.10: Gel Electropherogram of ethidium bromide stained 10% non-denaturing polyacrylamide gel for marker marker D4S2367 at 63.9cM chromosome 4q13-q21, of family B. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numbers indicate their positions within generation.



Family B

Lane 1.III-4 Lane 3.III-9 Lane 5.IV-13
Lane 2.IV-10 Lane 4.IV-11 Lne 6.IV:15

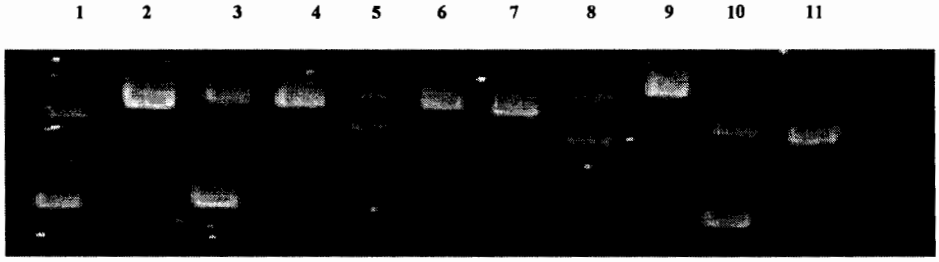
Figure 3.11: Gel Electropherogram of ethidium bromide stained 10% non-denaturing polyacrylamide gel for marker D14S306 at 41.9 cM on chromosome 14q12-q13 of family B. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numbers indicate their positions within generation.



Family B

Lane 1. III-4	Lane 5.IV-11	Lane 9. IV-18
Lane 2.III-5	Lane 6.IV-13	Lane 10.IV-2
Lane 3.IV-10	Lane 7.IV-15	Lane 11.IV-3
Lane 4.III-9	Lane 8.III-12	Lane 12.IV-9

Figure 3.12: Gel Electropherogram of ethidium bromide stained 10% non-denaturing polyacrylamide gel for marker D19S586 at 37.8 cM on chromosome 19 of family B. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numbers indicate their positions within generation.

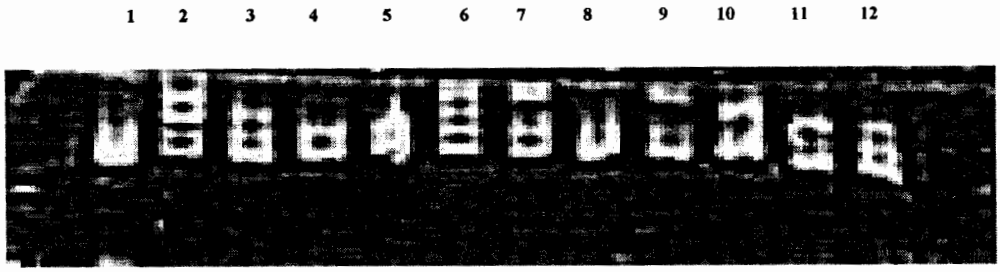


Family B

Lane 1. III-4	Lane 5.IV-11	Lane 9. IV-18
Lane 2.III-5	Lane 6.IV-13	Lane 10.IV-2
Lane 3.IV-10	Lane 7.IV-15	Lane 11.IV-3
Lane 4.III-9	Lane 8.III-12	Lane 12.IV-9

Figure 3.13: Gel Electropherogram of ethidium bromide stained 10% non-denaturing polyacrylamide gel for marker D22S420, at 25.5 cM on chromosome 22q12 of family B. The Roman numerals indicate the generation number of the

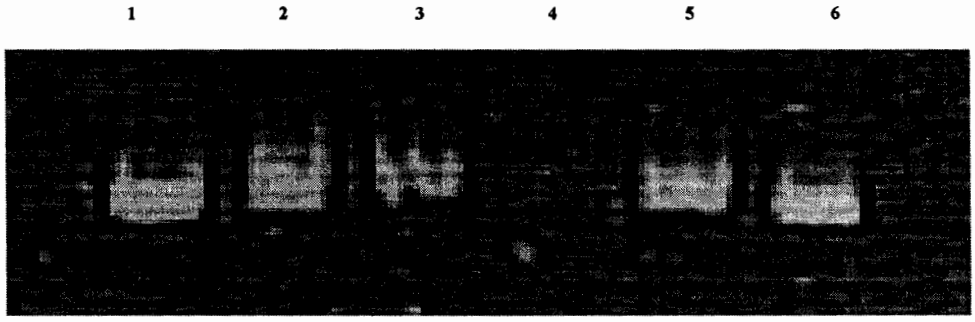
individuals within a pedigree while Arabic numbers indicate their positions within generation.



Family B

Lane 1. III-4	Lane 5.IV-11	Lane 9. IV-18
Lane 2.III-5	Lane 6.IV-13	Lane 10.IV-2
Lane 3.IV-10	Lane 7.IV-15	Lane 11.IV-3
Lane 4.III-9	Lane 8.III-12	Lane 12.IV-9

Figure 3.14: Gel Electropherogram of ethidium bromide stained 10% non-denaturing polyacrylamide gel for marker marker D4S3243 at 67.5cM chromosome 4q13-q21, of family B. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numbers indicate their positions within generation.

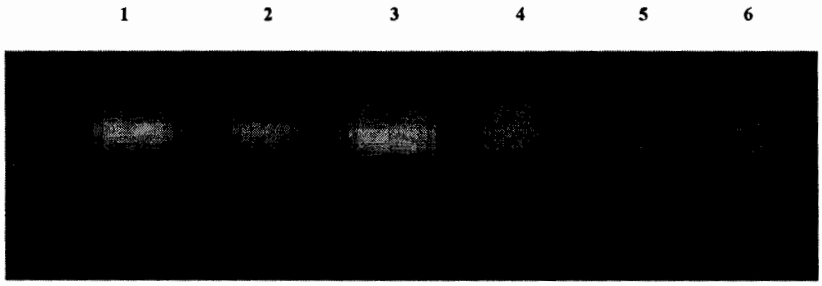


Family C

Lane 1.IV-4	Lane 3.III-7	Lane 5.IV-5
Lane 2.III-7	Lane 4.IV-6	Lane 5.IV-7

Figure 3.15: Gel Electropherogram of ethidium bromide stained 10% non-denaturing polyacrylamide gel for marker D19S586 at 37.8 cM on chromosome 19

of family C. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numbers indicate their positions within generation.



Family C

Lane 1.IV-4 Lane 3.III-7 Lane 5.IV-5
Lane 2.III-7 Lane 4.IV-6 Lane 5.IV-7

Figure 3.16: Gel Electropherogram of ethidium bromide stained 10% non-denaturing polyacrylamide gel for marker D19S433 on chromosome 19 of family C. The Roman numerals indicate the generation number of the individuals within a pedigree while Arabic numbers indicate their positions within generation.

Locus	Cytogenetic Location	Markers	Distance (cM)	Recombination Fraction						
				0.0	**0.01**	**0.05**	**0.1**	**0.2**	**0.3**	**0.4**
AIS4	4q13-q21	D4S2367	63.9	-3.47	-2.80	-1.77	-0.89	-0.11	-0.09	-0.07
		D4S3243	67.5	1.89	1.85	1.66	1.43	0.96	0.51	0.15
GCH1	14q22.1-q22.2	D14S306	41.9	-4.21	-3.48	-2.40	-1.47	-0.10	-0.07	-0.05
	19	D19S586	37.8	-5.47	-4.80	-3.77	-2.89	-0.11	-0.09	-0.06
COMT	22q12	D22S420	25.5	-4.21	-3.48	-2.40	-1.47	-0.15	-0.10	-0.08

Table 3.1: Two-point LOD scores between the phenotype and Vitiligo markers

Table 3.2: Analysis of Selected AIS4 Candidate Genes by using SUSPECT, PROSPECTR, Gene Wanderer, POSMED, G2D and Gene Distiller.

#	Gene	Description	Chromosomal Location	SUSPECT	PROSP-ECTR	Gene Wanderer	POSMED	G2D			Gene Distiller/ G2D
				Weighted Score	Score	Score	P Value	GO Score	R Score	E value T=1e-10	Functions
1	IGJ	immunoglobulin J polypeptide	71,740,131-71,751,114	10.13	0.52	0.0005990	1.40E-40	0.071170	0.000339	7e-23	Link two monomer units of either IgM or IgA.
2	IL8	Interleukin-8	74,825,122-74,828,295	11.25	0.59	0.006256	-	-	-	-	immune system chemotaxis
3	CXCL6	chemokine (C-X-C motif) ligand 6	74,921,277-74,923,340	12.71	0.60	0.0005536	-	-	-	-	immune system chemotaxis
	CXCL1	chemokine (C-X-C motif) ligand 1	74,953,973-74,968,249	12.53	0.60	0.0003201	-	-	-	-	immune system chemotaxis
	PPBP	pro-platelet basic protein (chemokine (C-X-C motif) ligand 7)	75,071,622-75,072,764	11.25	0.61	0.0007353	-	-	-	-	immune system chemotaxis
	CXCL5	chemokine (C-X-C motif) ligand 5	75,080,224-75,083,286	12.71	0.50	0.0002811	-	-	-	-	immune system chemotaxis
	CXCL3	chemokine (C-X-C motif) ligand 3	75,121,178-75,123,354	12.53	0.60	0.0002575	-	-	-	-	immune system chemotaxis
	CXCL2	chemokine (C-X-C motif) ligand 2	75,169,527-75,183,874	12.53	0.60	0.005963	-	-	-	-	immune system chemotaxis
4	EREG	epiregulin	75,449,724-75,473,341	11.45	0.52	0.005412	1.78E-18	-	-	-	Growth factor
	CXCL9	chemokine (C-X-C motif) ligand 9	77,141,647-77,147,686	9.12	0.60	0.0008122	5.71E-107	-	-	-	immune system chemotaxis
	CXCL10	chemokine (C-X-C motif) ligand 10	77,161,297-77,163,674	10.45	0.52	0.002478	9.62E-134	-	-	-	immune system chemotaxis
	CXCL11	chemokine (C-X-C motif) ligand 11	77,173,975-77,176,376	10.45	0.52	0.0007477	5.71E-107	-	-	-	immune system chemotaxis
	CXCL13	chemokine (C-X-C motif) ligand 13	78,651,930-78,752,010	10.45	0.41	0.001041	1.14E-27	0.056268	0.000514	-	immune system chemotaxis
5	FRAS1	Fraser syndrome 1	79,198,120-79,684,447	16.25	0.72	0.0001852	1.78E-18	-	-	-	immune system chemotaxis
6	FGF5	fibroblast growth factor 5	81,406,766-81,431,194	12.71	0.60	0.003664	1.64E-31	-	-	-	Growth factor

Table 3.3: AutoDock results of Catalase protein with all commonly used Drugs.

No	Compound	Intermol -ecular Energy kcal/mol	Internal Energy of Ligand kcal/mol	Torsional Free Energy kcal/mol	Cluster RMSD	Lowest Docked Energy kcal/mol	Estimated Free Energy of Binding	No of Hydrogen Bonds formed at best conformation	Residue which is forming Hydrogen Bond at best conformation	Energy calculated by Hex
1	**Monobenzone**	-7.43	-5.39e-04	+0.93	0.00	-7.43	-6.49	3	HIS 364 ARG 66(2)	-11.41
2	**Tacrolimus**	-18.22	+1.59	+2.18	0.00	-16.63	-16.04	3	ARG 66(3)	-30.05
3	**Methoxsalen**	-6.54	+0.06	+0.31	0.00	-6.48	-6.23	3	ARG 66 HIS 364(2)	-8.88
4	**L-Phenylalanine**	-7.87	-0.07	+0.93	0.00	-7.94	-6.97	4	ARG 363 ARG 66(3)	-19.72
5	**Psoralen**	-6.09	+0.00e+00	+0.00e+00	0.00	-6.09	-6.09	2	ASP 360 ARG 66	-22.88

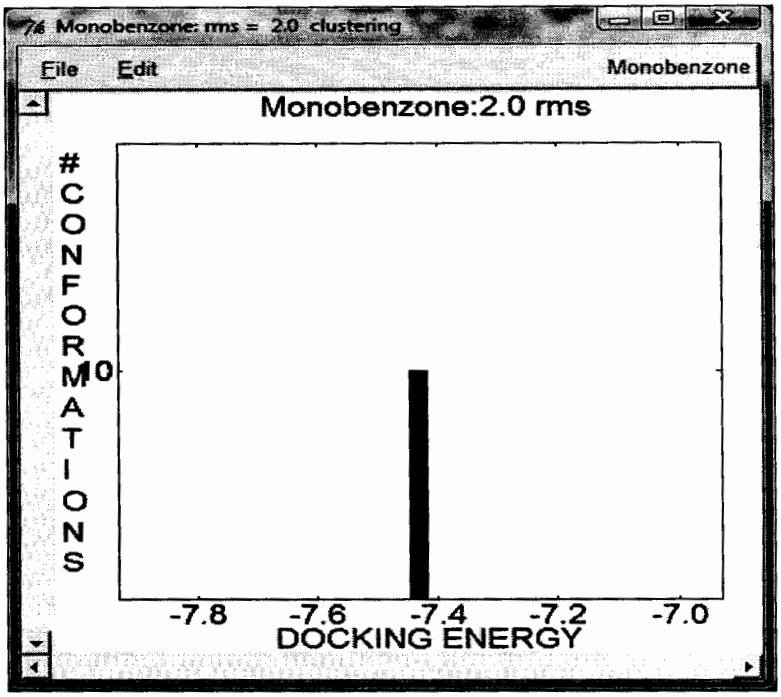


Figure 3.17: Clustering Histogram showing Docking Energy of all possible conformations with Monobenzone.

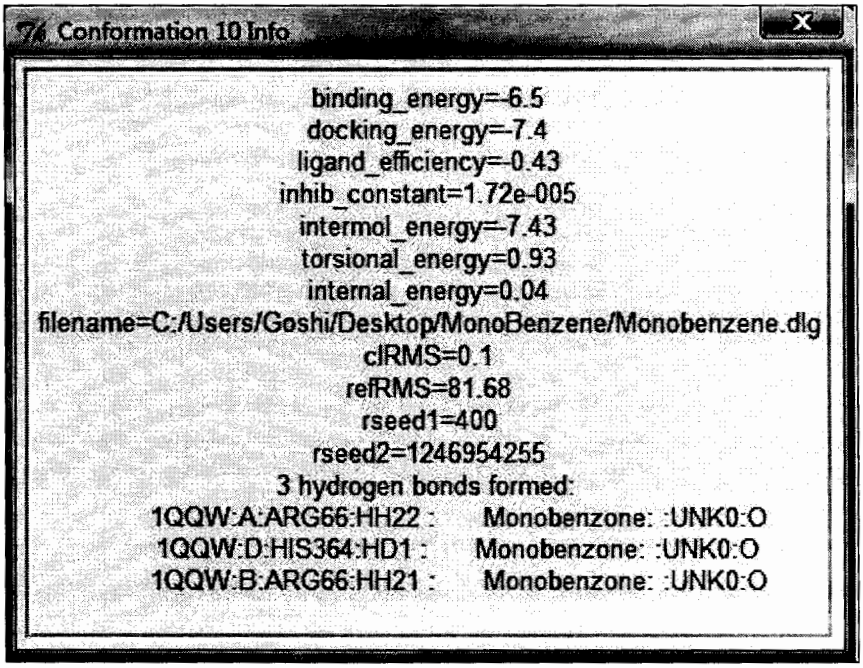


Figure 3.18: Window showing best conformation information about Docking Energy and number of Hydrogen bonds formed between Monobenzene and receptor 1QWW.

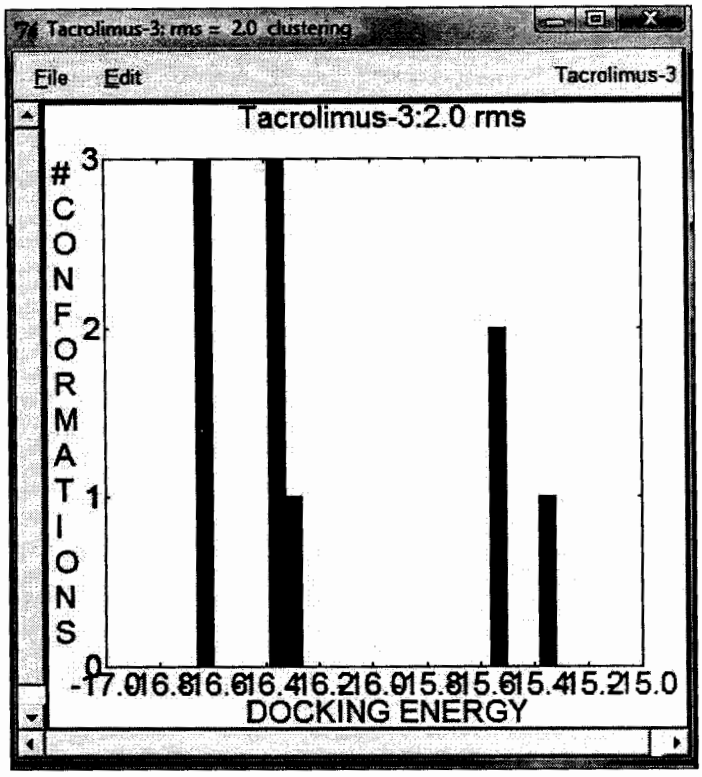


Figure 3.19: Clustering Histogram showing Docking Energy of all possible conformations with Tacrolimus.

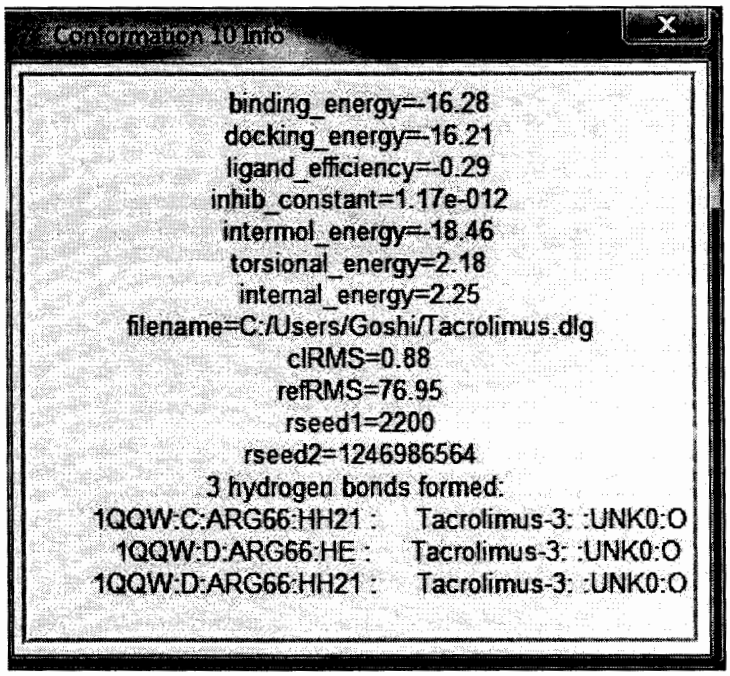


Figure 3.20: Window showing best conformation information about Docking Energy and number of Hydrogen bonds formed between Tacrolimus and receptor 1QWW.

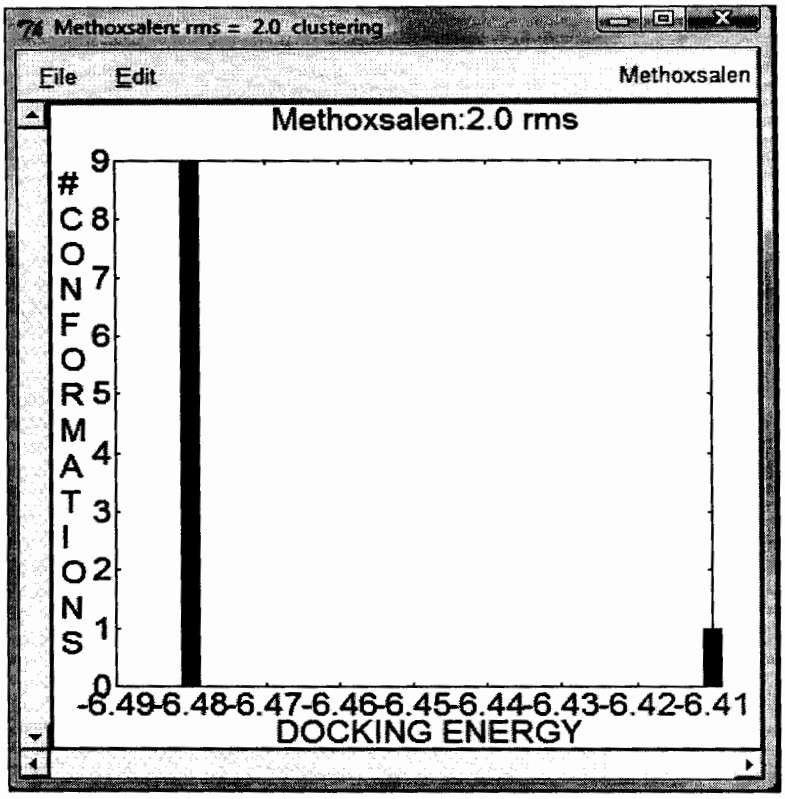


Figure 3.21: Clustering Histogram showing Docking Energy of all possible conformations with Methosalen.

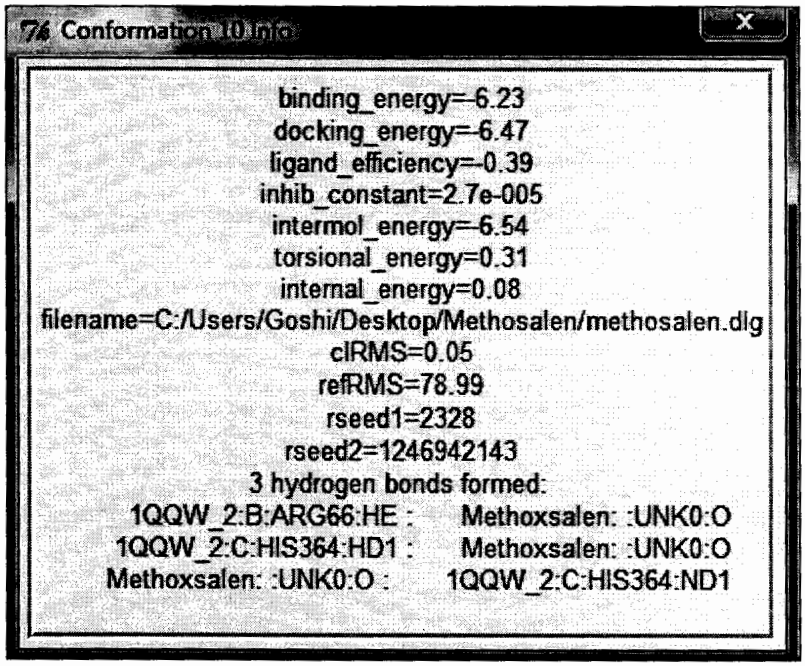


Figure 3.22: Window showing best conformation information about Docking Energy and number of Hydrogen bonds formed between Methosalen and receptor 1QQW.

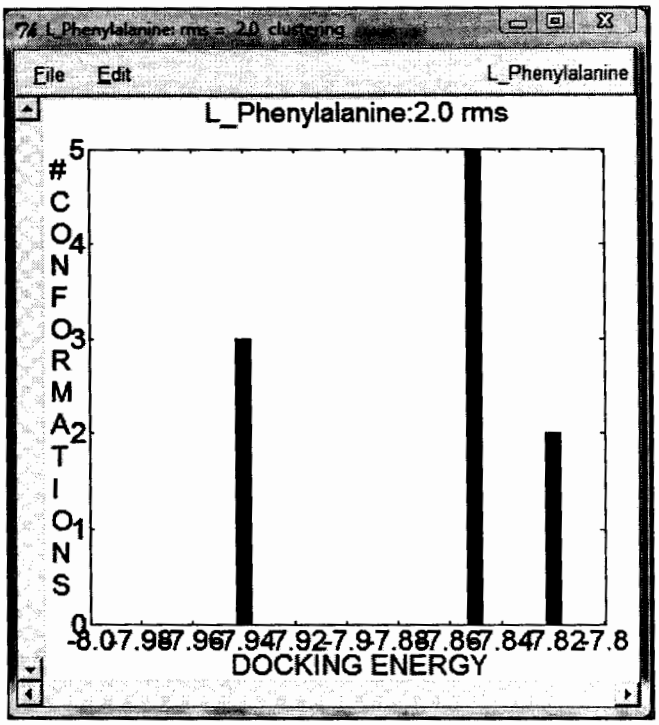


Figure 3.23: Clustering Histogram showing Docking Energy of all possible conformations with L_Phenyalanine.

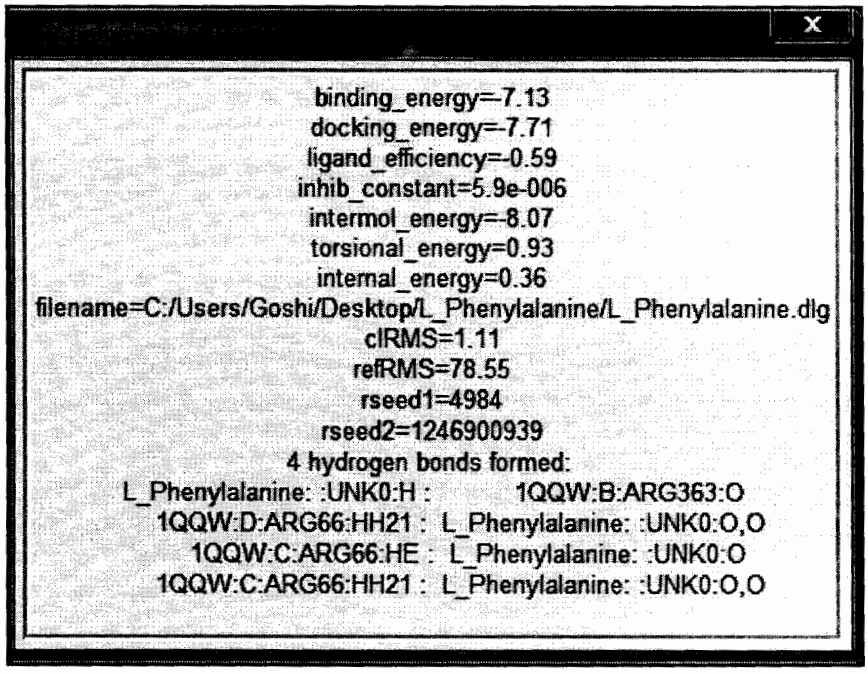


Figure 3.24: Window showing best conformation information about Docking Energy and number of Hydrogen bonds formed between L_Phenyalanine and receptor 1QQW.

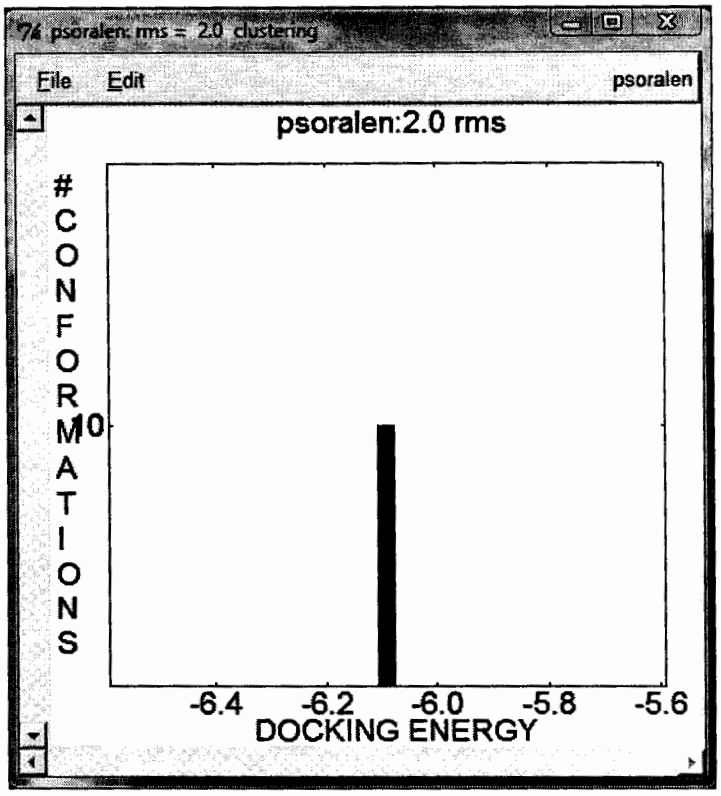


Figure 3.25: Clustering Histogram showing Docking Energy of all possible conformations with Psoralen.

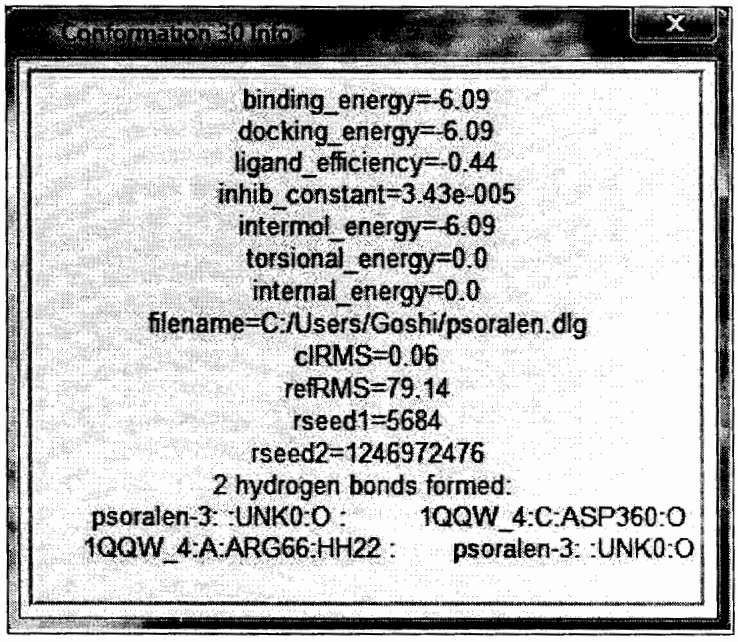
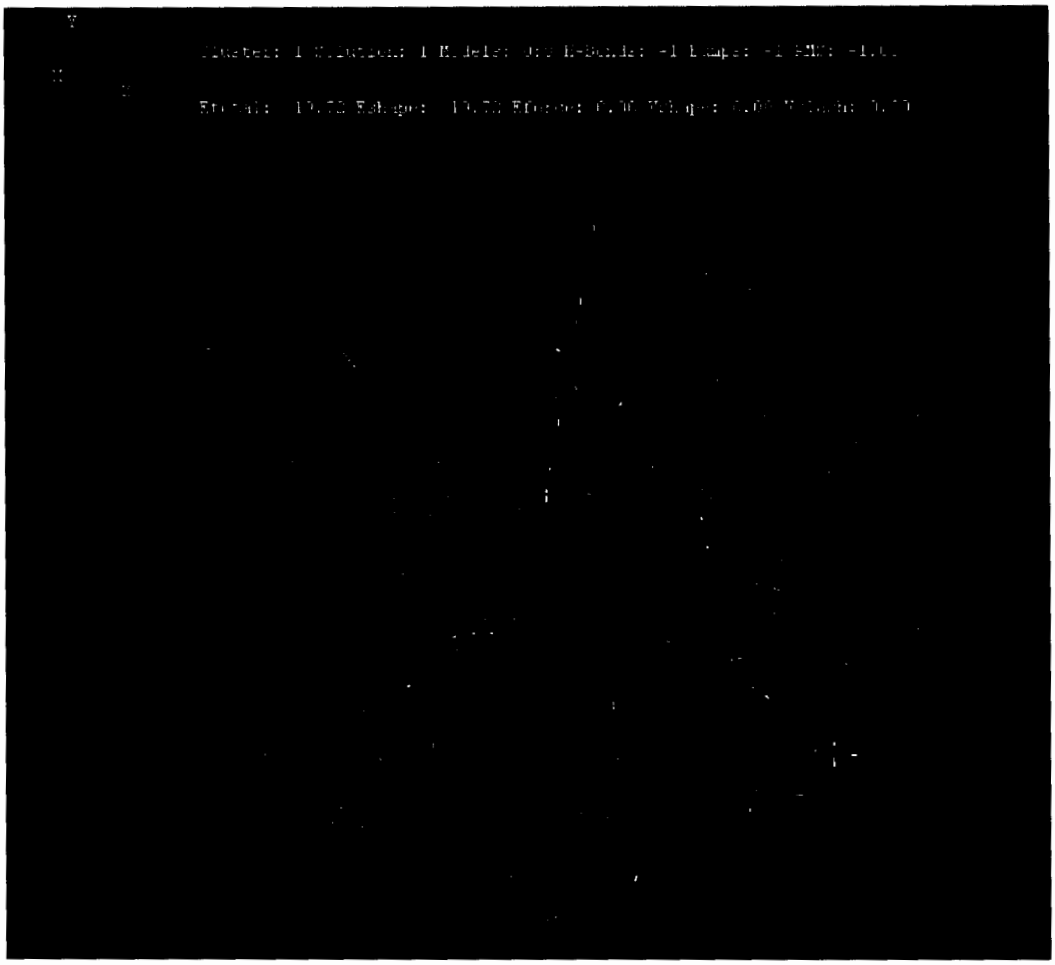


Figure 3.26: Window showing best conformation information about Docking Energy and number of Hydrogen bonds formed between Psoralen and receptor 1QQW.



3.27: 1QQW-L_Phenylalanine docking complex showing Total Energy of Complex as -19.72.

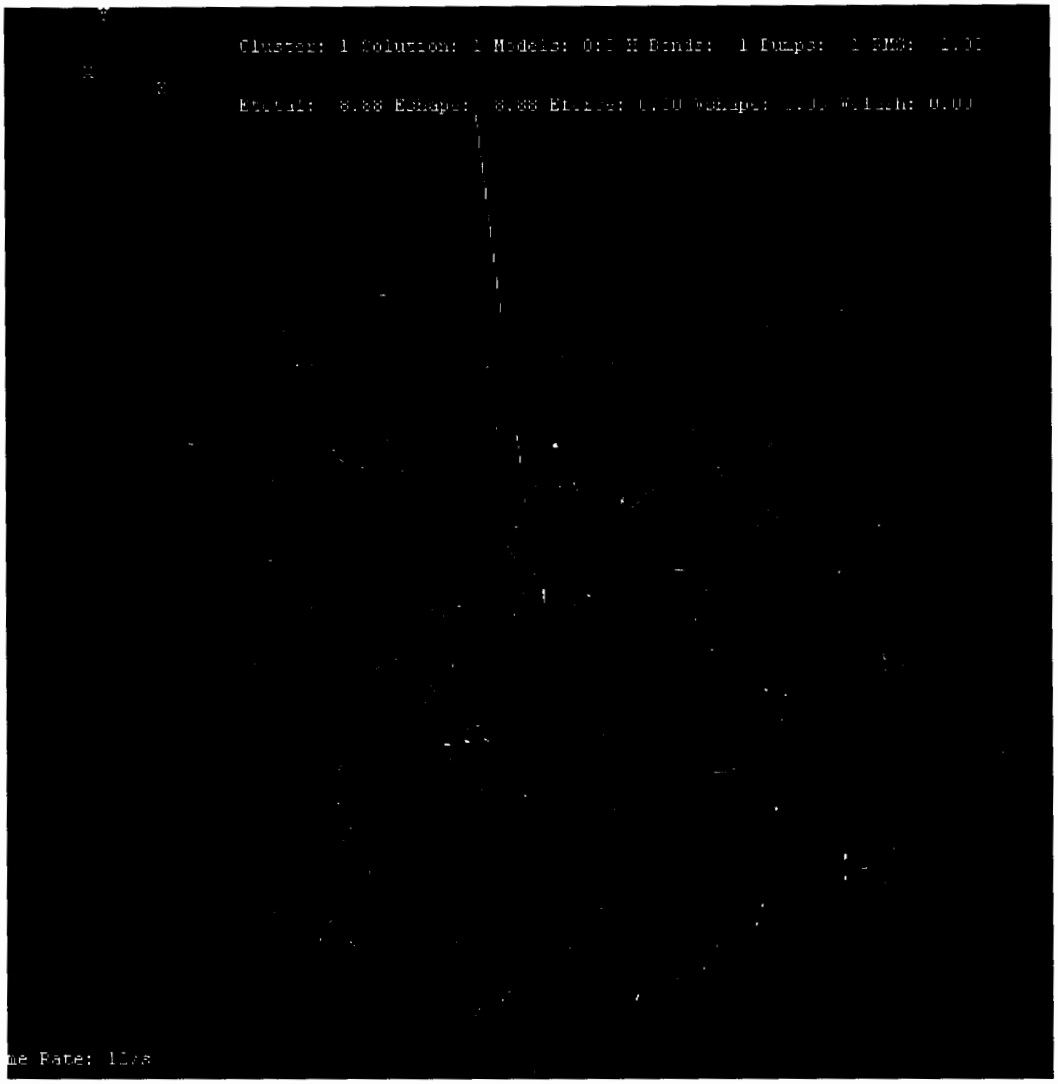


Figure 3.28: 1QQW-Methosalen docking complex showing Total Energy of Complex as -8.88.

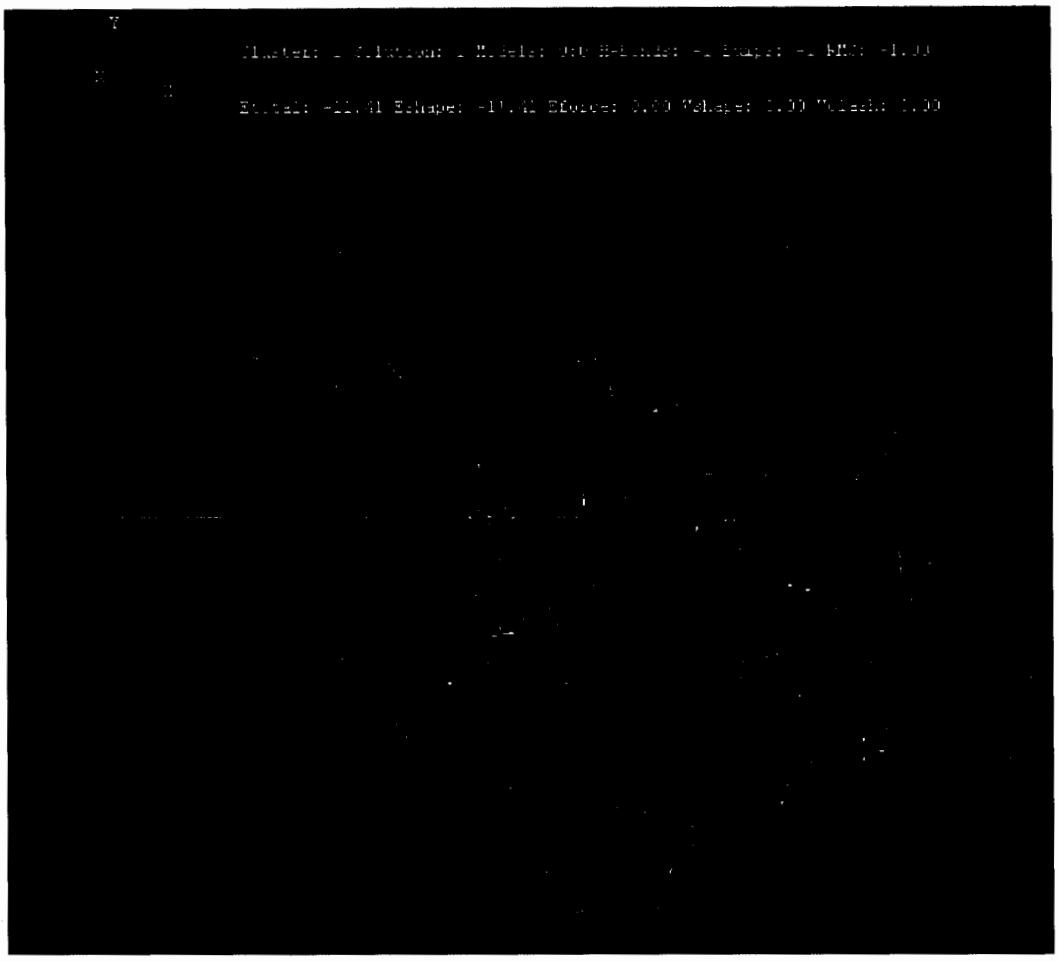


Figure 3.29: 1QQW- Monobenzene docking complex showing Total Energy of Complex as -11.41.

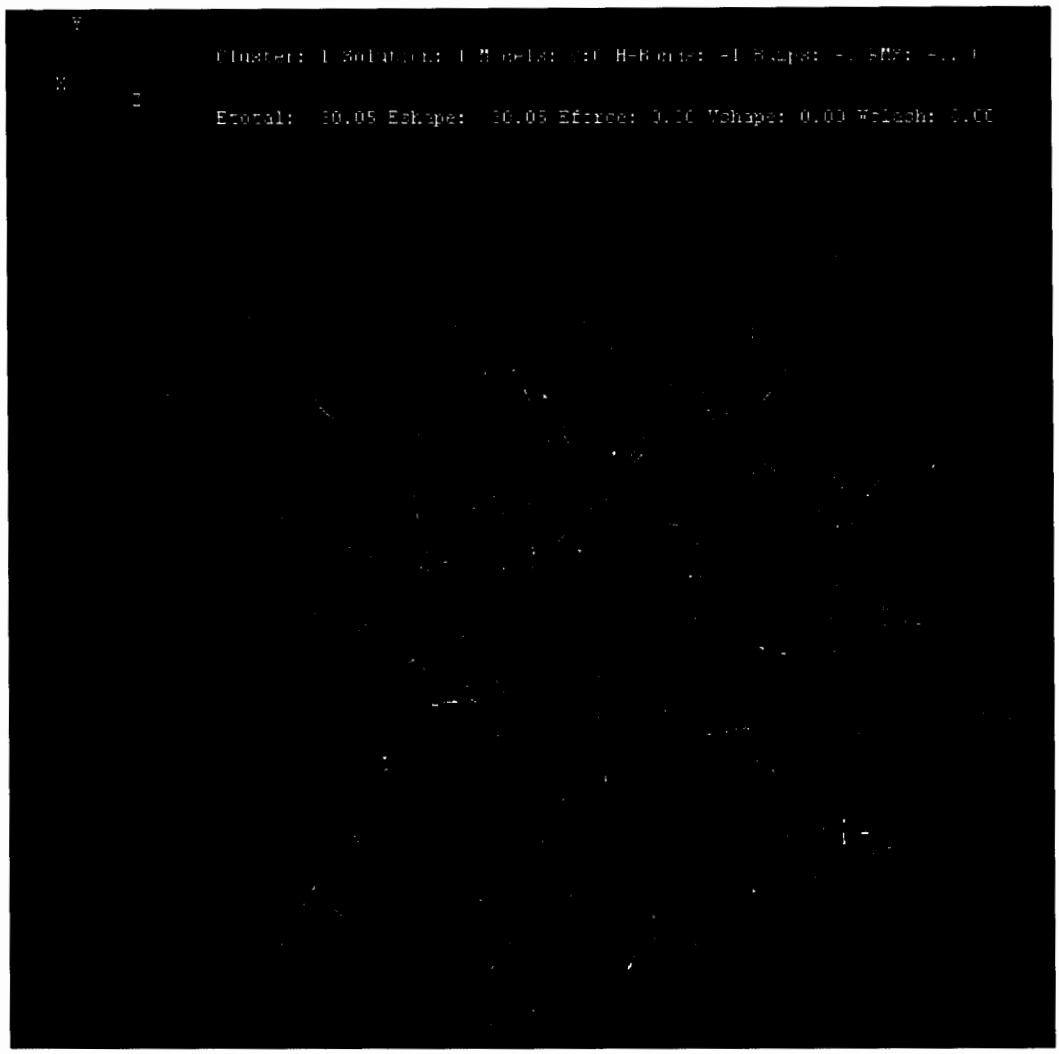


Figure 3.30: 1QQW- Tacrolimus docking complex showing Total Energy of Complex as -30.05.

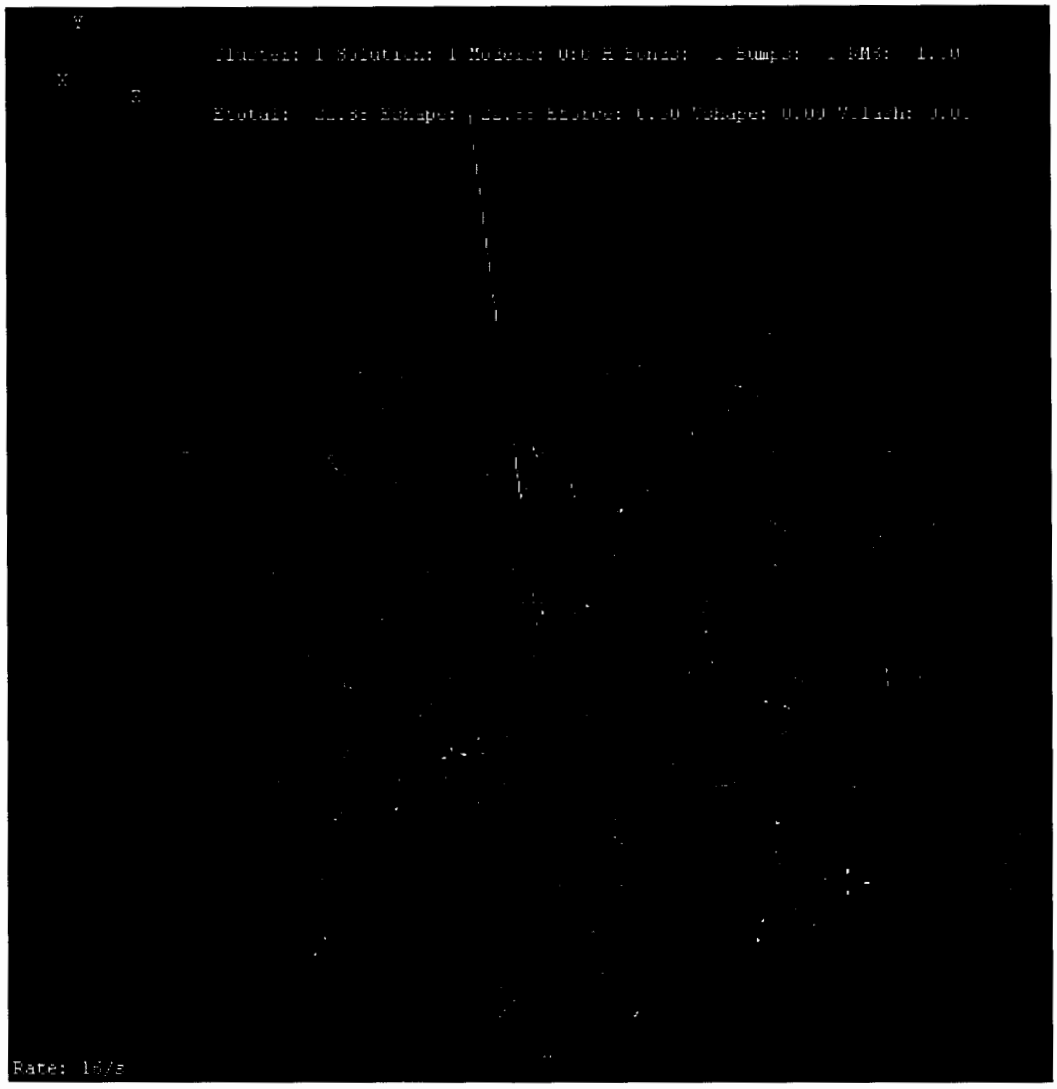


Figure 3.31: 1QQW- Psoralen docking complex showing Total Energy of Complex as -22.88.

Discussion

In Pakistan, it is found that population has stable communities, large sib-ships and a high consanguinity rate. Marriages within families are strongly favored due to the existence of various linguistic and racial levels, cultural, economic and custom differences.

In present study, three large inbred Pakistani families (Fig. 3.3, 3.4) with several affected individuals were ascertained from the province of Punjab in Pakistan. The reason is that these large families may provide an excellent opportunity to localize the skin pigment disorders in the human genome, to identify the primary gene and hence, to get to know the causal patho-mechanisms of the malformation. Therefore, these families were visited at their places of residence in order to get permission from them for conducting a clinical and molecular study of their disorder. In each family affected and normal subjects were examined to categorize the vitiligo patches on the basis of their shape and distribution over whole body. In order to infer the correct lineage and the inheritance pattern, an extended pedigree was drawn in each case with the help of the senior family members. Information was obtained about intermarriages, deceased and other related defects.

Vitiligo observed in Family A and C shows typical feature of generalized vitiligo in which white patches were observed to be scattered over whole body especially face, arms and legs etc. These families were analysed scrupulously in order to find any possibility of linkage to any of the known loci of vitiligo but unfortunately no linkage was witnessed and a significant negative LOD score was obtained which excluded all critical regions found at different loci of vitiligo. However, generalized vitiligo observed in Family B also showed white patches scattered over whole body especially face, arms and legs etc. Linkage studies on Family B demonstrated that linkage evidences were found on AIS4 locus which was assigned to chromosome 4q13-q21. This linkage result confirmed the previous work of Chen *et al.,* (2005) on AIS4, although this data do not contribute in narrowing this critical region. Gene(s) responsible for AIS4 has not been identified yet.

After linkage studies, an effort has been made to explore about genes found on the linked locus *i.e.* 4q13-q21. As mentioned by Risch (2000), linkage studies generate large data sets which are further analyzed to find subsets of most likely disease gene

candidates. Many computational approaches have been designed for this purpose. In this study, computational tools i.e G2D (Perez-Iratxeta *et al.,* 2005), SUSPECT, PROSPECTR (Adie *et al.,* 2006), GeneDistiller (Seelow *et al.,* 2008), GeneWanderer (Kohler *et al.,* 2008) and PosMed (Yoshida *et al.,* 2009) have been employed to gather a subset of genes that are positionally linked to the diseased locus and could be a cause of vitiligo.

The criteria for gene finding in SUSPECT and PROSPECTR is to find similarity with a known gene of vitiligo and rank them on the basis of their weighted Score which is a score given to each gene depending upon how significant the match is; based on how often the domain in question is found in the genome. High values indicate a possible relation to the disease under consideration. While in G2D, the criteria is to look for the source of protein first, homolog to be translated from candidate genomic interval, must be Homo sapiens; the locus of homolog protein should be preferably from already reported candidate disease genes. If a gene follows these criteria then the next step is to check the information regarding biological, functional and expression data. Gene evaluation was done on the basis of two scores *i.e.* Gene Ontology Score (GO Score) which is an average of the scores associated to each of the GO terms. High values of the score favour in the strong confidence of relationship to the disease under consideration and a Relative Score (R-Score) which is a score of a sequence according to GO scores. Values close to zero favour in close confidence about possible relation of the sequence to the disease. GeneWanderer evaluates a gene on the basis of a score which shows the strength of the prediction of the gene as a candidate for the disease in question. The criteria for GeneDistiller and PosMed are to select genes having gene-phenotype associations, gene expression patterns and protein-protein interactions.

The chromosomal region 4q13-q21 codes for more than 250 genes. A list of all cloned and known gene data from this interval was obtained from Ensembl database. In order to find best disease gene candidates, SUSPECT and PROSPECTR were used first which produced a list of more than 200 genes. These results were limited by selecting only the genes related to Markers D4S392 at q13.4, D4S3042 at q21.1, D4S2947 at q21.21 and D4S3243 at q21.21 under a recessive model of inheritance which gave maximum lod score for AIS4. Then G2D, GeneWanderer and GeneDistiller gave 100 candidates, PosMed produced 40 candidates. After complete analysis, the list of best

possible candidate disease genes was generated depending upon the criteria as mentioned earlier. The strong disease gene candidates, based on above analysis are presented in Table 3.3.

The present study has confirmed that the following genes are considered to be the most likely disease gene candidates which may be involved in causing vitiligo: Cluster of chemokine genes *i.e.* C-X-C motif ligand 6, C-X-C motif ligand 1, C-X-C motif ligand 7 (also known as pro-platelet basic protein), C-X-C motif ligand 5, C-X-C motif ligand 3, C-X-C motif ligand 9, C-X-C motif ligand 10, (C-X-C motif) ligand 11 and (C-X-C motif) ligand 13, Cytokines *i.e.* Interleukin-8 (IL8), autoantibodies *i.e.* immunoglobulin J polypeptide (IGJ), growth factors *i.e* epiregulin (EREG) , fibroblast growth factor 5 (FGF5) and Fraser syndrome 1 (FRAS1) which mainly function as chemotaxis and are involved in immune response. These genes share a strong homology with catalase (CAT) gene. Hence, they are considered to be the most strong vitiligo susceptible gene candidates found on AIS4 locus.

Depending upon the scores given by SUSPECT, PROSPECTR and GeneWanderer, Fraser syndrome 1 (FRAS1), fibroblast growth factor 5 (FGF5) and epiregulin (EREG) have the highest average score values so they are considered to be the most likely candidate genes which are more prone towards mutations to cause vitiligo while according to G2D scores, immunoglobulin J polypeptide (IGJ) is the most susceptible candidate gene for vitiligo with maximum GO score and minimum R score as compare to chemokine C-X-C motif ligand 13 (CXCL13). Their functional involvement is in the immune response, as a chemokine, cytokine or growth factor whose malfunction can result in vitiligo and are verified by possible associated pathways, OMIM, Generifs and GO associated terms given by GeneDistiller, POSMED and G2D.

I believe that genes presented in this study may serve as initial working candidates which may be analyzed further to identify such molecular mechanisms associated with generalized vitiligo on specific AIS4 locus in parallel with attempts to further narrow down the suspected regions by genetic linkage analysis.

Laboratory techniques, though, one of the most direct and reliable approaches for evaluating any drug or chemical compound but they are insufficient, to meet the daunting requirements of accurate risk assessment and informed decision making, as

their experimental procedures and development are very costly and can take four to five years to complete. But bioinformatics approaches are serving as one of the major player in field of drug industry.

In the present study antioxidants present in a Chinese and Indian herb known as psoralyea corylifolia (commomly known as Babchi in Pakistan) has been studied including Monobenzone, Tacrolimus, Methoxsalen, L-Phenylalanine and Psoralen. They have been analyzed computationally and docked with a mutated model of catalase protein (1QQW) by using AutoDock 3.0 and Hex 4.5 tools. The lowest docking energies, as calculated by AutoDock are: Tacrolimus (-16.04 kcal/mol), L-Phenylalanine (-6.97 kcal/mol), Monobenzone (-6.49 kcal/mol), Methoxsalen (-6.23 kcal/mol) and Psoralen (-6.09 kcal/mol). These results of AutoDock are in close agreement with the total energies of receptor-ligand complexes calculated by Hex which are as follows: Catalase-Tacrolimus complex (-30.05); Catalase-Psoralen complex (-22.88); Catalase-L-Phenylalanin complex (-19.72); Catalase-Monobenzone complex (-11.41) and Catalase-Methoxsalen complex (-8.88). The lowest binding affinities of the receptor with all the potential ligands show that Tacrolimus is the best potential candidate for a prosposed drug as an antioxidant to cure vitiligo.

Analysis of AutoDock and Hex results show that Tacrolimus has the lowest binding affinity with the receptor than rest of compounds. Conformational analysis shows that it forms three hydrogen bonds with receptor protein at ARG 66. Information regarding hydrogen bond formation is the strongest evidence of covalently bounded ligand with any residue existing within the binding pocket of receptor molecule. Psoralen forms two hydrogen bonds with ASP 66 and ARG 66 residues of the receptor protein with the second lowest binding energy, L-Phenylalanine forms four hydrogen bonds with ARG 363 and ARG 66 residues of the receptor protein with the third lowest binding energy, Monobenzone forms three hydrogen bonds with HIS 364 and ARG 66 residues of the receptor molecule with the fourth lowest binding energy. However, Methoxsalen forms three hydrogen bonds with ARG 66 and HIS 364 residues of the receptor protein with binding energy at the last rank.

So, Tacrolimus has the lowest intermolecular energy, internal energy of ligand and torsional free energy than the rest of the compounds as shown in Table 4.2, which

shows its best binding nature with receptor protein and gives full confidence to be called the best proposed potential drug target for the cure of vitiligo. These results are in close agreement with the work of Taher *et al.* (2009); Radakovic *et al.* (2009) and Lubaki et *al.* (2009). As consumption of antioxidants is essential for protection against oxidative stress, which is a major cause of vitiligo, I propose that Tacrolimus and other psoralea constituents are very much helpful in treating by restoring or cancelling out the effect of mutated catalase in order to overcome its reduced production in the body and helps in its scavenging hunger for free readicals found in the body.

Although computational analysis has favored Tacrolimus and other compounds as potential drug targets for treating vitiligo, there is need of clinical trials to be carried out in laboratories to identify specific antioxidants that have good therapeutic effects. As antioxidants are effective against certain free radicals and they tend to locate preferentially in different types of cells and tissues. So, it is also required to be check that which dosage fraction will be the best for the creation of any ointment or drug product which can show maximum effect in treating vitiligo.

REFERENCES

Abdallat A, Davis SM, Farrage J and McDonald WI (1980) Disordered pigmentation, spastic paraparesis and peripheral neuropathy in three siblings: a new neurocutaneous syndrome. *J. Neurol. Neurosurg. Psychiat,* **43:** 962-966.

Adie EA, Adams RR, Evans KL, Porteous DJ and Pickard BS (2006) Speeding disease gene discovery by sequence based candidates prioritization. *BMC Bioinformatics.* **6:** 55.

Adie EA, Adams RR, Evans KL, Porteous DJ and Pickard BS (2006) SUSPECTS: enabling fast and effective prioritization of positional candidates. *Bioinformatics.* **22:** 773-774; http://www.genetics.med.ed.ac.uk/suspects/.

Alkhateeb A, Stetler GL , Old W, Talbert J, Uhlhorn C, Taylor M, Fox A, Miller C, Dills DG, Ridgway EC, Bennett CD, Fain PR, and Spritz RA (2002) Mapping of an autoimmunity susceptibility locus (AIS1) to chromosome 1p31.3–p32.2. *Human Molecular Genetics.* **11:** 661-667.

Alkhateeb A, Fain PR and Spritz RA (2005) Candidate functional promoter variant in the FOXD3 melanoblast developmental regulator gene in autosomal dominant vitiligo. *J Invest Derm.* **125:** 388-391.

Anbar TS, Abdel-Rahman AT, Ghannam S, Hosam El-Din W and El- Khayyat MA (2006) Are Segmental and Non- Segmental Vitiligo Different Disease Entities? Clinical Profile of 1100 Vitiligo Patients. *Egyptian. Dermatology Online Journal.* **2.**

Anna MLD and Picardo M (2006) A review and a new hypothesis for non-immunological pathogenetic mechanisms in vitiligo. *Pigment Cell Res.* **19**: 406–411.

Arcos-Burgos M, Parodi E, Salgar M, Bedoya E, Builes JJ, Jaramillo D, Ceballos G, Uribe A, Rivera N, Rivera D, Fon-seca, I, Camargo M and Palacio LG (2002) Vitiligo: complex segregation and linkage disequilibrium analyses with respect to microsatellite loci spanning the HLA. *Hum Gene.* **110:** 334–342.

Bandyopadhyay D, Lawrence E, Majumder PP and Ferrell RE (2000) Vitiligo is not caused by mutations in GTP-cyclohydrolase I gene. *Clin Exp Dermatol.* **25:** 152–153.

Bennett RL, Steinhaus KA, Uhrich SB, O`Sullivan CK, Resta RG, Lochner-Doyle D, Markel DS, Vincent V, Hamanishi J (1995) Recommendation for standardized human pedigree nomenclature, Pedigree Standadization task force of the national society of genetic counselors. *Am J Hum Genet.* **56:** 745-752.

Bernstein FC, Koetzle TF, Williams GJB, Meyer Jr, EF, Brice MD, Rodgers JR, Kennard O, Shimanouchi T, Tasumi M (1977) The Protein Data Bank: A Computer-based Archival File for Macromodel Structures. *Journal of Molecular Biology.* **112:** 535-542; http://www.rcsb.org

Blomhoff A, Kemp EH, Gawkrodger DJ, Weetman AP, Husebye ES, Akselsen HE, Lie BA and Undlien DE (2005) CTLA4 polymorphisms are associated with vitiligo, in patients with concomitant autoimmune diseases. *Pigment Cell Res.* **18:** 55-58.

Carolina Perez-Iratxeta C, Wjst M, Bork P and Andrade MA (2005) G2D: a tool for mining genes associated with disease. *BMC Genetics.* **6:** 45; http://www.ogic.ca/projects/g2d_2/.

Casp CB, She JX and McCormack WT (2002) Genetic association of the catalase gene (CAT) with vitiligo susceptibility. *Pigment Cell Res.* **15:** 62–66.

Casp CB, She JX and McCormack WT (2003) Genes of the LMP/TAP cluster are associated with the human autoimmune disease vitiligo. *Genes Immun.* **4:** 492–499.

Chen JJ, Huang W, Gui JP, Yang S, Zhou FS, Xiong QG, Wu HB, Cui Y, Gao M, Li W, Li JX, Yan KL, Yuan WT, Xu SJ, Liu JJ and Zhang XJ (2005) A novel linkage to generalized vitiligo on 4q13-q21 identified in a genomewide linkage analysis of Chinese families. *Am J Hum Gene.* **76:** 1057-1065.

Chopra RN, Nayar SL and Chopra IC (1986) Glossary of Indian Medicinal Plants (Including the Supplement), *Council of Scientific and Industrial Research.*

Duke JA and Ayensu ES (1985) Medicinal Plants of China Reference Publications.

Eswar N and Sali A (2007) Comparative Modeling of Drug Target Proteins. In Comprehensive Medicinal Chemistry. In: Taylor JB, Triggle DJ, eds. *Elsevier Ltd, Oxford.* 215-236.

Ewart-Toland A, Enns GM, Cox VA, Mohan GC, Rosenthal P and Golabi M (1998) Severe congenital anomalies requiring transplantation in children with Kabuki syndrome. *Am. J. Med. Gene,* **80:** 362-367.

Fain PR, Gowan K, LaBerge GS, Alkhateeb A, Stetler GL, Talbert J, Bennett DC and Spritz RA (2003) A genomewide screen for generalized vitiligo: confirmation of *AIS1* on chro-mosome 1p31 and evidence for additional susceptibility loci. *Am J Hum Genet.* **72:** 1560–1564

Fain PR, Babu SR, Benneth DC and Spritz RA (2005) HLA class II haplotype DRB1*04–DQB1*0301 contributes to risk of familial generalized vitiligo and early disease onset. *Pigment Cell Res.* **19:** 51–57.

Fernandez DLFR (1997) Mutations in GTP-cyclohydrolase I gene and vitiligo. *Lancet.* **350:** 640.

Fitzpatrick (1997). Mechanisms of phototherapy in vitiligo. *Arch Dermatol.* **132:**1591-1592.

Garsuad AMB, Saint-Cyr I, Quist D, Arveiler B and Garsaud P (2001) Familial aggregation of vitiligo in the French West Indies (Isle of Martinique). *European Journal of Dermatology.* **11:** 554-556.

Genevieve D, Amiel J, Viot G, Le MM, Sanlaville D, Urtizberea A, Gerard M, Munnich A, Cormier-Daire V and Lyonnet S (2004) Atypical findings in Kabuki syndrome: report of 8 patients in a series of 20 and review of the literature. *Am. J. Med. Genet.* **129A:** 64-68.

Herane, M.I.MD (2003) Vitiligo and Leukoderma in Children. *Clin Dermatol.,* **21:** 283-295.

Hristakieva, E (2003) Vitiligo and other associated diseases. *Trakia journal of Sciences.* **3**: 60-62.

Huggins RH, Schwartz RA and Janniger CK (2005) Vitiligo. *Acta Dermatoven APA*. **14.**

Jin J, Cardozo T, Lovering RC, Elledge SJ, Pagano M and Harper JW (2004) Systematic analysis and nomenclature of mammalian F-box proteins. *Genes Dev.* **18:** 2573-2580.

Jin SY, Park HH, Li GZ, Lee HJ, Hong MS, Hong SJ, Park HK, Chung JH and Lee MH (2004) Association of angiotensin converting enzyme gene I/D polymorphism of vitiligo in Ko-rean population. *Pigment Cell Res.* **17:** 84–86.

Jin Y, Mailloux CM, Gowan K, Riccardi SL, LaBerge G, Bennett DC, Fain PR and Spritz RA (2007) NALP1 in vitiligo-associated multiple autoimmune disease. *New Eng J Med.* **356:** 1216-1225.

Johansson CM, Zunec R, Garcia M, Scherbarth HR, Tate GA, Paira S, Navarro SM, Perandones CE, Gamron S, Alvarellos A, Graf CE, Manni J and 11 others (2004) Chromosome 17p12-q11 harbors susceptibility loci for systemic lupus erythematosus. *Hum. Genet.* **115:** 230-238.

Kanehisa M, Bork P (2003) Bioinformatics in the Post sequence era. *Nature Genet.* **33:** 305-310.

Kemp EH, Ajjan RA, Waterman EA, Gawkrodger DJ, Cork MJ, Watson PF and Weetman AP (1999) Analysis of a microsatellite polymorphism of the cytotoxic T-lymphocyte antigen- 4 gene in patients with vitiligo. *Br J Dermatol.* **140:** 73–78.

Kim YC, Kim W and Hann SK (1996) The Genetics of Vitiligo in Korean Patients. *Korean J Dermatol.* **34(6):** 968-972.

Kohler S, Bauer S, Horn D and Robinson PN (2008) Walking the Interactome for Prioritization of Candidate Disease Genes. *The American Journal of Human Genetics*. **82:** 949-958.

Lathrop GM, Lalouel JM, Julier C and Ott J (1984) Strategies for multilocus linkage analysis in humans. *Proc Natl Acad Sci.* **81:** 3443-3446.

Labrune P, Assathiany R, Penso D and Odievre M (1992) Progressive vitiligo, mental retardation, facial dysmorphism, and urethral duplication without chromosomal breakage or immunodeficiency. *J. Med. Genet,* **29:** 592-594.

Le Poole IC, Sarangarajan R, Zhao Y, Stennett LS, Brown TL, Sheth P, Miki T and Boissy RE (2001). 'Vit1', (sic) a novel gene associated with vitiligo. *Pigment Cell Res.* **14:** 475-484.

Lison M, Kornbrut B, Feinstein A, Hiss Y, Boichis H and Goodman RM (1981).

Lubaki LJ, Ghanem G, Vereecken P, Fouty E, Benammar L, Vadoud-Seyedi J, Dell'anna ML, Briganti S, Picardo M and Heenen M (2009). Time-kinetic study of repigmentation in vitiligo patients by tacrolimus or pimecrolimus. *Arch Dermatol Res.*

Majumder PP, Das SK and Li CC (1988) A genetical model for vitiligo. *Am. J. Hum. Genet.* **43:** 119-125.

Majumder PP, Nordlund JJ and Nath SK (1993) Pattern of familial aggregation of vitiligo. *Arch. Derm.* **129:** 994-998.

Margaret RW and Wayne TM (2007). The Role of Genetics in Vitiligo Susceptibility. Reported on June 1, 2006 – May 31, 2007. (http://www.avrf.org)

McGaughran J, Aftimos S, Jefferies C and Winship I (2001) Clinical phenotypes of nine cases of Kabuki syndrome from New Zealand. *Clin. Dysmorph.* **10:** 257-262.

Morris GM, Goodsell DS, Halliday RS, Huey R, Hart WE, Belew RK, Olson AJ (1998) Automated Docking Using a Lamarckian Genetic Algorithm and an Empirical Binding Free Energy Function. *Journal of Computation Chemistry.* **19:** 1539-1662; http://autodock.scripps.edu/.

Na GY, Lee KH, Kim MK, Lee SJ, Kim DW and Kim JC (2003) Polymorphisms in the melanocortin-1 receptor (MC1R) and agouti signaling protein (ASIP) genes in Korean vitiligo patients. *Pigment Cell Res.* **16:** 383–387.

Nagamine K, Peterson P, Scott HS, Kudoh J, Minoshima S, Heino M, Krohn KJE, Lalioti MD, Mullis PE, Antonarakis SE, Kawasaki K, Asakawa S, Ito F and Shimizu N (1997) Positional cloning of the APECED gene. *Nat Genet.* **17:** 393–398.

Nath SK, Kelly JA, Namjou B, Lam T, Bruner GR, Scofield RH, Aston CE and Harley JB (2001) Evidence for a susceptibility gene, SLEV1, on chromosome

17p13 in families with vitiligo-related systemic lupus erythematosus. *Am J Hum Genet.* **69:** 1401-1406.

Nath SK, Majumder PP and Nordlund JJ (1994) Genetic epidemiology of vitiligo: multilocus recessivity cross-validated. *Am. J. Hum. Genet.* **55:** 981-990.

Nikos GG, Samia A, David JG, Philip FW, Anthony PW and Kemp EH (2006) Analysis of allelic variants in the catalase gene in patients with the skin depigmenting disorder vitiligo. *Biochemical and Biophysical Research Communications,* **345:** 1586-1591

Poole ICL, Sarangaranjan R, Zhao Y, Stennett LWS, Brown TL, Sheth P, Miki T and Boissy RE (2001) 'VIT1', A Novel Gene Associated with Vitiligo. *Pigment Cell Res.* **14:** 475–484.

Potapenko AY (1998). Application of antioxidants in investigation and optimization of photochemotherapy. *Membr Cell Biol.* **12:** 269-278.

Prahalad S, Kingsbury DJ, Griffin TA, Cooper BL, Glass DN, Maksymowych, WP and Colbert RA (2001) Polymorphism in the MHC-encoded LMP7 gene: association with JRA without functional significance for immunoproteasome assembly. *J Rheumatol.* **28:** 2320-2325

Risch NJ (2000). Searching for genetic determinants in the new millennium. *Nature*. **405**: 847-856.

Radakovic S, Breier-Maly J, Konschitzky R, Kittler H, Sator P and Hoenigsmann H, Tanew A (2009) Response of vitiligo to once- vs. twice-daily topical tacrolimus: a controlled prospective, randomized, observer-blinded trial. *J Eur Acad Dermatol Venereol.* **23(8):** 951-3.

Ritchie DW and Graham JLK (2000) Protein Docking Using Spherical Polar Fourier Correlations, *Proteins: Struct Func Genet.* **39:** 178-194.

Rozycki DL, Ruben RJ, Rapin I and Spiro AJ (1971) Autosomal recessive deafness associated with short stature, vitiligo, muscle wasting and achalasia. *Arch. Otolaryng,* **93:** 194-197.

Schallreuter KU and Pittelkow MR (1989). Defective calcium uptake system in vitiligo. *Arch Dermatol Res.* **281:** 40-44.

Schallreuter KU, Wood JM and Berger J (1991). Low catalase levels in the epidermis of patients with vitiligo. *J Invest Dermatol* **97:**1081-1085.

Schallreuter KU, Wood JM and Pittelkow MR (1994) Regulation of melanin biosynthesis in the human epidermis by tetrahydrobiopterins. *Science* **263:**1444-1446.

Schallreuter *et al.,* 2008. *Experimental Dermatology*, **17:** 139–160

Seelow D, Schwarz JM and Schuelke M (2008) GeneDistiller-Distilling Candidate Genes from Linkage Intervals. *PLoS ONE.* **3:** 3874.

Spritz RA, Gowan K, Bennett DC and Fain PR (2004) Novel vitiligo susceptibility loci on chromosome 7 (AIS2) and 8 (AIS3), confirmation of SLEV1 on chromosome 17, and their roles in an autoimmune diathesis. *Am J Hum Genet.* **74:** 188-191.

Spritz RA (2007) The genetics of generalized vitiligo and associated autoimmune diseases. *Pigment Cell Res.* **20:** 271–278.

Stein LD (2003) Integrating biological databases. *Nature. Rev. Genet.* 4: 337–345.

Tastan HB, Akar A, Orkunoglu FE, Arca E and Enal A (2004) Association of HLA Class I Antigens and HLA Class II Alleles with Vitiligo in a Turkish Population. *Pigment Cell Res.* **17:** 181–184.

Taher ZA, Lauzon G, Maguiness S, Dytoc MT (2009) Analysis of interleukin-10 levels in lesions of vitiligo following treatment with topical tacrolimus. *Br J Dermatol.*

Tiffin N, Adie E, Turner F, Brunner HG, van Driel MA, Oti M, Lopez-Bigas N, Ouzounis C, Perez-Iratxeta C, Andrade-Navarro MA, Adeyemo A, Patti ME, Semple CAM and Hide W (2006) Computational disease gene identification: a concert of methods prioritizes type 2 diabetes and obesity candidate genes. *Nucleic Acids Research.* **34:** 3067-3081.

Tursen U, Kaya TI, Erdal ME, Derici E, Gunduz O and Ikizoilu G (2002) Association between catechol-O-methyltransferase polymorphism and vitiligo. *Arch Dermatol Res.* **294:** 143–146.

Van DMA, Cuelenaere K, Kemmeren PP, Leunissen JA, Brunner HG and Vriend G (2005) GeneSeeker: extraction and integration of human disease-related information from web-based genetic databases. *Nucleic Acids Res.* **33:** 758–761.

Wishart DS, Knox C, Guo A C, Shrivastava S, Hassanali M, Stothard P, Chang Z and Woolsey J (2006) DrugBank: a comprehensive resource for *in silico* drug discovery and exploration. *Nucleic Acids Research.* **34:** D668-D672

Witkop CJ (1979) Depigmentations of the general and oral tissues and their genetic foundations. *Ala. J. Med. Sci,* **16:** 330-333.

Yeung HC (1985). Handbook of Chinese Herbs and Formulas.

Yildirim M, Baysal V, Inaloz HS, Kesici D and Delibas N. The role of oxidants and antioxidants in generalized vitiligo (2003) *J Dermatol.* **30(2):**104-8.

Yoshida Y, Makita Y, Heida N, Asano S, Matsushima A, Ishii M, Mochizuki Y, Masuya H, Wakana S, Kobayashi N and Toyoda T (2009) PosMed (Positional Medline): prioritizing genes with an artificial neural network comprising medical documents to accelerate positional cloning. *Nucleic Acids Res.* **1:** 37; http://omicspace.riken.jp/).

Zamani M, Spaepen M, Sghar SS, Huang C, Westerhof W, Nieuweboer-Krobotova L and Cassiman JJ (2001) Linkage and association of HLA class II genes with vitiligo in a Dutch population. *Br J Dermatol.* **145:** 90–94.

Zhang XJ, Liu HS, Liang YH, Sun LD, Wang JY, Yang S, Liu JB, Gao M, He PP, Cui Y and Yang Q (2004) Association of HLA class I alleles with vitiligo in Chinese Hans. *J Dermatol Sci.* 35: 165–168.

Zhu M and Zhao S (2007) Candidate Gene Identification Approach: Progress and Challenges. *Int J Biol Sci.* **3:** 420-427.